Satdarshan P. S. Monga
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Molecular Pathology of Liver Diseases

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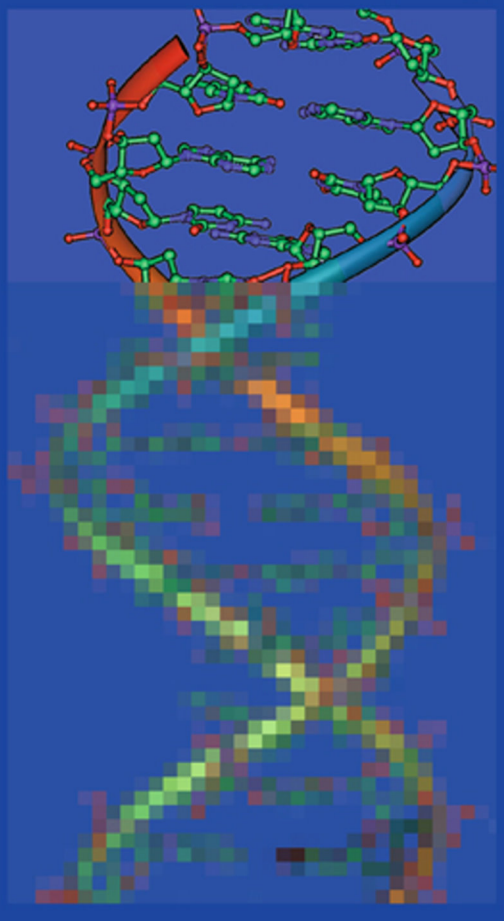

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Satdarshan P.S. Monga
Editor

Molecular Pathology of Liver Diseases

Springer

Editor
Satdarshan P.S. Monga
Director-Division of Experimental Pathology
Associate Professor
Division of Experimental Pathology and Department
of Pathology Division of Gastroenterology, Hepatology
and Nutrition, Department of Medicine
University of Pittsburgh, School of Medicine
200 Lothrop Street, S421-BST Pittsburgh
Pennsylvania, 15261, USA
mongss@UPMC.EDU

ISSN 1935-987X e-ISSN 1935-9888
ISBN 978-1-4419-7106-7 e-ISBN 978-1-4419-7107-4
DOI 10.1007/978-1-4419-7107-4
Springer New York Dordrecht Heidelberg London

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Printed on acid-free paper

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Acknowledgments

I would like to dedicate this textbook to my late mother, an outstanding Obstetrician and Gynecologist who selflessly served her patients. The textbook is also incomplete without thanking many individuals without whom this venture would not have been possible. I would like to acknowledge the support of my wife Dr. Dulabh Kaur Monga and my children, Jappmann Kaur Monga and Jayvir Singh Monga for their incessant support and understanding throughout this undertaking. I would also like to acknowledge the patience of my laboratory members, including the graduate students, postdoctoral fellows, and technicians during the editing phase of the textbook. I would also like to thank my past assistant Ms. Candace Smigla and present assistant Ms. Lorrin Bowser for their incredible support and in keeping the materials organized and updated for efficient completion of this project. This book would not have been possible without the support of Ms. Barbara Lopez-Lucio, who very efficiently and patiently worked with me throughout the prepublication proceedings. Last but not by any means the least, I would like to acknowledge the time and effort of all the authors without whom this textbook would not have been possible at all. Despite their overwhelming commitments and tremendous responsibilities, my colleagues so graciously contributed an outstanding array of material that comprises the heart and soul of this textbook. With great humility I thank you all!

Satdarshan P.S. Monga, MD

Series Preface

The past two decades have seen an ever-accelerating growth in knowledge about molecular pathology of human diseases which received a large boost with the sequencing of the human genome in 2003. Molecular diagnostics, molecular-targeted therapy, and genetic therapy are now routine in many medical centers. The molecular field now impacts every field in medicine, whether clinical research or routine patient care. There is a great need for basic researchers to understand the potential clinical implications of their research whereas private practice clinicians of all types (general internal medicine and internal medicine specialists, medical oncologists, radiation oncologists, surgeons, pediatricians, family practitioners), clinical investigators, pathologists and medical laboratory directors, and radiologists require a basic understanding of the fundamentals of molecular pathogenesis, diagnosis, and treatment for their patients.

Traditional textbooks in molecular biology deal with basic science and are not readily applicable to the medical setting. Most medical textbooks that include a mention of molecular pathology in the clinical setting are limited in scope and assume that the reader already has a working knowledge of the basic science of molecular biology. Other texts emphasize technology and testing procedures without integrating the clinical perspective. There is an urgent need for a text that fills the gap between basic science books and clinical practice.

In the *Molecular Pathology Library* series, the basic science and the technology is integrated with the medical perspective and clinical application. Each book in the series is divided according to neoplastic and nonneoplastic diseases for each of the organ systems traditionally associated with medical subspecialties.

Each book in the series is organized to provide specific application of molecular pathology to the pathogenesis, diagnosis, and treatment of neoplastic and nonneoplastic diseases specific to each organ system. These broad section topics are broken down into succinct chapters to cover a very specific disease entity. The chapters are written by established authorities on the specific topic from academic centers around the world. In one book, diverse subjects are included that the reader would have to pursue from multiple sources in order to have a clear understanding of the molecular pathogenesis, diagnosis, and treatment of specific diseases. Attempting to hunt for the full information from basic concept to specific applications for a disease from varied sources is time-consuming and frustrating. By providing this quick and user-friendly reference, understanding and application of this rapidly growing field is made more accessible to both expert and generalist alike.

As books that bridge the gap between basic science and clinical understanding and practice, the *Molecular Pathology Series* serves the basic scientist, the clinical researcher, the practicing physician, or other health care provider who require more understanding of the application of basic research to patient care, from "bench to bedside." This series is unique and an invaluable resource to those who need to know about molecular pathology from a clinical, disease-oriented perspective. These books are indispensable to physicians and health care providers in multiple disciplines as noted above, to residents and fellows in these multiple disciplines as well as their teaching institutions and to researchers who increasingly must justify the clinical implications of their research.

Philip T. Cagle, MD
Series Editor

Preface

Molecular Pathology of Liver Diseases is the fifth volume in the *Molecular Pathology Library Series* by Springer. Owing to the improved understanding of the cellular and molecular mechanisms of diseases, academic medicine is undergoing an evolution. Novel molecular tests not only aid in a disease diagnosis, but also can be extrapolated for disease prognosis. Similarly, modulation of biological pathways for the treatment of a disease is becoming a reality. The molecular structure and function of a normal and abnormal gene product enables the determination of highly relevant diagnostic, therapeutic, and prognostic information. In essence, the physicians and scientists alike are inundated with basic and translational information on the mechanisms of health and disease. It is of great significance to generate resources capable of: (1) bridging molecular biology and pathology, and clinical medicine; (2) providing a unique educational resource to the academic physicians and researchers keeping abreast of the timely advances, evolving modalities, and shifting paradigms; and (3) providing fundamental concepts in organ-based molecular pathobiology. *Molecular Pathology of Liver Diseases* is a compilation of a broad range of topics in liver health and disease to serve as a unique, timely, and comprehensive resource for practicing physicians, researchers, and trainees in the ever-evolving field of hepatic pathobiology, as we move forward into an era of integrative and personalized medicine.

Molecular Pathology of Liver Diseases integrates the traditional knowledge of physiological and pathological processes in the liver with a balanced emphasis on fundamental concepts, timely advances in cellular and molecular mechanisms, and applied pathology. The textbook is organized into several sections, each of which includes an array of chapters that progressively and cohesively elaborate on pertinent liver biology and pathology. The first three sections discuss the cellular composition of the liver along with their specialized functions, and further dissect the molecular basis of the cellular processes that are so unique to the liver. The next section examines the mechanisms that are commonly implicated in the cellular and molecular basis of several hepatic pathologies, followed eventually by a section each on a multitude of nonneoplastic and neoplastic diseases of the liver. Thus, these sections provide an expansive understanding of hepatic physiology, whose aberrations have pathological consequences.

The textbook is written and presented as a one-stop and comprehensive reference on liver pathobiology for basic, translational and clinical researchers, and physicians. As is vividly reflected by the diversity of the contributing authors from various disciplines, I would also anticipate this textbook to be of value to pathologists, hepatologists, surgeons, oncologists, molecular biologists, physiologists, biochemists, and toxicologists with interest in the Liver. This textbook is also suitable for medical students, graduate students, residents, and fellows with an interest in liver biology. The format of the textbook is meant to serve as a ready reference to relevant topics in the liver, thus providing a practical disease-based integrative resource on the molecular pathology of liver disease.

Satdarshan P.S. Monga, MD
Director, Division of Experimental Pathology
Associate Professor of Pathology and Medicine
University of Pittsburgh, SOM

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Contributors

N. Volkan Adsay, MD
Professor and Vice-Chair, Director of Anatomic Pathology,
Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA

Gianfranco D. Alpini, PhD
VA Scholar AwardeeProfessor of Internal Medicine and Systems Biology and Translational Medicine, Holder of the Nicholas C. Hightower Endowed Chair of Gastroenterology, Director of the Scott & White Digestive Research Center Division Research, Central Texas Veterans Health Care System, Scott & White and Texas A & M University Health Science Center College of Medicine, Temple, TX, USA

Domenico Alvaro, MD
Full Professor of Gastroenterology, Head of the Division of Gastroenterology D. Alvaro Polo Pontino, Italy, University of Rome, Sapienza, Rome, Italy

Udayan Apte, PhD, DABT
Department of Pharmacology, Toxicology and Therapeutics,
University of Kansas Medical Center, Kansas City, Kansas, USA

Alla Arzumanyan, PhD
Assistant Director of Biotechnology Center,
Department of Biology, Temple University, Philadelphia, PA, USA

Yusuf Bayraktar, MD
Professor of Medicine and Gastroenterology, Department of Internal Medicine, Gastroenterology Section, Hacettepe University, Ankara, Turkey

Jaideep Behari, MD, PhD
Assistant Professor, Department of Medicine, Division of Gastroenterology, Hepatology, and Nutrition, University of Pittsburgh, Pittsburgh, PA, USA

Antonio Benedetti, MD
Director, Clinic of Gastroenterology, Universita Politecnica Delle Marche – Azienda Ospedaliero – Universita “Ospedali Riuniti”, Ancona, Italy

D. Hunter Best, BS, PhD
Assistant Professor (clinical) and Assistant Medical Director of Molecular Genetics, Department of Pathology, University of Utah School of Medicine/ARUP Laboratories, Salt Lake City, UT, USA

Jorge A. Bezerra, MD
Professor of Pediatrics, Department of Gastroenterology, Hepatology and Nutrition, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA

Sangeeta N. Bhatia, MD, PhD
M.I.T., E19-502D; 77 Massachusetts Ave., Cambridge, MA 02139

Jayme Borensztajn, MD, DPhil
Professor, Department of Pathology, Feinberg School of Medicine,
Northwestern University, Chicago, IL, USA

Marie Annick Buendia, PhD
Director of Research, Oncogenesis and Molecular Virology Unit,
Institut Pasteur, Paris, France

Yvonne Chao, BS
Graduate Student, Department of Pathology, University of Pittsburgh,
Pittsburgh, PA, USA

Gaurav Chaturvedi, PhD
Research Assistant Professor, Department of Molecular and Integrative Physiology,
University of Kansas Medical Center, Kansas City, KS, USA

Alice A. Chen, BS
Graduate student, Department of Health Sciences & Technology,
Massachusetts Institute of Technology, Cambridge, MA, USA

Mingyi Chen, MD, PhD
Department of Pathology and Laboratory Medicine, Loma Linda University Medical Center,
Loma Linda, CA, USA

Onpan Cheung, MD, MPH
Fellow, Division of Gastroenterology and Hepatology, Department of Medicine,
Virginia Commonwealth University, Richmond, VA, USA

John Y. L. Chiang, PhD
Department of Integrative Medical Sciences, Northeastern Ohio Universities Colleges of
Medicine and Pharmacy, Rootstown, OH, USA

Callisia N. Clarke, MD, BA
Resident (PGY-3), Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Marcia M. Clayton, BS
Technician, Department of Biology, Temple University, Philadelphia, PA, USA

William B. Coleman, BS, PhD
Professor and Director of Graduate Studies, Department of Pathology and Laboratory
Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, USA

Sabine Colnot, PhD
Department of Endocrinology, Metabolism and Cancer,
INSERM U567, Institut Cochin, Université Paris Descartes, Paris, France

Amedeo Columbano, PhD
Professor of Pathology, Department of Toxicology, School of Medicine,
University of Cagliari, Cagliari, Italy

Francisco Javier Cubero, PhD
Postdoctoral Researcher, Department of Internal Medicine III,
University Hospital Aachen (RTWH), Aachen, Germany

Albert J. Czaja, MD
Mayo Clinic College of Medicine, 200 First Street SW Rochester, Minnesota 55905

Mariana D. Dabeva, MD, PhD
Associate Professor, Department of Medicine, Albert Einstein College
of Medicine of Yeshiva University, New York, NY, USA

Anthony J. Demetris, MD
Department of Pathology, Thomas E. Star21 Transplantation Institute, Pittsburgh, PA, USA

Wen-Xing Ding, PhD
Assistant Professor, Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, Kansas City, KS, USA

Jonathan A. Dranoff, MD
Associate Professor of Medicine, Department of Internal Medicine/Digestive Diseases and Yale Liver Center, Yale University School of Medicine, New Haven, CT, USA

Jean-François Dufour, MD
Professor, Department of Visceral Medicine, University of Berne, Berne, Switzerland

Monique Fabre, MD
Associate Professor of Pathology, Head of Liver Pathology Unit, Paul Brousse Hospital, Department of Pathology, Paul Brousse and Bicêtre Hospitals, Le Kremlin-Bicêtre, France

Jia Fan, MD, PhD
Chairman, Department of Liver Surgery, Liver Cancer Institute, Shanghai, P.R. China

Giammarco Fava, MD
Division of Gastroenterology Azienda Ospedaliero-Universitaria, "Ospedali Riuniti", Ancona, Italy

Mark A. Feitelson, PhD
Principal Investigator, Department of Biology, Temple University, Philadelphia, PA, USA

Ira J. Fox, MD
Professor, Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA

Samuel W. French, MD
Division of Anatomic Pathology, Department of Pathology, Harbor UCLA Medical Center, Torrance, CA, USA

Heather L. Francis, BS
Research Associate, Department of Research and Education/Systems Biology and Translational Medicine, Scott & White Hospital/Texas A&M College of Medicine, Temple, TX, USA

Chandrashekhar R. Gandhi, MSc, PhD
University of Pittsburgh, No. 1542, 200 Lothrop Street, Pittsburgh, PA 15213, USA

Eugenio Gaudio, MD
Director, Division of Anatomy, University of Rome "Sapienza", Rome, Italy

David A. Geller MD
Richard L. Simmons Professor of Surgery and Co-Director, Department of Surgery, UPMC Liver Cancer Center, University of Pittsburgh, Pittsburgh, PA, USA

M. Eric Gershwin, MD
Professor, Department of Internal Medicine, Rheumatology, University of California, Davis, Davis, CA, USA

Gregory J. Gores, MD
Professor of Medicine and Chair, Department of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, Rochester, MN, USA

Theodorus B. M. Hakvoort, PhD
Biochemist, AMC Liver Center, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Caroline Hora, MD
PhD Student, Department of Clinical Pharmacology and Visceral Research, University of Berne, Berne, Switzerland

Jiansheng Huang, MD, PhD
Research Assistant Professor, Department of Pathology, Northwestern University, Chicago, IL, USA

Kumiko Isse, MD, PhD
Post-Doctorial Fellow, Department of Pathology, Thomas E. Starzl Transplantation Institute, Pittsburgh, PA, USA

Hartmut Jaeschke, PhD, MSc
Professor, Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA

Klaus H. Kaestner, PhD, MS
Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Sanjay Kakar, MD
Associate Professor, Department of Anatomic Pathology, University of California, San Francisco and San Francisco VA Medical Center, San Francisco, CA, USA

Nora Katabi, MD
Assistant Attyending Pathologist, Department of Pathology, Memorial Sloan Kettering Center, New York, NY, USA

Naoya Kobayashi, MD, PhD
Associate Professor, Department of Gastroenterological Surgery, Transplant and Surgical Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Eleonore S. Köhler, PhD
Assistant Professor, Department of Anatomy & Embryology, NUTERIM School for Nutrition, Toxicology and Metabolism of Maastricht, Maastricht, The Netherlands

Kris V. Kowdley, MD
Director,
Center for Liver Disease, Virginia Mason Medical Center and Benaroya Research Institute, Seattle, WA, USA

Partha Krishnamurthy, PhD
Assistant Professor,
Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, MO, USA

Manoj Kumar, MBBS, MD, DM
Assistant Professor,
Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, India

Abigale Lade, BS
Graduate Student Researcher, Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA

Wouter H. Lamers, MD, PhD
AMC Liver Center, Academic Medical Center, University of Amsterdam, Meibergdreef 69–71, 1105 BK, Amsterdam, The Netherlands NUTRIM School for Nutrition, Toxicology and Metabolism of Maastricht University Medical Center, Universiteitssingel 50, 6229 ER, Maastricht, The Netherlands

Giovanna Maria Ledda-Columbano, PhD
Professor of Pathology,
Department of Toxicology, School of Pharmacy, University of Cagliari, Cagliari, Italy

Frederic P. Lemaigre, MD, PhD
de Duve Institute, Université catholique de Louvain, Brussels, Belgium

Alex B. Lentsch, PhD
Professor and Vice Chairman for Research,
Department of Surgery, University of Cincinnati College of Medicine,
Cincinnati, OH, USA

Zhaorui Lian, MD, PhD
Staff Scientist,
Department of Biology, Temple University, Philadelphia, PA, USA

Chih-Wen Lin, MD
Research Fellow,
Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Keith D. Lindor,
MD Professor of Medicine,
Department of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA

Ton Lisman, PhD
Associate Professor, Section of Hepatobiliary Surgery and Liver Transplantation
and Surgical Research Laboratory, Department of Surgery,
University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Joseph Locker, MD, PhD
Department of Pathology, Albert Einstein College of Medicine,
Bronx, New York, NY, USA

Ivano Lorenzini, MD
Director, Division of Gastroenterology, Azienda Ospedaliero – Universitaria, Ancona, Italy

John G. Lunz III, PhD
Post-Doctorial Fellow, Department of Pathology, Thomas E. Starzl Transplantation Institute, Pittsburgh, PA, USA

Harmeet Malhi, MBBS
Assistant Professor of Medicine, Division of Gastroenterology and Hepatology,
Mayo Clinic College of Medicine, Rochester, MN, USA

Pranoti Mandrekar, PhD
Research Associate Professor, Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

Marco Marzioni, MD
Assistant Professor of Gastroenterology, Department of Gastroenterology, Ospedali Riuniti University Hospital, Università Politecnica delle Marche, Ancona, Italy

George K. Michalopoulos, MD, PhD
Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA

Kisha Mitchell, MBBS
Assistant Professor, Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

Yoshiaki Mizuguchi, MD, PhD
Post-Doctorial Fellow, Department of Pathology, Thomas E. Starzl Transplantation Institute, Pittsburgh, PA, USA

Hiroyuki Nakai, MD, PhD
Assistant Professor, Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Nalu Navarro-Alvarez, MD, PhD
Postdoctoral Fellow, Department of Surgery, Transplantation Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA

James E. Nelson, PhD
Research Scientist, Benaroya Research Institute, Seattle, WA, USA

Michael Oertel, PhD
Assistant Professor, Department of Medicine, Albert Einstein College of Medicine of Yeshiva University, New York, NY, USA

David H. Perlmutter, MD
Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh of UPMC,
Pittsburgh, PA, USA

Christine Perret, PhD
Group Leader, Department of Endocrinology, Metabolism and Cancer, INSERM U567, Institut Cochin, Université Paris Descartes, Paris, France

Robert J. Porte, MD, PhD
Professor of Hepatobiliary Surgery and Liver Transplantation, Section of Hepatobiliary Surgery and Liver Transplantation and Surgical Research Laboratory, Department of Surgery, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Dirk Raddatz, MD, MD
Department of Gastroenterology and Endocrinology, University of Göttingen, Göttingen, Germany

Giuliano Ramadori, MD
Director, Department of Internal Medicine Gastroenterology, University Clinic, Göttingen, Germany

Janardan K. Reddy, MBBS, MD
Department of Pathology, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA

Helena M. G. P. V. Reis, MBA, PhD
Executive Director, MIT–Portugal Program, Lisbon, Portugal

Juan Carlos Roa, MD
Director, Molecular Pathology Laboratory and Director of Postgrade and Research, Department of Pathology, Facultad de Medicine, Universidad de la Frontera, Temuco, Arucania, Chile

Arun J. Sanyal, MBBS, MD
Department Chair, Department of Gastroenterology, Hepatology and Nutrition, Medical College of Virginia, Richmond, VA, USA

Shiv K. Sarin, MBBS, MD, DM
Director, Professor and Head, Department of Gastroenterology, G.B. Pant Hospital and Project Director, Institute of Liver and Biliary Sciences, New Delhi, India

Michael L. Schilsky, MD
Associate Professor of Medicine and Surgery, Medical Director, Adult Liver Transplant, Division of Digestive Diseases and Section of Transplantation and Immunology, Yale New Haven Transplant Center, Yale New Haven Medical Center, New Haven, CT, USA

Stewart Sell, MD
Senior Research Physician, Department of Molecular Medicine, Wadsworth Center, Ordway Research Institute and University at Albany, Albany, NY, USA

Carlo Selmi, MD, PhD
Assistant Professor, Department of Internal Medicine, IRCCS Institute Clinico Humanitas, University of Milan, Rozzano, Italy

David A. Shafritz, MD
Department of Medicine, Cell Biology and Pathology, Marion Bessin Liver Research Center, Albert Einstein College of Medicine of Yeshiva University, New York, NY, USA

Jing Shan, BS
Graduate student, Department of Health Sciences & Technology, Massachusetts Institute of Technology, Cambridge, MA, USA

Vijay H. Shah, MD
Consultant,
Division of Gastroenterology and Hepatology, Department of Internal Medicine, Mayo Clinic, Rochester, MN, USA

Ying-Hong Shi, MD
Attending Surgeon, Department of Liver Surgery, Liver Cancer Institute, Shanghai, P.R. China

Marina G. Silveira,MD
Fellow, Department of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA

Sumit K. Singla, MD
Resident Physician, Department of Internal Medicine, Mayo Clinic, Rochester, MN, USA

Mirela-Patricia Sirbu-Boeti, MD
Research Associate, Department of Surgery, University of Pittsburgh School of Medicine/ Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA

Kyle Soltys, MD
Assistant Professor, Department of Surgery, Thomas E. Starzl Transplantation Institute/ Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA

Alejandro Soto-Gutierrez, MD, PhD
Department of Surgery, University of Pittsburgh School of Medicine/Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA

Susan Specht,
Research Technician, Department of Pathology, Thomas E. Starzl Transplantation Institute, Pittsburgh, PA, USA

Kelly R. Stevens, PhD
Postdoctoral Fellow, Harvard-MIT Department of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge MA, USA

Donna Beer Stolz, PhD
Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, USA

Bill S. Sun, MD
Research Scientist, Department of Biology, Temple University, Philadelphia PA, USA

Jiaren Sun, PhD
Associate Professor, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA

Arief A. Suriawinata, MD
Atomic Pathology Section Chief, Department of Pathology, Dartmouth Hitchcock Medical Center and Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, NH, USA

Gyongyi Szabo, MD, PhD
Professor of Medicine, Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

John M. Taylor, PhD
Senior Member, Fox Chase Cancer Center, Philadelphia, PA, USA

Amit D. Tevar, MD
Attending–Transplant Surgery, Department of Surgery, University of Cincinnati, Cincinnati, OH, USA

Nikolai A. Timchenko, PhD
Department of Pathology, Baylor College of Medicine, Houston, TX, USA

Michael H. Trauner, MD
Professor of Medicine, Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria

Christian Trautwein, MD
Director, Department of Internal Medicine III, University Hospital Aachen (RTWH), Aachen, Germany

Kartik Trehan, MSE, MSE
Graduate student Harvard-MIT Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge,MA, USA

Debbie Trinder, PhD
Research Professor, Fremantle Hospital, School of Medicine and Pharmacology and Western Australian Institute for Medical Research, University of Western Australia, Fremantle, WA, Australia

Michael Tsapakos, MD
Director, Body MRI, Department of Medicine-Radiology, Dartmouth Hitchcock Medical Center and Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, NH, USA

Gregory J. Tsongalis, PhD
Director, Molecular Pathology, Department of Pathology, Dartmouth Hitchcock Medical Center and Norris Cotton Cancer Center, Dartmouth Medical School, Lebanon, NH, USA

Allan Tsung, MD
Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA

Gregory H. Underhill, PhD
Research Scientist, Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA, USA

Steven A. Weinman, MD, PhD
Professor, Department of Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA

Alan Wells, MD, DMSc
Thomas Gill Professor of Pathology and Vice-Chair for Laboratory Medicine, Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA

Rebecca G. Wells, MD
Assistant Professor of Medicine and Pathology and Laboratory Medicine, Department of Medicine (Gastroenterology), University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Qian Wu, PhD
Post-Doctoral Associate, Department of Pathology, University of Pittsburgh, Pittsburgh, PA 19104, USA

Xiao-Ming Yin, MD, PhD
Professor, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh PA, USA

Jessica Zucman-Rossi, MD, PhD
Department of Oncology, Inserm U674, Université Paris Descartes, 27 rue Juliette Dodu, Paris 75010, France

Part I
Liver Cells and Functions

Chapter 1
Gross and Cellular Anatomy of the Liver

Allan Tsung and David A. Geller

Gross Anatomy of the Liver

The liver is the largest organ in the body and has an extraordinary spectrum of functions. Weighing approximately 1,500 g, it sits in the right upper abdominal cavity beneath the diaphragm, and is protected by the rib cage. It is reddish-brown in color and is surrounded by a thin connective tissue capsule known as Glisson's capsule. Traditionally, the liver is grossly separated into the right and left lobes by the external landmark of the falciform ligament, a ligament that runs along the umbilical fissure and anchors the liver to the anterior abdominal wall (Fig. 1.1). However, a more accurate description of lobar anatomy of the liver is based on its blood supply. The right and left lobes of the liver can be divided by a plane from the gallbladder fossa to the inferior vena cava, known as Cantlie's line [1]. The right lobe typically accounts for 60–70% of the liver mass, with the left lobe (and caudate lobe) making up the remainder. The caudate lobe lies to the left and anterior of the inferior vena cava. The right lobe can be further divided into anterior and posterior segments, while the left lobe can be divided by the falciform ligament into a medial segment (quadrate lobe), and a lateral segment.

A significant advance in our understanding of the liver anatomy came from the studies of the French surgeon and anatomist, Claude Couinaud, in the early 1950s. Couinaud enumerated the liver with eight segments to more accurately describe its functional anatomy (Fig. 1.2). Each segment is supplied by a single portal triad composed of a portal vein, hepatic artery, and bile duct. The caudate lobe is referred to as segment 1. Segments 2 and 3 comprise the left lateral segment, while segment 4 is the left medial segment. Thus, the left lobe is made up of the left lateral segment (Couinaud's segments 2 and 3) and the left medial segment (segment 4). Segment 4 can be sub-divided into segment 4A and segment 4B. Segment 4A is cephalad and just below the diaphragm, spanning from segment 8 to the falciform ligament adjacent to segment 2. Segment 4B is caudad and adjacent to the gallbladder fossa. The right lobe is comprised of segments 5–8, with segments 5 and 8 making up the right anterior lobe, and segments 6 and 7 the right posterior lobe [2, 3].

Additional functional anatomy can also be detailed based on the distribution of the hepatic veins. There are three hepatic veins (right, middle, and left) that pass obliquely through the liver to drain the blood to the suprahepatic inferior vena cava (IVC) and eventually to the right atrium. The three hepatic veins run in corresponding scissurae (fissures) and divide the liver into four sectors [4]. The right hepatic vein runs along the right scissura and separates the right posterolateral sector from the right anterolateral sector. The main scissura contains the middle hepatic vein and separates the right and left livers. The left scissura contains the course of the left hepatic vein, and separates the left posterior and left anterior sectors. The hepatic vein branches "bisect" the portal branches inside the liver parenchyma (e.g., the right hepatic vein runs between the right anterior and posterior portal veins; the middle hepatic vein passes between the right anterior and left portal vein; and the left hepatic vein crosses between the segment III and II branches of the left portal vein). These hepatic vein branches drain specific areas of the liver back to the IVC. The right hepatic vein drains segments 5–8; the middle hepatic vein drains segments 4 as well as segments 5 and 8; and the left hepatic vein drains segments 2 and 3. The caudate lobe is unique, as its venous drainage feeds directly into the IVC. In addition, the liver usually has a few, small, variable, and short hepatic veins that directly enter the IVC from the undersurface of the liver. The left and middle hepatic veins form a common trunk ~95% of the time before entering the IVC, while the right hepatic vein inserts separately (in an oblique orientation) into the IVC. There is a large, inferior, and accessory right hepatic vein (15–20% of cases) that runs in the hepatocaval ligament. Obstruction of the hepatic venous outflow has significant pathological relevance and is discussed in Chap. 48.

The liver is situated in the right upper abdominal cavity and is held in place by several ligaments: the round, falciform, triangular, and coronary ligaments (Fig. 1.1). The round ligament

A. Tsung (✉)
Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: tsunga@upmc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_1, © Springer Science+Business Media, LLC 2011

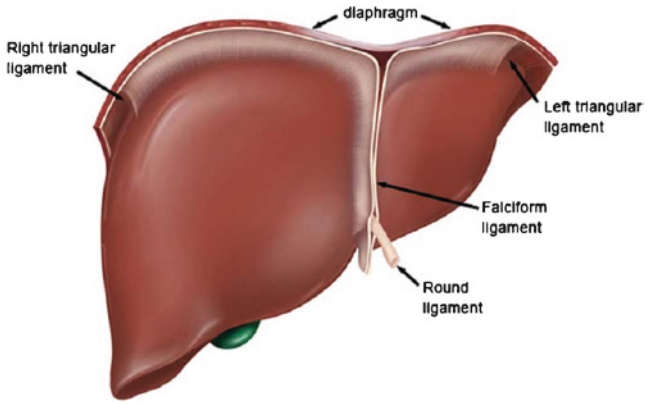

Fig. 1.1 Hepatic structure: Ligaments suspending liver to diaphragm and abdominal wall. Brunicardi FC, Andersen DK, Billiar TR (ed). *Schwartz's Principles of Surgery, 9e.* New York: McGraw-Hill

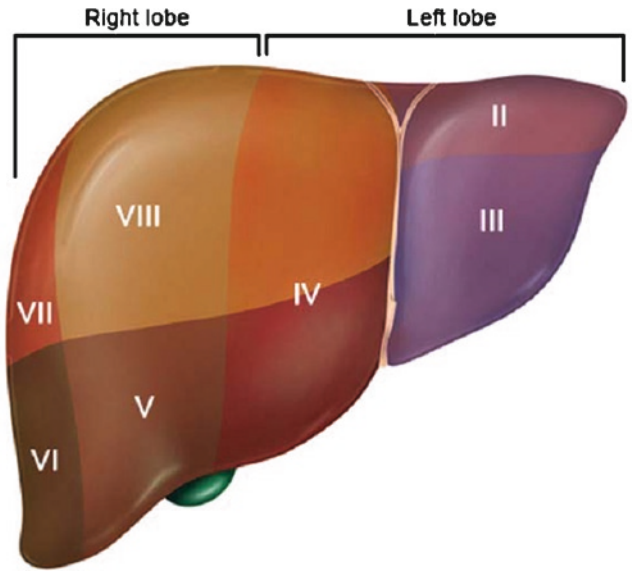

Fig. 1.2 Couinaud's liver segments (I through VIII). The left lobe includes segments II–IV, the right lobe includes segments V–VIII, and the caudate lobe is segment I. Brunicardi FC, Andersen DK, Billiar TR (ed). *Schwartz's Principles of Surgery, 9e.* New York: McGraw-Hill

is the remnant of the obliterated umbilical vein and enters the left liver hilum at the front edge of the falciform ligament. The falciform ligament separates the left lateral and left medial segments along the umbilical fissure. Deep in the plane between the caudate lobe and the left lateral segment is the fibrous ligamentum venosum, which is the obliterated ductus venosus and is covered by the plate of Arantius. The left and right triangular ligaments secure the two sides of the liver to the diaphragm. Extending from the triangular ligaments anteriorly on the liver are the coronary ligaments. The right coronary ligament also extends from the right undersurface of the liver to the peritoneum overlying the right kidney, thereby anchoring the liver to the right retroperitoneum. Centrally and just to the left of the gallbladder fossa, the liver attaches via the hepatoduodenal and the gastrohepatic ligaments. The hepatoduodenal ligament is known as the porta hepatis and contains the common bile duct, the hepatic artery, and the portal vein. From the right side and deep (dorsal) to the porta hepatis, is the foramen of Winslow, also known as the epiploic foramen.

The liver's interface with the digestive system allows for its crucial role in the processing of absorbed nutrients through the metabolism of glucose, lipids, and proteins.

The liver has a dual blood supply consisting of the hepatic artery and the portal vein. The hepatic artery delivers ~25% of the blood supply, and the portal vein ~75%. All nutrition absorbed by the intestinal system reaches the liver through the portal vein, except the complex lipids which are transported mainly by lymphatics. The portal vein is formed by the confluence of the splenic vein and the superior mesenteric vein. The inferior mesenteric vein usually drains into the splenic vein upstream from the confluence. The main portal vein traverses the porta hepatis before dividing into the left and right portal vein branches. The left portal vein typically branches from the main portal vein outside of the liver with a sharp bend to the left, and consists of the transverse portion, followed by a 90° turn at the base of the umbilical fissure to become the umbilical portion before entering the liver parenchyma. The left portal vein then divides to give off the segment 2, and segment 3 that branches to the left lateral segment, as well as the segment 4 feedback branches that supply the left medial segment. The left portal vein also provides the dominant inflow branch to the caudate lobe (although branches can arise from the main and right portal veins also) usually close to the bend between the transverse and umbilical portions. The division of the right portal vein is usually higher in the hilum and may be close to (or inside) the liver parenchyma at the hilar plate.

The hepatic artery arises from the celiac axis (trunk) which gives off the left gastric, splenic, and common hepatic arteries. The common hepatic artery then divides into the gastroduodenal artery and the hepatic artery proper. The right gastric artery typically originates off of the hepatic artery proper, but this is variable. The hepatic artery proper divides into the right and left hepatic arteries. This "classic" or standard arterial anatomy is only present in ~75% of cases, with the remaining 25% having variable anatomy. The most common hepatic arterial variant is a replaced or accessory right hepatic artery coming off the superior mesenteric artery 18–22% of the time. When there is a replaced or accessory right hepatic artery, it traverses posterior to the portal vein and then takes up a right lateral position before diving into the liver parenchyma. The left hepatic artery is replaced (or accessory) off of the left gastric artery in 12–15% of cases, and runs obliquely in the gastrohepatic ligament anterior to the caudate lobe before entering the hilar plate at the base of

the umbilical fissure. Other less common variants (~2% each) are an early bifurcation of the left and right hepatic arteries, as well as a completely replaced common hepatic artery coming off the superior mesenteric artery.

In addition to the portal vein and hepatic artery, the biliary system is the third component of the portal triad. Bile is a complex fluid containing organic and inorganic substances dissolved in an alkaline solution that flows from the liver, through the biliary system, and into the small intestine. The main components of bile are water, electrolytes, and a variety of organic molecules including bile pigments, bile salts, phospholipids (lecithin), and cholesterol. The two fundamental roles of bile are to aid in the digestion and absorption of lipids and lipid-soluble vitamins, and to eliminate waste products (bilirubin and cholesterol) through secretion into bile, and elimination in feces. In between meals, bile is stored in the gallbladder and concentrated through the absorption of water and electrolytes. Upon entry of food into the duodenum, bile is released from the gallbladder to aid in digestion. About one liter of bile can be produced by the human liver daily. However, more than 95% of the bile salts secreted in bile are reabsorbed in the intestine and then excreted again by the liver (enterohepatic circulation). Please see Chap. 12 for a detailed discussion on bile-acid metabolism.

Within the hepatoduodenal ligament, the common bile duct lies anteriorly to the right. It gives off the cystic duct to the gallbladder, and becomes the common hepatic duct before dividing into the right and left hepatic ducts. In general, the hepatic ducts follow the arterial branching pattern inside the liver. The bifurcation of the right anterior hepatic duct usually enters the liver above the hilar plate, while the right posterior duct dives behind the right portal vein and can be found on the surface of the caudate process before entering the liver. The left hepatic duct typically has a longer extra-hepatic course before giving off segmental branches behind the left portal vein at the base of the umbilical fissure. There is a considerable variation that occurs in 20–30% of cases for the hepatic duct confluence with accessory or aberrant ducts (Fig. 1.2).

Cellular Anatomy of the Liver

The liver consists of many different cell types. Broadly, they can be classified as parenchymal cells (hepatocytes) and non-parenchymal cells (NPC). The hepatocytes form the structural basis of the liver and make up the majority of the mass of the liver. The NPCs include other various cell types including Kupffer cells, sinusoidal endothelial cells, stellate cells, periportal fibroblasts, and hepatic dendritic cells. The characteristics and functions of these cell types will be covered in more detail in subsequent chapters.

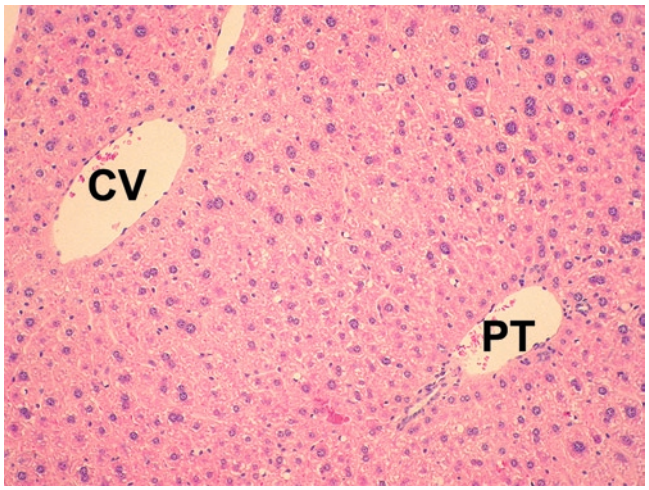

Fig. 1.3 Histology of normal mouse adult liver showing a central vein (CV) and a portal triad (PT)

The liver is made of functional units called liver lobules [5]. These polygonal masses of tissue consist of three to six portal triads at the periphery and a central vein in the center of the lobule. All portal triads of each lobule consist of a venule branch of the portal vein, an arteriole branch of the artery, and bile duct (Fig. 1.3). Originating from the porta hepatis outside the liver, the common bile duct, the hepatic artery, and the portal vein continue to travel together as they continue to branch, supplying the various segments, and ultimately culminate at the portal triad of a lobule. The portal-vein venules of each portal triad then branch into distributing veins that run around the periphery of each lobule, as well as branches than enter the lobules to form the sinusoids. The liver sinusoids are blood vessels with fenestrated, discontinuous endothelium that serve to provide oxygen and nutrients to the surrounding hepatocytes. The sinusoids are positioned radially in the liver lobule, and ultimately converge and form the central vein at the center of the lobule (Fig. 1.3). Similarly, as the hepatic artery continues to branch to form portal arterioles, these vessels also branch into the sinusoids to provide arterial blood to the cells. The central vein of each lobule is a thin walled vein consisting only of endothelial cells. As it is supplied by more sinusoids and exits each lobule, it forms larger veins, and ultimately these veins converge to form the three hepatic veins that drain into the IVC. The portal venous system is a valveless system. Thus, portal pressure in normal physiology is low (3–5 mmHg). However, in the setting of portal hypertension, the pressure can be quite high (20–30 mmHg). This results in decompression to the systemic circulation through portacaval anastomoses, and can produce symptoms of varices seen in settings of cirrhosis.

The direction of blood flow of each liver lobule is from the periphery to the center. The liver can be divided into three zones, based upon oxygen and nutrient supply [5]. Zone 1 is the periportal area where the blood from hepatic

arterioles and portal venules enters, zone 3 is the centrilobular area located around central veins, where oxygenation is poor, and zone 2 is located in between. With zonal arrangement, oxygen, and nutrients absorbed from the digestive system reach the hepatocytes located at the periphery of the lobule first, and then reach the cells located in the center. This lobular structure accounts for structural and functional differences between the periportal and centrolobular cells, as well as differences in their response to various stress or disease states. A greater discussion on liver zonation is presented in Chap. 2.

The hepatocytes are the building blocks of the liver lobule. They are arranged radially with sinusoids separating the plate of cells making up the lobule. The space of Disse is a subendothelial area that separates the endothelial cells from the hepatocytes. Every hepatocyte is in contact with the wall of the sinusoids through the space of Disse as well as with the surface of surrounding hepatocytes. This architecture of hepatocytes lined with the sinusoids consisting of a discontinuous layer of fenestrated endothelial cells allows for bi-directional permeability and exchange of materials from both the hepatocyte and blood. In addition, many nonparenchymal cells of the liver are located in the sinusoids and space of Disse, allowing for efficient immune surveillance and clearance, as well as other metabolic functions.

Bile is a complex fluid containing organic and inorganic substances dissolved in an alkaline solution that flows from the liver, through the biliary system, and into the small intestine. The main components of bile are water, electrolytes, and a variety of organic molecules including bile pigments, bile salts, phospholipids (lecithin), and cholesterol. The two fundamental roles of bile are to aid in the digestion and absorption of lipids and lipid-soluble vitamins, and to eliminate waste products (bilirubin and cholesterol) through secretion into bile and elimination in feces. Bile is produced by hepatocytes and secreted through the biliary system. Between each adjacent hepatocytes are tubular structures consisting of bile canaliculi. These canaliculi are the initial segments of the bile duct system. Similar to the portal vein and hepatic artery branches, except in the opposite direction, the bile canaliculi form networks and terminate in the portal triads at the periphery of the liver lobule. The canaliculi coalesce to from larger bile ductules and eventually make the right and left hepatic ducts, which drain into the intestine through the common bile duct [6].

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Chapter 2
Liver Zonation

Sabine Colnot and Christine Perret

Introduction

Maintenance of liver homeostasis relies on the metabolic function of this organ. To carry out these metabolic functions at a maximal possible efficiency, hepatocytes are both quiescent and highly specialized. They specialize as a function based on their position along the porto-central axis of the liver lobule that determines their fate as either "periportal" (PP), or "perivenous" (PV) hepatocytes. This zonation of function mainly affects ammonia detoxification, glucose/energy metabolism, and xenobiotic metabolism. Over the last 30 years, since the initial discovery of liver zonation, the mechanisms by which this zonation is established and maintained have been widely investigated. The Wnt/β(beta)-catenin developmental pathway has been recently shown to play a key role in this functional heterogeneity of mouse hepatocytes. It is activated in perivenous hepatocytes, partly due to the absence, in the perivenous area, of adenomatous polyposis coli (APC), a tumor suppressor gene product. APC is a negative regulator of Wnt signaling, also described as the "zonation-keeper" of the liver lobule. The Wnt pathway induces the PV genetic program and represses the PP genetic program. The ras/mapk/erk pathway acts in a reciprocal manner to counterbalance Wnt signaling and favors a PP genetic program. More recently, a cross-talk between the transcription factor Hnf4α(alpha) and Wnt signaling has been proposed as a potential mechanism of liver zonation.

The Liver Lobule, the Zonated Unit of the Liver

The liver occupies a strategic position for efficient overall metabolic function in the body. As described in Chap. 1, it receives its supply of hydrophilic nutrients through the portal vein; these nutrients are absorbed by the intestine. It then delivers metabolized products to the other organs through the central vein. The hepatic artery located in the vicinity of the portal vein, within the portal triad (composed of the bile duct, portal vein, and hepatic artery), supplies the liver with blood enriched in oxygen. Blood flow within the liver determines the organization of the anatomical unit of the hepatic parenchyma, the liver cell plate, which is located within the liver lobule or acinus. Here, the blood flows from the portal vein and the hepatic artery (in a 75–25% ratio) to the centrilobular vein, while bile moves from the pericentral area to the periportal one. The liver cell plate consists of 15–25 hepatocytes that extend from the portal triad to the hepatic venule. This structure carries out metabolic functions mostly through specialized hepatocytes, which act in isolation, or together with non parenchymal cells (Fig. 2.1).

Thirty years ago, K. Jungermann demonstrated that hepatocytes, although being histologically indistinguishable, were specialized, and their function differed depending on their position along the porto-central axis of the liver cell plate [1]. Nearly six to eight PP hepatocytes (zone 1) surround the portal triad and are in close contact with the afferent blood. Nearly two to three PV hepatocytes (zone 3) are found close to the efferent centrilobular vein. A less well defined midlobular population of six to ten hepatocytes (zone 2) has also been described. Jungermann proposed the concept of "zonation." According to this concept, opposing or complementary metabolic pathways are carried out within distinct non-overlapping regions of the liver lobule to maintain optimal metabolic homeostasis [2].

Metabolic Zonal Functions

Not all hepatic processes are strictly zonal. The synthesis of large amounts of serum proteins, such as the transthyretin and transferrin transporters appears to occur in all hepatocytes. Albumin is also synthesized in all hepatocytes, with a higher concentration in the periportal area. The most studied zonated functions currently consist of glucose metabolism,

S. Colnot (✉)
Department of Endocrinology, Metabolism and Cancer, INSERM U567, Institut Cochin, Université Paris Descartes, Paris, France
e-mail: sabine.colnot@inserm.fr

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_2, © Springer Science+Business Media, LLC 2011

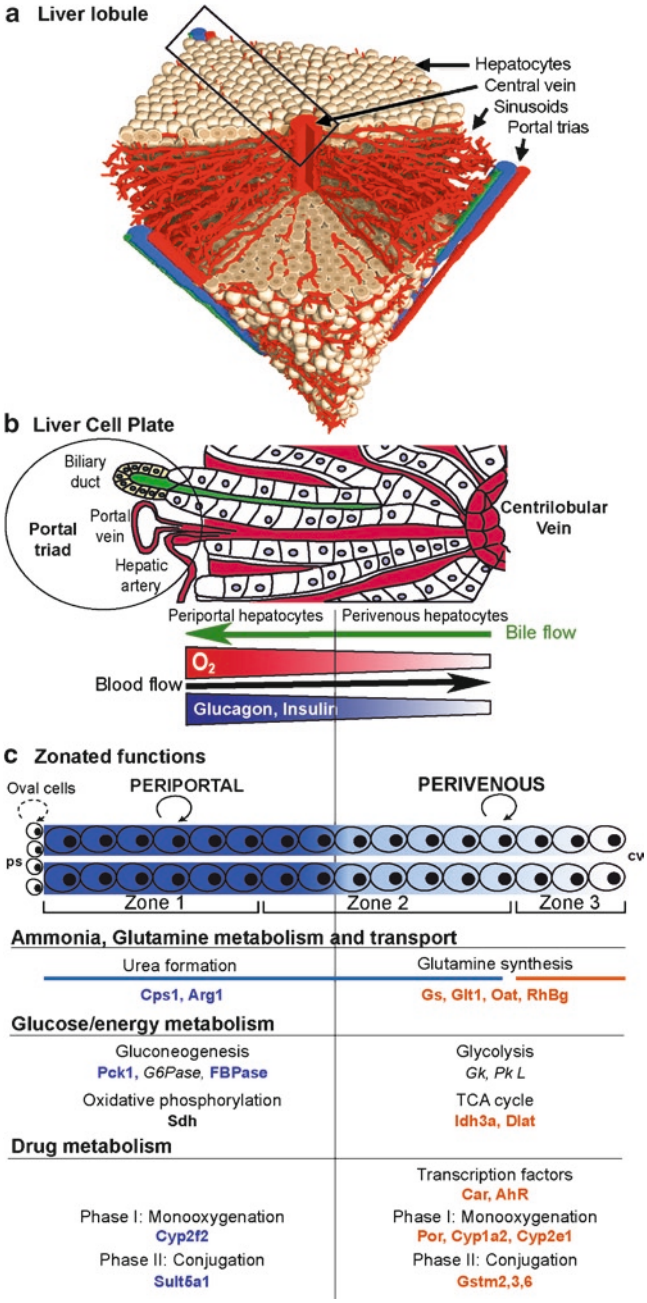

Fig. 2.1 Structure and functions of the zonated liver lobule. (**a**) Three-dimensional structure of the liver lobule. The liver lobule is centered around a branch of the centrilobular vein, limited at each end by the portal triad consisting of a branch of the portal vein, the hepatic artery, and a bile duct. The sinusoids and the vasculature are depicted in red (Hoehme et. al. [61]) (**b**) The liver cell plate, with blood circulation indicated in red. Bile is shown in green and circulates in the opposite direction to blood. The concentration of oxygen and hormones decreases along a continuous gradient from the PP area to the PV area. (**c**) Zonal functions. The proliferation of hepatocytes is achieved mostly by division of the mature hepatocytes themselves (*circled arrow*), which do not migrate along the portocentral axis, with proliferation from oval cells observed only rarely (*dotted circled arrow*). The zonated metabolic systems include the ammonia detoxification system, glucose and energy metabolism, and xenobiotic metabolism. The proteins involved in each type of zonal metabolism are indicated. The proteins displaying zonation regulated at the posttranscriptional level are shown in italics. The PV positive Wnt targets are shown in orange, and the PP negative targets are shown in blue. These targets were identified by microarray analysis on PV and PP hepatocytes [3], and on β(beta)-catenin-activated hepatocytes ([12] and unpublished data). *Gk* glucokinase; *PkL* liver-specific pyruvate kinase; *Sdh* succinate dehydrogenase; *Idh3a* isocitrate dehydrogenase 3a; *Dlat* dihydrolipoamide S-acetyltransferase; *Gstm* glutathione S-transferase mu; *Sult5a1* sulfotransferase family 5A, member 1

ammonia detoxification, and the metabolism of drugs and xenobiotics (Fig. 2.1). Other zonated processes include: lipid metabolism, with lipogenesis occurring perivenously and fatty-acid degradation periportally [2, 3]; Cyp7a1-mediated synthesis of bile acids derived from cholesterol, showing clear PV zonation [3, 4]; and the metabolism of several amino acids [3], the catabolism of histidine and serine being mostly periportal, and glutamine synthesis (associated with ammonia detoxification) being perivenous.

Glucose metabolism provides a historical example of compartmentalized metabolism. Jungermann showed that gluconeogenesis was mostly periportal, with gradual accumulation of phosphoenolpyruvate carboxykinase (Pepck1) in this region, whereas glycolysis was mostly perivenous (glucokinase and pyruvate kinase L, but not their respective RNAs, being perivenous) [1]. However, the concentration gradients of these enzymes differ depending on nutritional status, suggesting that nutrients and hormones play a role in the zonation of these components of glucose metabolism. Please see Chap. 8 for a more detailed review of carbohydrate metabolism in the liver.

Ammonia detoxification is subject to zonation and has been extensively studied by groups led by Gebhardt, Lamers, and Haussinger [5–7]. One of the major roles of the liver is the removal of harmful ammonia arriving from the intestine via the portal vein (Fig. 2.2). Ammonia is first metabolized by PP hepatocytes, through a high-capacity/low-affinity system, to generate urea. This involves the enzymes carbamoylphosphate synthetase (Cps1) and arginase 1 (Arg1). Residual ammonia is then converted to glutamine by perivenous hepatocytes, through a low-capacity/high-affinity system involving the perivenous enzyme glutamine synthetase (Gs). Chapter 9 discusses protein metabolism.

Metabolism of drugs and xenobiotics also displays well defined zonation, occurring mostly in the PV area. The cytochrome P450 system is responsible for the conversion of xenobiotics into excretable products. This involves monooxygenation followed by conjugation with either glucuronic or sulfuric acid. Monooxygenation mainly occurs in the PV zone, with glucuronidation as the major conjugation reaction in these cells, whereas sulfation is the predominant conjugation reaction in PP cells [2]. Chapter 11 discusses detoxification functions of the liver.

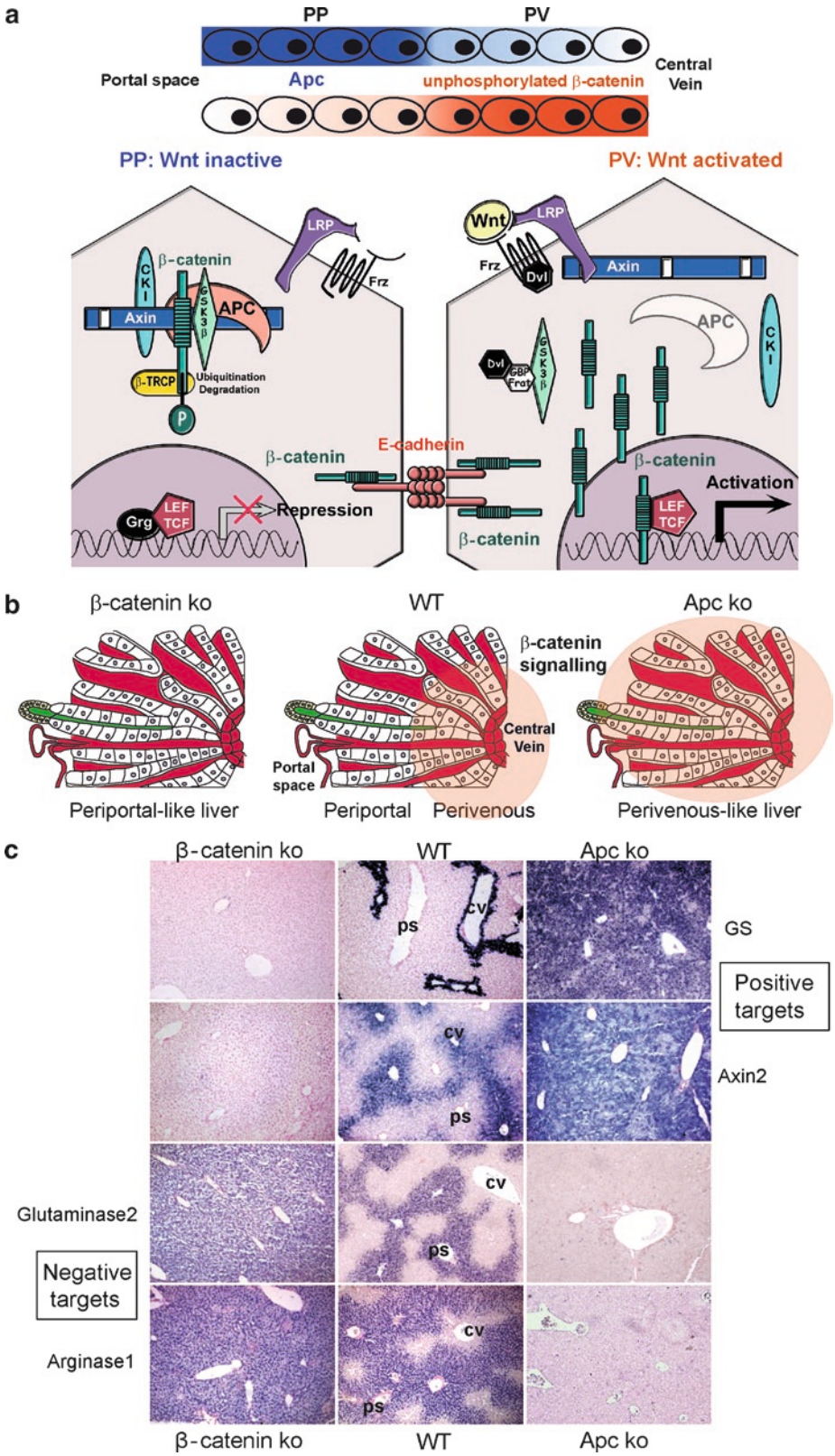

Fig. 2.2 Zonation of Wnt signaling in the liver. (**a**) Localization of Wnt partners. PP hepatocytes are enriched in APC, allowing the accumulation of active unphosphorylated β(beta)-catenin in PV hepatocytes. A schematic diagram of a PP hepatocyte in which Wnt is inactive is shown on the left, and the consequences of Wnt activation in a PV hepatocyte are shown on the right. (**b**) Mouse models of liver-specific β(beta)-catenin inactivation (β(beta)-catenin ko) or activation (Apc ko). (**c**) In situ hybridization showing the distribution of PV positive target gene expression and of PP negative target gene expression along the portocentral axis of the liver, in β(beta)-catenin-null, wild-type, and APC-null livers. The Axin2 gene is a universal target gene of β(beta)-catenin, and the signal generated for this gene on in situ hybridization is thought to correspond to the area of β(beta)-catenin activation

Possible Mechanisms for Zonation

Until recently, the mechanisms underlying zonation were poorly understood even if a number of hypotheses have been put forward.

The developmental hypothesis suggests that periportal and perivenous hepatocytes are derived from different embryonic origins, and distinct lineages. However, evidence for this is lacking; indeed, the perinatal liver is not zonated and coexpresses perivenous and periportal mRNAs, such as those encoding Gs, and Cps1 [8]. Zonation is only observed in the first week following birth in mice.

The streaming liver theory is based on the observation that periportal hepatocytes are more prone to proliferate (see section on proliferation). According to this model, hepatocytes are derived from the periportal area, where putative hepatic stem cells reside. Hepatocytes then migrate further along the portocentral axis to become perivenous, gaining a particular metabolic profile as they mature. However, cell-tracing studies have shown this not to be the case; instead, hepatocyte renewal seems to occur in both parts of the lobule [9].

The blood hypothesis offers a more feasible explanation, with aspects still to be explored. Blood entering the sinusoid is a mixture of blood from the hepatic artery and portal vein, and is rich in oxygen. The low oxygen tension in the hepatic venules [10] thus gives rise to a steep oxygen gradient across the sinusoid. Blood then perfuses hepatocytes in the plate sequentially. This means that the composition of blood changes as hepatocytes in the plate are perfused. Thus, hepatocytes located in different parts of the liver cell plate are exposed to different microenvironments. Accordingly, in this model, zonation may be determined by the concentrations of oxygen, hormones, drugs, or metabolites in the blood. However, changes in the hormone or oxygen content of afferent blood reverses the patterns of glycolysis and gluconeogenesis, and such changes do not affect ammonia detoxification [2]. This led to the definition of "dynamic zonal metabolism," which can be applied to the somewhat plastic processes of glucose and drug metabolism. By contrast, stable zonal metabolic systems, such as ammonia detoxification, cannot easily be reversed or lost.

Zonation is subjected to transcriptional control. Nevertheless, functional zonation is mainly controlled by differential expression of genes encoding the enzymes responsible for the functions concerned. This control may involve transcriptional or posttranscriptional regulation along the portocentral axis (Fig. 2.1). Transcriptional mechanisms seem to be crucial, as shown by microarray studies. These studies characterized mRNAs from the PP and PV hepatocytes, and confirmed that zonation of glucose, ammonia, and drug metabolism was mainly under the control of mRNA levels [3].

The identification of the PV glutamine synthetase gene as a direct target of β(beta)-catenin in liver suggested that β(beta)-catenin may be one of the *trans*-acting factors mediating liver zonation [11]. Studies of murine models with β(beta)-catenin signaling activated or inactivated in the liver have shown that the β(beta)-catenin pathway is one critical aspect involved in liver zonation [12–14].

The Wnt/β(Beta)-Catenin Pathway

The Wnt/β(beta)-catenin pathway, which has been strongly conserved through evolution, plays an important role in development and has been implicated in tumorigenesis in various tissues [15]. A detailed account on this pathway is available in Chap. 20. Wnt signaling is initiated by the binding of secreted ligands (Wnts) to frizzled receptors (fz), leading to the activation of canonical or non-canonical signaling. Canonical signaling activates β(beta)-catenin-mediated gene transcription [16] (Fig. 2.2). In the absence of Wnt signaling, β(beta)-catenin plays a role in cell adhesion, through interaction with cadherins. β(beta)-catenin is kept at low concentrations in the cytosol through its phosphorylation by CK1 and GSK3 kinases, within a degradation complex comprising two tumor suppressors, APC and axin. Phosphorylated β(beta)-catenin is ubiquitinylated by β(beta)-TrCP, resulting in it being targeted for degradation by the proteasome. Wnt binding to fz receptors together with LRP5/6 coreceptor activation, causes the dissociation of the β(beta)-catenin degradation complex; unphosphorylated β(beta)-catenin then accumulates and is translocated into the nucleus, where it associates with Lef/Tcf transcription factors to regulate the transcription of its target genes.

The Wnt pathway was initially described for its role in developmental processes, but has also been implicated in the maintenance of stem-cell compartments in adults [17]. These physiological, developmental, and oncogenic effects are mediated by the regulation of different genetic programs, depending on the temporal and spatial contexts.

β(Beta)-Catenin, A Master Signaling Molecule Orchestrating Metabolic Zonation in the Liver

The link between the Wnt pathway and the liver was initially established through the demonstration that β(beta)-catenin-activating mutations frequently occur during liver carcinogenesis [18]. These mutations define a particular carcinogenic route. Indeed, tumors in which β(beta)-catenin is activated display a specific transcriptome, which strongly favors PV

metabolism [13, 19–21]. More recent work has shown this signaling pathway to play a role in liver development and physiology, with a major role in determining the final patterning of the adult liver [12].

Initial observations suggesting a role for β(beta)-catenin in establishing liver zonation was based on the complementary distribution patterns of active β(beta)-catenin in PV hepatocytes and of the negative regulator APC in PP hepatocytes, in wild-type adult mice [12] (Fig. 2.2). Mice were then generated, in which APC was inactivated specifically in the liver. The subsequent activation of β(beta)-catenin signaling in these mice induced a PV genetic program throughout the entire lobule and repressed the PP genetic program. These findings were confirmed in mice producing Dkk1, a Wnt inhibitor, or with inactivation of β(beta)-catenin gene expression in the liver lobule. The PV genetic program was switched off in these mice, whereas the PP genetic program was active throughout the lobule [12, 14, 22] (Fig. 2.2).

The zonal metabolic pathways affected by changes in β(beta)-catenin signaling include those mediating ammonia metabolism potentially explaining the hyperammonemia observed in mice with aberrant hepatocytic β(beta)-catenin signaling. β(beta)-catenin exerts strict positive control over the PV genes encoding glutamine synthetase (Gs, or glul), Glt-1 (Eaat2 or slc1a2; a transporter of glutamate), and RhBg, a transporter of ammonia. It negatively regulates the glutaminase 2 (Gls2), arginase 1 (Arg1), and carbamoylphosphate synthase (Cps1) PP genes with Arg1 and Cps1 being key enzymes of the urea cycle [11, 12] (Figs. 2.1 and 2.2).

Drug metabolism is strongly controlled by the Wnt pathway. A study by Hebrok and colleagues demonstrated that mice with specific deletion of β(beta)-catenin in the liver were insensitive to intoxication with carbon tetrachloride (CCl_4), presumably due to the absence of CypP450-detoxifying enzymes in these mice [14]. Wnt signaling controls the expression of two major CypP450 enzymes, Cyp2e1 which metabolizes ethanol, and Cyp1a2 [13, 14, 23]. The aryl-hydrocarbon receptor (Ahr) and the constitutive androstane receptor (Car) are two PV proteins that act as both xenosensors and as transcription factors. They control the expression of drug-metabolizing enzymes induced by β(beta)-catenin in the liver [13, 14, 23]. Whether β(beta)-catenin signaling has a direct effect on the transcription of detoxifying enzymes or whether it acts indirectly involving Car and Ahr is yet unclear.

Mice with liver-targeted activation of the Wnt pathway have altered glucose metabolism and develop hypoglycemia [24]. This hypoglycemia may be caused by impaired gluconeogenesis, a PP process, through the negative regulation of the Pepck and FBPase genes in β(beta)-catenin-activated livers [12, 24]. β(beta)-catenin signaling may also modify the energetic profile of the hepatocyte. In APC null livers, ATP energy supply seems to be provided through glycolysis rather than through mitochondrial oxidative phosphorylation due to the upregulation of lactate dehydrogenase protein and activity, together with the downregulation of two mitochondrial subunits ATP5α(alpha)1 and ATP5β(beta) by β(beta)-catenin [24]. The potential zonation of these new β(beta)-catenin targets remains to be investigated.

These data clearly demonstrate that β(beta)-catenin signaling is a key pathway in the control of liver metabolism. APC appears to be the "zonation-keeper" of the liver, consistent with its specific distribution along the portocentral axis of the lobule and the major effects of its inactivation in mice.

The Consequences of Disrupting Zonation

The effects of activation of β(beta)-catenin signaling differ from the effects of loss of β(beta)-catenin signaling in mice. β(beta)-Catenin-null livers become periportal-like, but with no major effect on phenotype; however, APC-null livers, become perivenous-like, leading to death of the animals, mainly through lethal hyperammonemia arising from the lack of urea-cycle enzymes [12] (Fig. 2.2).

The fact that mice with loss of β(beta)-catenin signaling in the liver are viable implies that the perivenous functions of the hepatocytes are not essential for normal liver homeostasis. These mice are only more sensitive to nutritional dysfunction, with a protein overload leading to mild hyperammonemia, due to defects in perivenous enzymes and ammonia transporters, presumably including GS and RhBg [14]. This suggests that the perivenous genetic program is a scavenger system, which is not required for basal metabolism, but is activated in certain contexts. By contrast, mice with β(beta)-catenin activation in all the hepatocytes of the liver lobule rapidly die. Whether this is due to an essential role for periportal proteins in basal metabolism necessary for life, or to the disruption of liver homeostasis caused by aberrant expression of perivenous proteins throughout the lobule, remains unknown.

Establishment of Liver Zonation by β(Beta)-Catenin and Wnts

The role of β(beta)-catenin during liver development remains a matter of debate. β(beta)-catenin may play a limited role in hepatogenesis. Hepatogenesis occurs as a multistep process during embryonic development, beginning with the emergence of the liver bud from endodermal cells derived from the ventral foregut [25]. Liver bud formation requires

distinct signals emitted by different cell types. The role of β(beta)-catenin in this initial step is controversial, as this molecule seems to antagonize liver bud formation in *Xenopus,* but is required for this process in Zebrafish [26, 27]. At later stages of development, the Wnt pathway affects liver growth, probably in a restricted time-window, controlling the proliferation and differentiation of immature hepatoblasts into the biliary lineage [28, 29]. Wnt signaling also stimulates liver growth after birth [30]. Given that liver zonation appears in early postnatal development, Wnt signaling has to be correlated with the emergence of a compartmentalized liver.

Adenovirus-mediated liver transfer of Dickkhopf-1 (Dkk1), a molecule that blocks Wnt signal transduction, also blocks the physiological activation of β(beta)-catenin signaling in the PV area of the liver. Thus, β(beta)-catenin PV signaling requires Wnts [12]. Nineteen Wnt factors have been identified in mice, some of which are expressed in the liver [31]. However, the Wnt factors involved in zonal gene expression and the cells that produce them (presumably in contact with PV hepatocytes) are still unknown. The most likely sources of Wnt are endothelial cells, specifically those surrounding the centrilobular vein, or the PV hepatocytes themselves; this also remains to be resolved.

β(Beta)-Catenin and Hepatocyte Proliferation

The PV compartment was long thought to be resistant to proliferative signals and prone to hepatocellular necrosis or apoptosis [32]. Moreover, the oval "stem" cells recruited in very specific conditions for liver regeneration are located in the PP area [33]. The Wnt pathway has been implicated in the proliferation and self-renewal of stem cells; thus, the physiological activation of Wnt signaling in perivenous hepatocytes was unexpected.

This phenomenon must be considered in the context of liver homeostasis, a unique process, which differs, for example, from homeostasis in the intestine. In the intestine, a pool of β(beta)-catenin-activated crypt stem cells is responsible for the constant renewal of the epithelium. This renewal is essential, as enterocytes have a lifespan of 5–7 days. This essential role of the Wnt pathway has been demonstrated in mice showing major consequences of the loss of APC in the small intestine, in particular the enlargement of the crypt compartment [34, 35]. By contrast, the liver cells are quiescent and hepatocytes have a turnover rate estimated between 148 and 400 days. These cells very rarely divide during their lifespan. When such divisions do occur during a physiological process or following injury (as in the experimental model of 2/3 partial hepatectomy), it is the mature hepatocyte itself that enters the cell cycle. Hepatocytes are renewed independently of their location along the portocentral axis, even if the rate of replication is faster in the PP hepatocytes than in intermediate or PV hepatocytes (Fig. 2.1) [9]. The recruitment of oval "stem" cells for regeneration can be promoted using drugs to block the proliferation of mature hepatocytes [33]. Under such conditions, Wnt/β(beta)-catenin signaling may play an active role in the activation and proliferation of oval cells [36, 37].

Paradoxically, despite the fundamental metabolic role of physiological Wnt signaling in the quiescent liver, aberrant β(beta)-catenin activation throughout the liver leads to marked hepatocellular hyperproliferation [21, 38, 39], and the focal activation of β(beta)-catenin may induce liver cancer formation [21]. The Wnt pathway also plays an important role in liver regeneration, as deletion of the β(beta)-catenin gene in mice delays S-phase by one day after partial hepatectomy. This provides further evidence for the involvement of the Wnt pathway in controlling hepatocyte proliferation.

The set of genes involved in hepatocyte proliferation has yet to be elucidated. Target genes with a potential effect on hepatocyte proliferation, such as those encoding Reg1a and Reg3a/Hip/Pap, have been identified, but functional studies have not yet been performed [40]. In the intestine, the Wnt pathway controls cell proliferation through a cell-autonomous phenomenon, in which *c-myc* is a critical target gene [41]. By contrast, *c-myc* plays no role in liver cell proliferation [21, 38, 42, 43]. Cyclin-D1 has been shown to be a target of the pathway and is critical in G1 to S-phase transition. In many scenarios in liver biology, cyclin-D1 expression and subsequent proliferations are known to be regulated by β(beta)-catenin. Such examples include liver regeneration and development [29, 44]. Moreover, β(beta)-catenin-dependent hepatocyte proliferation is not cell autonomous, and only occurs when Wnt is activated above a critical threshold (activation in more than 70% of hepatocytes) [12, 21]. Future studies will now need to identify β(beta)-catenin target genes mediating this cell non-autonomous and threshold-dependent proliferation. This will provide a better understanding of both β(beta)-catenin-dependent liver carcinogenesis and the control of liver-cell renewal in a zonated-quiescent or regenerating liver.

Integration of β(Beta)-Catenin Signaling with Other Regulators of Zonal Liver Functions

Microarray studies comparing the Wnt targetome in various cell types show less than 5% of β(beta)-catenin targets to have a ubiquitous distribution [45]. This tissue-specificity of

β(beta)-catenin signaling responses suggest that the molecular mechanisms involved in this signaling are also dependent on cell type. In particular, the mechanisms underlying β(beta)-catenin-induced repression of the PP genetic program need to be elucidated.

Work by Schwarz and colleagues provided some initial insight into the mechanisms of β(beta)-catenin signaling in the liver. This group identified a Ras/Mapk/Erk signal in the periportal hepatocyte, which is activated by blood-borne molecules. This pathway favors the expression of a PP genetic program and blocks the PV program [46]. In this model, the Ras and β(beta)-catenin signaling pathways are antagonistic. However, in addition to this apparent physiological antagonism, cooperation between β(beta)-catenin and the Ras pathway appears to accelerate liver tumorigenesis [47]. Further characterization of the interaction between the β(beta)-catenin and Ras pathways could therefore help our understanding of liver physiopathology.

Hnf4 α(Alpha) Is a Liver-Enriched Factor Specializing β(Beta)-Catenin Transcriptome

Recent studies have suggested that Hnf4α(alpha) is a master player in establishing the molecular network that drives liver zonation. Hnf4α(alpha) had been previously described as a major transactivator of genes associated with liver differentiation and metabolism [48–52]. A pioneering genome-wide study revealed 12% of the promoters in liver bound Hnf4α(alpha), of which 80% were transcriptionally active [51]. However, Hnf4α(alpha) displays a relatively homogenous distribution throughout the liver lobule and controls transcription of both zonated and not zonated genes. Thus, despite its fundamental roles in the liver, Hnf4α(alpha) seemed an unlikely candidate for controlling liver zonation [53].

Two recent studies have demonstrated that Hnf4α(alpha) is a modulator of the zonal expression of genes [54, 55], acting through cross-talk with the Wnt pathway [54].

The first of these studies involved the analysis of zonal gene expression in Hnf4α(alpha)-null livers. Stanulovic et al. showed reexpression of some (but not all) of the PV genes, such as *Gs* and *Oat* in the PP hepatocytes of these mice [55]. This concept was further tested in fetal rodent hepatocytes, which can be differentiated in culture to display a hepatocyte phenotype [54]. In this model, only PP genes were transcribed, and the activation of Wnt pathway by the GSK3β(beta) inhibitor 6-bromoindirubin-3′-oxime (BIO) redirected the expression profile towards the PV program. The authors showed that one member of the Tcf/Lef family of transcription factors, Lef1, can interact with Hnf4α(alpha). They used chromatin immunoprecipitation assays to evaluate binding to regulatory elements in vivo and found that Hnf4α(alpha) and Lef1 interaction was required for *Gs* expression, whereas Hnf4α(alpha) binding alone seemed to repress the expression of this gene. Conversely, on three periportal promoters, the authors show that Hnf4α(alpha) alone activates these genes in the PP area, while the presence of β(beta)-catenin in the PV zone leads to the replacement of Hnf4α(alpha) by Lef1, silencing their expression (Fig. 2.3).

A genome-wide analysis of targets bound by Tcf4 in colon cancer cell lines identified several Hnf4α(alpha)-binding sites present at a high frequency in the vicinity of Tcf4-binding sites, and this reinforces the link between Hnf4α(alpha) and Wnt dependent transcriptions in epithelial tissues [56].

The cross-talk between Hnf4α(alpha) and Wnt signaling to ensure proper liver zonation should be extended on other hepato-specific genes. But, it opens up new perspectives in this domain, raising several new questions: What are the mechanisms determining whether Hnf4α(alpha) activates or silences target genes? Why is Hnf4α(alpha) not sufficient to activate the transcription of PV genes, such as the gene encoding glutamine synthetase, despite the recognition of its binding motif? How does β(beta)-catenin control the equilibrium between Lef1 and Hnf4α(alpha) binding to chromatin?

Conclusion

Studies over the last three years have provided considerable insight into the molecular mechanisms involved in liver zonation. In particular, these studies have established the involvement of β(beta)-catenin pathway and Hnf4α(alpha) transcription factor. The complex zonal organization of the liver is of particular interest in the study of hepatocarcinogenesis. The overall patient prognosis and disease course of HCC in β(beta)-catenin mutated versus non-mutated HCC remains unsettled. Two routes of hepatocarcinogenesis have been described, based on analyses of tumor transcriptome profiles [20, 57, 58]. The first of these is linked to a high level of genomic instability, with frequent LOH and loss of p53 [59]. The second carcinogenic pathway defines a different type of tumor progression, with maintenance of a stable chromosome profile during HCC development. Tumors undergoing this second route of progression are enriched in β(beta)-catenin mutations, and are well-differentiated tumors with a predominant activation of PV gene transcription. Immunostaining of these tumors with GS antibodies is now used as a marker of these β(beta)-catenin-mutated well-differentiated HCCs [19, 60]. The effect of this particular metabolic PV transcriptome on β(beta)-catenin-depen-

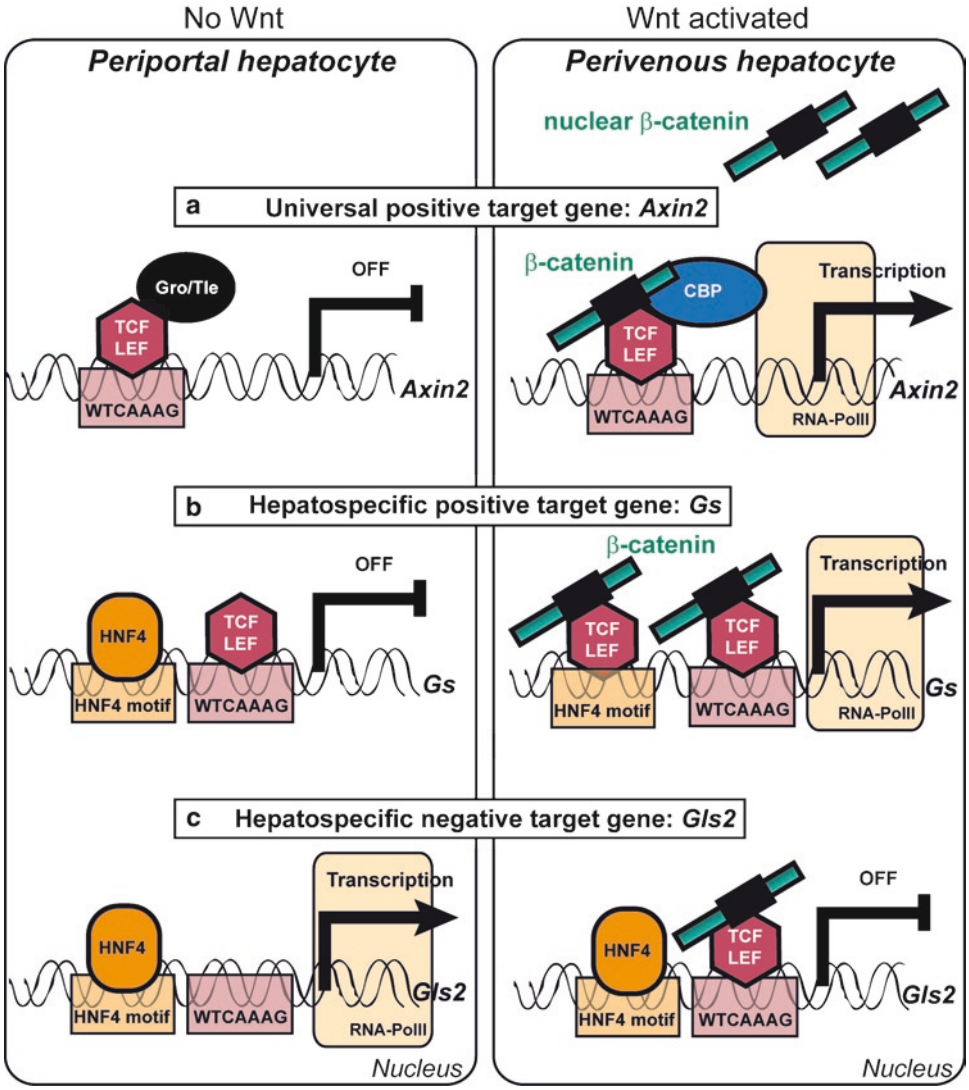

Fig. 2.3 *Trans*-acting partners of β(beta)-catenin and Lef/Tcf factors in the nuclei of PV versus PP hepatocytes. (**a**) Conventional interaction of β(beta)-catenin with Lef/Tcf factors on an example of universal target gene (Axin2). In the absence of β(beta)-catenin in PP nuclei, Lef/Tcf factors, bound to its recognition motif (WTCAAAG) recruit Groucho/Tle cofactors to repress the transcription of genes. In the presence of β(beta)-catenin, Lef/Tcf factors recruit CBP coactivator that establish a link to the preinitiation complex and the RNA Polymerase II to activate the transcription of genes. (**b**) For the transcription of the hepatospecific β(beta)-catenin target gene Gs, Lef1 factor is recruited to both Lef/Tcf and Hnf4 motifs in presence of β(beta)-catenin. In the absence of β(beta)-catenin, Hnf4 and Lef1 bind to their respective motif, and this has a repressive impact on transcription. (**c**) The transcription of the hepatospecific negative β(beta)-catenin target Gls2 is mediated by Hnf4 in the PP nuclei. The binding of Lef1 on its recognition motif in PV chromatin inhibits this Hnf4-dependent transcription

dent hepatocarcinogenesis is yet to be determined. This metabolic profile may offer new perspectives for targeted therapeutic strategies for the treatment of this subset of β(beta)-catenin-activated HCCs. This would represent a novel and unanticipated use of our current knowledge of liver zonation.

Acknowledgments We warmly thank Drs Jan Hengstler (IFADo, Dortmund, Germany), Stefan Hoehme and Dirk Drasdo (INRIA, France) for providing their three-dimensional reconstruction of the liver lobule (Fig. 2.1a) [61]. This work was supported by INSERM, CNRS and the "Ligue Nationale Contre le Cancer" (LNCC, Comité de Paris, équipe Labellisée 2008), the ANR-07-PHYSIO and the CANCERSYS European network.

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Chapter 3
Hepatocytes

Alejandro Soto-Gutierrez, Nalu Navarro-Alvarez, and Naoya Kobayashi

Introduction

The ability of the liver to regenerate was recognized by the Greeks in the ancient myth of Prometheus, the Titan god of forethought, who gave fire to the mortals and angered Zeus. Zeus then punished him for his crime by having him bound to a rock while a great eagle ate his liver every day only to have it grow back to be eaten again the next day [1]. In contrast to other solid organs, the human liver has the unique ability to regenerate after toxic injury, chronic inflammation, and surgical resection and is able to restore its original mass, cellular structure and functions [1–3].

The liver is responsible for the synthesis of serum proteins; intermediary metabolism of amino acids, lipids, and carbohydrates; and detoxification of xenobiotic compounds. Liver receives all exiting circulation from the small and most of the large intestine, as well as spleen and pancreas, through the portal vein. Its "strategic" location in relation to the food supply via the portal vein, and the unique gene-and protein-expression patterns of hepatocytes (the main functional cells of the liver) allow it to function as a master metabolic organ. These functions are performed primarily by its functional unit, hepatocytes. However, hepatocyte function (e.g., gene expression profile and biochemical activities) is not identical among all hepatocytes. Hepatocytes perform different roles depending on their physical location within the hepatic lobule [4] and it is discussed comprehensively in Chap. 2.

The liver of mammals receives the portal venous blood flow from the gastrointestinal tract and about 30% of the resting arterial circulation [5, 6]. Lymph flow from the liver is estimated to be 25–50% of the total thoracic duct flow, or approximately 0.5 ml/kg of liver/minute [5, 6].

A. Soto-Gutierrez (✉)
Department of Surgery, University of Pittsburgh School of Medicine/ Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA
e-mail: sotogutierreza@upmc.edu

The hepatocellular parenchyma accounts for 60% of the total cell population and 80% of the total volume of the organ, with the lobular parenchyma representing approximately 93%, the hepatic veins 4%, and the portal triads 3% of the hepatic parenchyma. Nonparenchymal cells comprise 30–35% of the total number of liver cells, but only 6% of the total liver volume. Almost half (40%) of the nonparenchymal cells are fenestrated endothelial cells. The remainder consists of phagocytic Kupffer cells (33%), extraluminal stellate cells (22%), biliary epithelial cells (4%), natural killer cells (1%), adrenergic or peptidergic nerve cells in primates and dogs, and mast cells in the dog [7].

The adult mammalian liver is composed of diverse cell types that arise from various embryologic origins. In this chapter, the discussion of the "hepatocytes" focuses on their physiology, functions, and regenerative capacities. Finally, we discuss briefly about the alternative sources of hepatocytes from stem cells.

Hepatocyte Structure

Polarity

The architecture of the liver parenchyma is unique when compared with other epithelia. In the adult liver, the hepatocyte is structurally and functionally polarized and has three distinct membrane domains: sinusoidal (basal), lateral, and canalicular (apical). Sinusoids carry the blood flow from the portal vein and hepatic artery to the central vein, and are lined with the endothelial cells, Kupffer cells (resident macrophages), and fat cells. Canalicular surfaces form the bile canalicular network that transports bile produced by hepatocytes to the bile ducts. Lateral plasma membranes fuse alongside bile canaliculi to form zonulae occludens (tight junctions) that occlude the apical domain from the basolateral surface, and thus from the blood-bile barrier. Intermediate junctions, desmosomes, and gap junctions, also on lateral domains provide cohesive strength and functional communication between hepatocytes [8].

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_3, © Springer Science+Business Media, LLC 2011

Ultrastructive

An analysis of the fine structural features of hepatocytes and nonparenchymal cells has been undertaken using electron microscopic techniques. Hepatocytes normally show a typical large round-cell nucleus along with cytoplasmic organelles including both smooth (SER) and rough (RER), endoplasmic reticulum and many mitochondria (M) and dense glycogen granules appear to be distributed throughout the cytoplasm. The basal surface of the hepatocyte, neighboring the sinusoidal capillary space, displays a profuse elaboration of microvilli in the space of Disse (D). The space of Disse is situated just under the endothelial layer of the sinusoidal capillary. Adjacent hepatocytes are separated by an intercellular space with occasional intercellular junctional complexes.

Nonparenchymal cells have also been detected with electron microscopic methods. Kupffer cells can be encountered frequently, either situated upon underlying endothelial cells or as part of the lining of the sinusoidal capillaries. These cells display large numbers of lysosomes. Kupffer and endothelial cells display no specialized contacts. Stellate cells can be identified by the presence of prominent intracellular lipid droplets and filamentous material. These stellate cells are situated between endothelial cells and hepatocytes, or can be intercalated between hepatocytes as well. Endothelial cells can be identified by their elongated and flattened nuclei and by the presence of fenestrations in the cytoplasmic processes that form much of the lining of the sinusoidal capillaries. These fenestrations have diameters of approximately 125–175 nm. At sites where cell processes from apparent adjacent endothelial cells meet, specialized adhesive intercellular-junctional complexes can be identified [9].

Isolation and Culture of Hepatocytes

Hepatocytes are harvested using modifications of the two-step collagenase perfusion technique described by Berry, and Friend [10] and Strom et al. [11]. In general, the liver is perfused with a buffer containing ethylenediaminetetraacetic acid (EDTA), or ethylene glycol tetraacetic acid (EGTA), followed by a second, similarly constituted, buffer that contains collagenase. Each lot of collagenase must be assessed for its ability to release hepatocytes. Individual lots of collagenase that function well to isolate hepatocytes in mice may not function well in other species. Following perfusion with collagenase, the capsule of the liver is torn and the parenchymal cells are separated from the connective tissue using a nylon gauze filter. Hepatocytes are then washed several times and separated by centrifugation at $50 \times g$. The isolated cells after perfusion can be utilized for various applications such as storage, cell transplantation or primary culture.

For storage, isolated hepatocytes can be resuspended in ViaSpan or cryopreserved using dimethyl sulfoxide (DMSO). Viability is usually assessed based on the ability of cells to exclude trypan blue. Unfortunately, this measure is a poor guide to hepatocyte function and engraftment potential. In vitro characteristics of isolated hepatocytes have not been shown to correlate with engraftment, and the ability of the cells to adhere to tissue culture plates 24 h after seeding is a better in vitro assay of engraftment potential [12]. The number of cells needed to treat liver failure or cure inherited disorders of metabolism however, is variable for each condition, and has yet to be determined. In animal experiments, hepatocytes are transplanted fresh, but they can be used following cryopreservation. Additional details on preparation of cells for transplantation are provided in Chap. 21. The ability to preserve and bank hepatocytes, either by cryopreservation or tissue culture offers several theoretical advantages. These include: (1) donor/recipient tissue matching, (2) possible immunologic modulation of donor cells, (3) pooling of multiple donors, if needed, to increase cell numbers for transplantation, and (4) allowing the transplant procedure to be performed semi-electively. Unfortunately, human hepatocyte viability following cryopreservation is quite variable using present technology, and, despite recent advances which have made long-term maintenance of hepatocyte growth in culture possible, successful tissue culture often requires the use of biomatrices which may adversely affect hepatocyte use for transplantation [13, 14].

Ultrastructural studies in human isolated hepatocytes have demonstrated that hepatocytes are easily distinguishable from other contaminant cells because of their large number of mitochondria, microvillous borders, and bile canaliculus remnants. Microvilli surround the entire surface of the liver cell, indicating a complete loss of polarity, probably due to the loss of intercellular contact during isolation, whereas in undisrupted liver tissue they are confined to the bile canaliculus, sinusoidal surfaces, and perisinusoidal compartment.

With completely altered configuration and with total loss of cell polarity caused by the isolation procedure, scientists have attempted to reconstitute some basic aspects of their usual ultrastructure and polarity (bile canaliculi-like expansions, intercellular junctions, and polarized distribution of organelles, peribiliary bodies, and Golgi fields) by cell culture procedures. Cultured hepatocytes are used extensively as an experimental system to study liver functions and diseases, chemical toxicity, and xenobiotic metabolism. One major application of the system is to obtain data on species comparison of chemical toxicity, metabolism, and xenobiotic metabolism [15, 16]. Provided that a good scientific understanding of the correlation between in vitro cell culture and in vivo findings has been established, studies on chemical

toxicity and metabolism with primary cultures of human hepatocytes may yield information that would allow a more accurate extrapolation of data obtained from laboratory animals to humans.

The goal of hepatocyte culture is to create a culture system in which the cells can respond to various extracellular signals in a manner that is physiologically relevant to the in vivo circumstance. Therefore, researchers have investigated methods of hepatocyte culture aimed for maintenance, and improvement of hepatic function using extracellular matrix (ECM), growth factors, cytokines, and hormones [13, 17–19].

The establishment of long-term cultures of primary hepatocytes has long been desired and many efforts have been undertaken to achieve this goal. There is a strong need for robust long-term in vitro screening models, the use of which reduces the number of animals used in drug development. Today, cultures of primary human and animal hepatocytes have been adopted for a variety of pharmacological and toxicological experiments, allowing for the study of chronic effects in vitro. Although in vitro experimental models can never resemble the complexity of a whole organism, their simplicity provides the ability to specifically manipulate and analyze single parameters.

Culturing hepatocytes in a sandwich configuration between two layers of gelled ECM proteins, with collagen I and matrigel being the most commonly used, has dramatically prolonged the longevity of cultures displaying hepatocyte-specific functions [20, 21]. In addition, medium formulation (e.g., the addition/omission of serum, or specified hormone mixtures) has a significant influence on the morphological development and cell survival of hepatocytes in culture [18, 22–24].

Conventional monolayer hepatocytes quickly adopt their polygonal shape and establish extensive cell-cell contacts, whereas in sandwich culture, this takes markedly longer, being still mostly spherical and singular. In general, conventional monolayers appear more flattened than sandwich-cultured cells, a result of the lack of a three-dimensional ECM environment.

By overlaying monolayer cultures of primary hepatocytes with collagen gel, it is possible to obtain cultures displaying some features of the in vivo geometry. This configuration of hepatocytes in collagen sandwich cultures permits to maintain active expression of liver-specific features over extended periods of time (e.g., bile canaliculi-like structures). Cells cultured as a collagen sandwich in serum-free medium do not significantly spread out, and polygonal cell formats, clear plasma membrane boundaries, and stable bile canaliculi-like networks are still evident after 72 h of cultures. In contrast, hepatocytes incubated in serum-containing medium noticeably deteriorate, and lose cytoplasmic integrity and stability of their canaliculi-like structures. Thus, the use of collagen type I gel as ECM component for hepatocyte sandwich cultures is appropriate for long-term cultures. It is a fact that primary hepatocytes cultured in serum-free collagen sandwich cultures stay morphologically unchanged for a few weeks and offer the ability to investigate alterations in cellular structures induced by chemical treatment with the use of high content imaging [21]. In addition to the ECM application and media formulation, cell density also has some influence on the morphology of hepatocytes in culture [25, 26]. Since the polar differentiation of hepatocytes in culture is only visible in cell aggregates, cell density should always be close to confluency, about 90%.

Hepatocyte Functional Characterization

Hepatocytes, the functional unit of a liver, perform many important functions including detoxification, synthesis, and metabolism and these are discussed in-depth in forthcoming chapters. Here, we discuss some features that define hepatocytes both structurally and functionally. The availability of a homogeneous source of human hepatocytes is considered the most precious tool for toxicity screening. In addition to hepatotoxicity, hepatocytes provide a renewable, cell-based assay to examine other key factors of compound attrition such as the metabolism of xenobiotics by CYP450 enzymes, drug–drug interactions, and system for studying hepatic metabolism of xenobiotics, hepatotoxicity and the activity of drug transporters as well as regenerative medicine (Fig. 3.1).

The possibility of having a permanent source of human hepatocytes from stem cells opens exciting new possibilities for pharmacology and toxicology, as well as for cell therapy. However, the nature of the "hepatocyte-like cells" should be analyzed very carefully under several constrictions and a clear definition of the term *hepatocyte* has to be implemented. The expression of hepatocyte markers, such as alphafetoprotein, albumin, or cytokeratin 18, as well as induction of an epithelial phenotype and inducible cytochrome P450 has been reported in several works.

Properties such as epithelial morphology and expression of some hepatocyte markers are necessary, but not sufficient to consider a cell as a hepatocyte. Albumin expression and cytochrome (CYP) P450 are examples of this. In fact, hepatocytes are the only cell type that secretes albumin. However, the conclusion that any albumin-secreting or expressing cell necessarily represents a hepatocyte can be still be premature. For example, it is possible that stem-cell–derived cell types express albumin together with a limited number of hepatocyte markers, but this does not mean that they also express the necessary set of hundreds of genes that make up a true hepatocyte. Moreover, CYP enzymes are not exclusively limited to hepatocytes. Indeed, CYP induction has been reported for lung, colon, small intestine epithelial cells, white adipose tissue, and several other cell types [16, 27]. Therefore,

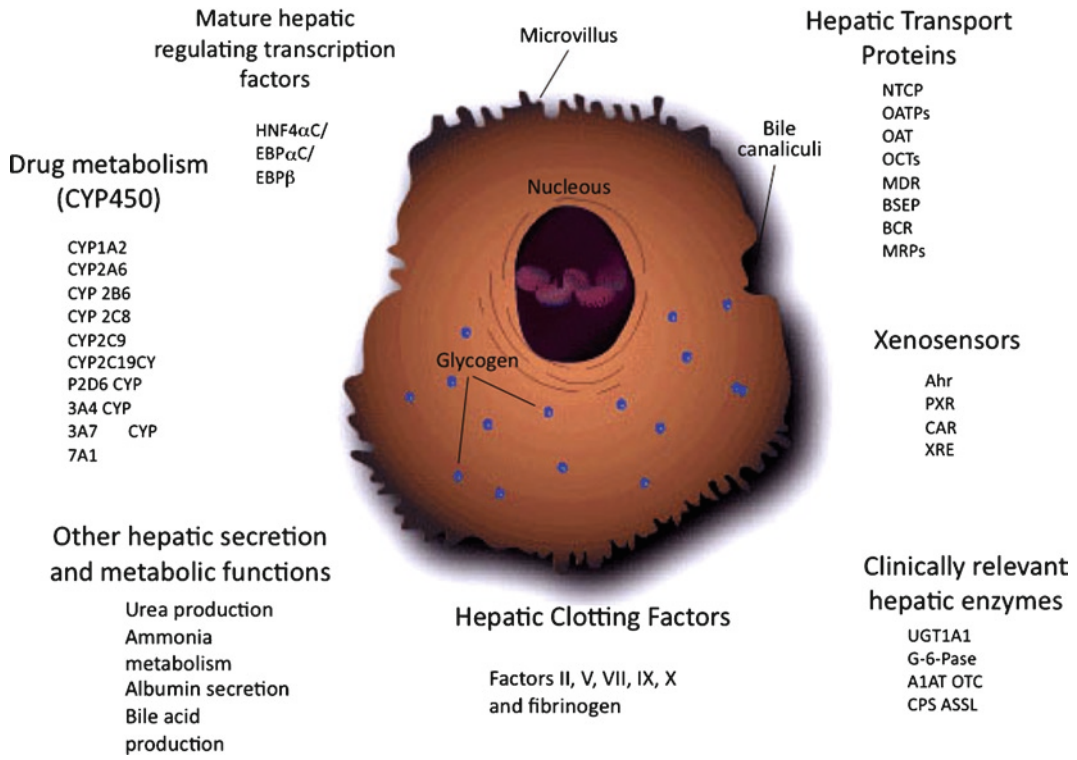

Fig. 3.1 Hepatocyte characterization

it is possible to unequivocally define whether a candidate cell is a hepatocyte or not. Thus, the definition of hepatocyte should include qualitative studies where the presence/absence of hepatocyte markers are demonstrated together with an enzymatic activity evaluation.

With the well known knowledge on the biology and usage of primary human hepatocytes [27], it seems reasonable to introduce additional criteria to define if a cell is a true hepatocyte or only shares several characteristics with a hepatocyte. In this perspective, it is also important to understand what properties a hepatocyte or the substitute hepatocyte-like cell should have. The resulting hepatocyte-like cells should be compared with human fetal and mature human liver, and we should define endpoints to measure in stem cell-derived hepatocytes, the level of hepatic maturation. Finally, we need fast and easy tests that provide relevant and robust information of the hepatic capacities of the produced stem cell-derived hepatocytes.

From a functional point of view, any candidate hepatocyte-like cell type should represent a minimal set of hepatic functions of a true hepatocyte. Here, we present a battery of relevant studies for the analysis of enzyme activities of stem cell-derived hepatocytes: (a) Analysis of expression of genes identified in mature livers, (b) metabolism of xenobiotics and endogenous substances (hormones and ammonia); (c) synthesis and secretion of albumin, clotting factors, complement, transporter proteins, bile, lipids, and lipoproteins; and (d) storage of glucose (glycogen), fat soluble vitamins A, D, E, and K, folate, vitamin B12, copper, and iron. Finally, a convincing in vivo experiment to prove hepatocellular differentiation is to restore liver function in animal models by means of repopulation assays. However, any repopulation experiment may only evaluate that a certain hepatic cell type has the capacity to generate hepatocytes in vivo. Thus, testing a defined battery of activities and comparing them with primary hepatocytes remains the only feasible option for evaluating the in vitro potential of stem cell-derived hepatocyte cultures as appropriate surrogates for primary human hepatocytes (Fig. 3.1).

The entire hepatic drug-metabolizing enzyme system in an integrated form provides an in vitro model that is a very useful tool for anticipating drug metabolism and drug hepatotoxicity in man. Cytochrome P450s (CYPs) are mixed function monooxygenases and the major enzymes in phase I metabolism of xenobiotics. Depending on the nature of the xenobiotic, this oxidative metabolism results in inactivation and facilitated elimination, activation of pro-drugs, or metabolic activation [28]. Evaluation of CYP for specific measurements in hepatocytes classified as phase 1 metabolism may include CYP1A2, CYP2A6, CYP 2B6, CYP 2C8, CYP2C9, CYP2C19, CYP2D6, CYP 3A4, CYP 3A7, and CYP 7A1. The major site of CYP expression is the liver and CYP 3A4 is the most abundant CYP isoenzyme in human adult liver. The enzymes of greatest importance for drug metabolism belong to the families 1–3, responsible for 70–80% of all phase I dependent

metabolism of clinically-used drugs [29]. Studies performed in primary human hepatocytes point to the CYP 3A4 as an important marker for hepatocytes, as this enzyme is the most abundant CYP enzyme in the human liver. CYP 3A4 activity can be measured using 6-beta hydroxytestosterone. It has been reported to be quantitative, sensitive, and specific test for 3A4 [16, 29].

CYP expression and activity present large interindividual variations due to polymorphisms [30]. Moreover, CYPs can be induced several fold or inhibited by specific drugs, resulting in additional, although transient, variability of metabolic activity. Inducibility of CYPs is a mature liver function that must also be observed in useful stem cell-derived hepatocytes. CYPs are inducible by exposure to phenobarbital, rifampicin, and to a lesser extent steroid hormones [16]. The CYP2C family also represents a significant proportion of total P450s (2C9, 2C8, 2C19, and 2C18), representing about 20% of the total P450 and metabolizes many drugs [31], thus making this enzyme subfamily important to monitor. CYP1A2 is a minor enzyme in the liver and only a small number of drugs (4%) are metabolized by this enzyme [16, 31]. However, it is involved in the bioactivation of pro-carcinogens and is therefore considered to be an important enzyme to test. CYP2B6 is emerging as an important enzyme in drug-drug interactions despite a previously reported low abundance in the liver (0.2% of total P450) [32]. However, once thought to be of minor importance and uninducible in humans, CYP2B6 may actually constitute at least 5% of the total P450, contribute to the metabolism of more than 25% of all pharmaceutical-drug metabolism and exhibit high inducibility [33]. CYP3A7 is mainly expressed in fetal liver even at mid-gestation, although in rare cases, CYP3A7 mRNA has been detected in adults. CYP3A7 activity can be induced by hydroxy progesterone caproate metabolism. The CYP3A forms have demonstrated an equal or reduced metabolic capability for CYP3A5 compared with CYP3A4, and a significantly lower capability for CYP3A7. Thus, active metabolism can be detected for both CYPs 3A7/3A4. CYP7A1, cholesterol 7a-hydroxylase, is found exclusively in the liver, where it catalyzes the first step in the major pathway responsible for the synthesis of bile acids [34]. The expression of this enzyme is subject to feedback regulation by sterols and is thought to be coordinately regulated with enzymes in the cholesterol supply pathways, including the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and synthase [34] (Fig. 3.1).

Hepatic transport proteins and mainly measurement of bile acids can serve as indicators of hepatic function. However, all hepatic functions do not mature at the same rate, and some hepatic transporters are expressed early in the development and may not be exclusive for liver. There is evidence that they are expressed in the intestine, kidney, brain, and other organs [35]. Some important hepatic transport proteins can be classified as follows: (a) the solute carrier (SLC) family, comprising among others such as Na+-taurocholate co-transporting polypeptides (NTCP), organic anion-transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs); and (b) the ATP-binding cassette (ABC) transporter family, including the multi drug resistance proteins (MDR), bile salt export pump (BSEP) (both belong to the ABCB family), breast cancer resistance protein (BCRP, belonging to the ABCG or White family) and (c) the multi drug resistance associated proteins (MRPs), belonging to the ABCC family.

The basolateral NTCP transports bile acids from the space of Disse into hepatocytes. Human NTCP accepts most physiological bile acids while at the canalicular membrane the efflux of bile acids by the BSEP mediates concentrative excretion [36].

Sensors like the aryl hydrocarbon receptor (Ahr), pregnane X receptor (PXR), and the constitutive androstane receptor (CAR) are integral to the regulation and induction of the main P450s and their analysis may provide a strong evidence of the maturation state of stem cell-derived hepatocytes due to their up-regulation during liver development. These receptors control the expression of CYP1A (Ahr), CYP2, and CYP3A (PXR and CAR), families. Once activated, the receptors form heterodimers with other factors, such as Arnt (Ahr nuclear translocator) and retinoid X receptor (RXR for both PXR and CAR) and then bind to the target xenobiotic response elements (XRE) located in both the proximal and distal P450 gene promoters, resulting in the transcription of the respective CYP isoform [37].

The demonstration of expression of transcription factors regulating hepatic development and maturation is useful (HNF4α, C/EBPα, C/EBPβ), but not as useful as the CYPs for measuring maturation because they are expressed at near adult liver levels even at mid gestation.

Microarray data suggest that HNF1α binds to 222 target genes in human hepatocytes corresponding to 1.6% of the genes assayed. HNF6 bound to 227 (1.7%), and HNF4α bound to 1,575 (12%) of the genes, which means that HNF4α bound to nearly half of the active genes in hepatocytes [38] (Fig. 3.1).

The differentiated state of the hepatocytes is regulated by a coordinated interplay of hepatocyte-specific transcriptional factors, including HNF-4 and C/EBPα [38]. HNF-4 is involved in hepatocyte specific expression of serum proteins, such as albumin and transferrin, and of cytochrome P450 proteins. In primary cultures of rat hepatocytes, the expression of C/EBPα is rapidly reduced within a few days of culture, resulting in reduced hepatic functions. It has been demonstrated that the maintenance of C/EBPα, HNF-4, nuclear factor-kappa B (NFκB), and activator protein-1 (AP-1) contributed to the prolonged expression of liver-specific proteins in human hepatocyte cultures [39].

Additionally, analysis of some hepatic clotting factors (II, V, VII, IX, X, and fibrinogen), albumin production, urea production or ammonia metabolism and glycogen storage may provide additional robust evidence of an effective hepatic maturation in case the stem cell-derived hepatocytes are evaluated for their hepatic functional capacity (Fig. 3.1).

The presence of hepatic enzymes with clinical implications would be useful in the process of hepatic maturation categorization, for example UDP-glucuronosyltransferase (UGT1A1), an enzyme of the glucuronidation pathway that transforms small lipophilic molecules, such as steroids, bilirubin, hormones, and drugs, into water-soluble, excretable metabolites [40]. Another important enzyme that is present in mature hepatocytes is glucose-6-phosphatase (G-6-Pase)1, which catalyzes the hydrolysis of glucose 6-phosphate to glucose, which is the terminal step of both hepatic gluconeogenesis and glycogen breakdown [41]. In addition, alpha-1-antitrypsin (A1AT) is an example of a clinically relevant enzyme that is present in a mature stage of hepatocytes. As a member of the serpin superfamily of proteins, A1PI is a potent inhibitor of serine proteases, especially neutrophil elastase, which degrades connective tissue in the lung [42]. The A1AT gene is expressed in cells of several lineages, with expression being highest in hepatocytes.

Urea cell cycle related enzymes might be important when hepatic function of stem cell-derived hepatocytes is to be evaluated. Ornithine transcarbamylase (OTC) gene is expressed exclusively in liver and intestinal mucosa, and is located in the mitochondria and is part of the urea cycle as well as carbamyl-phosphate synthetase I (CPS) and argininosuccinate synthetase (ASSL) [43] (Fig. 3.1).

Epithelial to Mesenchymal Transitions of Hepatocytes

Successful liver repair results in replacement of damaged hepatocytes cells with healthy new epithelial cells; this process is recognized as liver regeneration. These responses differ depending on the severity and chronicity of liver injury. For example, residual mature hepatocytes and cholangiocytes proliferate to restore liver mass after acute partial hepatectomy, while liver progenitors are involved in the repair of chronically injured livers, or in special kind of injuries [44]. This repair of chronic liver injury also variably involves changes in mesenchymal cells and they may also lead to hepatic inflammation, vascular remodeling, and fibrosis, and result in hepatic architectural distortion and liver dysfunction and eventually culminate in cirrhosis [45].

By concept, epithelial cells (hepatocytes) are adherent cells that closely attach to each other, forming coherent layers in which cells exhibit apico-basal polarity as illustrated above in the hepatocyte structure section. On the other hand, mesenchymal cells are non-polarized cells, capable of moving as individual cells because they lack intercellular connections. Thus epithelial to mesenchymal transition (EMT) describes the process by which cells gradually lose typical epithelial characteristics and acquire mesenchymal traits. Moreover, mesenchymal to epithelial transition (MET) refers to the reverse process. It is important to emphasize that these transitions refer to changes in cell shape and adhesive properties.

Key epithelial features that are eventually lost during EMT include typical epithelial expression and distribution of proteins that mediate cell-cell and cell-matrix contacts, as well as the cytoskeletal organization that is responsible for normal epithelial polarity. In contrast, mesenchymal characteristics that are ultimately gained during EMT include the ability to migrate and invade the surrounding matrix. This migratory/invasive phenotype requires induction of mesenchymal filaments, cytoskeletal rearrangements, and increased production of factors that degrade ECM [46]. These alterations of EMT (or its reversal) require a carefully-orchestrated series of events that eventually lead to wide-spread changes in gene expression. This is regulated both at the level of gene transcription and via various post-transcriptional mechanisms [47].

Recent literatures have demonstrated that EMT/MET are involved in (a) embryogenesis/development, (b) wound healing/tissue regeneration/organ fibrosis, and (c) neoplasia.

The determination of whether or not EMT occurs in situ, and how significant this process might be to outcomes of liver injury (e.g., regeneration or fibrosis), is actually a very difficult task. Chronic epithelial degeneration is thought to provoke patchy epithelial-mesenchymal transitions that involve relatively small numbers of cells [48]. During culture, several resident adult liver cells appear capable of undergoing EMT and/or MET, raising the possibility that EMT/MET might be involved in liver regeneration. However, despite considerable effort to deploy state-of-the-art technology to determine if (and how) such phenotypic transitions influence the outcomes of liver injury, the issue remains controversial. The existing data that might be helpful in resolving the role of epithelial-mesenchymal transitions in adult liver repair has been derived so far from a few studies using transgenic mice, each of which likely marked distinct types of liver cells and their resultant progeny [49, 50]. Data interpretation is made further difficult by the fact that the published studies used different models of injury and examined outcomes at different time points. These studies (work in Alb-Cre/LacZ mice and GFAP-Cre/GFP mice) provide compelling in vivo evidence that EMT/MET do occur in certain types of adult liver injury, although the exact cell types that are capable of this response remain unclear. Also, when it occurs, EMT does appear to correlate with changes in hepatic matrix production/accumulation, although it has not yet been proven that the EMT-derived fibroblastic cells actually generate matrix.

There is also a growing immunohistochemical evidence for EMT/MET in various human liver diseases, including primary biliary cirrhosis, biliary atresia, alcoholic and nonalcoholic fatty liver disease [51, 52]. Further research is needed to evaluate this theory. The resulting knowledge may be important in designing novel diagnostic and therapeutic strategies to prevent, and treat chronic liver damage.

Alternative Production of Hepatocytes from Stem Cells

Liver regeneration capacity results mainly from mitotic division of mature hepatocytes, bile duct, and endothelial cells as mentioned above. But under special circumstances, hepatic progenitors called oval cells are activated to expand and take part in liver regeneration when the proliferative capacity of the mature native hepatocytes is impaired, and a stimulus of regeneration is present [14, 53]. Hepatic oval cells express markers associated with immature liver cells, such as alphafetoprotein; mature hepatocytes, such as albumin; hematopoietic stem cells such as c-kit, Thy-1, CD34, and Sca-1 [53]. These bi-potential progenitors, isolated from the liver, differentiate into mature hepatocytes after transplantation into the liver [14, 53, 54]. However, currently it is uncertain whether such cells can be maintained and expanded in vitro to be used in lieu of adult hepatocytes for clinical application; its contribution to carcinogenesis is not yet clear. Nonetheless, oval cells isolated from the adult liver represent a promising source for cell-based therapy.

Bone marrow cells are an attractive source for extrahepatic stem cells. Hepatocytes can, in fact, be generated from hematopoietic stem cells and the mechanism by which hematopoietic cells can directly affect hepatic regeneration includes both transdifferentiation and fusion of a cell of hematopoietic origin with defective hepatocytes [14, 55, 56]. Their capacity to integrate into injured livers of animals has been reported to be low and the clinical potential to correct liver disease remains to be determined from either bone marrow or adipose tissue mesenchymal stem cells (MSCs) [57]. Another mechanism reported to alleviate liver injury using MSCs is the secretion of soluble factors. Recent studies directed by our group have found that injection of conditioned-medium from MSCs or the use of a liver assist device can reduced hepatocytes death and increase hepatocytes replication [58]. However, the exact mechanism and the type of liver injury that can be treated by this strategy remains to be determined.

Another source of hepatocytes are fetal human liver progenitor cells, and these cells have shown enormous replication and differentiation potential, including the capacity to generate mature hepatocytes after transplantation in immunodeficient animals, [59] mainly due to the extended reconstitution of telomerase activity which maintains chromosomal integrity during cell division even after cryopreservation [60]. Oertel et al. have used fetal rat liver cells to show that at least three distinct subpopulations of hepatoblasts exist between embryonic days 12–14. Based on histochemical markers, one population appeared to be bipotential and the other two harbored either unipotent hepatocytes, or biliary epithelial cell phenotypes [61, 62]. The same group has reported that a phenomenon known as cell competition is responsible for the liver repopulation where the transplanted fetal liver cells are capable of repopulating the liver by inducing apoptosis in neighboring host hepatocytes that proliferate more slowly than the transplanted cells [61]. So that if the engrafted hepatocytes possess a greater proliferative capacity than the host hepatocytes, the engrafted cells would grow preferentially in response to mitotic stimuli, progressively competing out the host hepatocytes [61]. Nevertheless, the supply of fetal human tissues is also limited and the possibility of oncogenic perturbations needs further study. Moreover, it will be important to determine whether cells derived from fetal livers before 20 weeks of gestational age, when most elective abortions are performed, would express differentiated hepatocellular function after transplantation [63]. Clinical transplantation of fetal hepatocytes in patients with acute liver failure has been documented, but resulted in modest clinical decrease in serum bilirubin [64, 65]. Further studies need to be performed to corroborate clinical significance.

At this point, most published embryonic stem cells-differentiation protocols that generate hepatocyte-like cells have reported in general a limited functionality and not complete maturity. Major liver repopulation has been difficult to document. However, it has been reported that by selecting for cells expressing markers such as the asialoglycoprotein receptor, it is possible to isolate the most hepatocyte-like cells in the culture with repopulating capacity [66–68]. It appears likely that conditions and protocols will be developed for the practical production of embryonic stem cell-derived hepatocytes. Nevertheless, major advances have been difficult to achieve. The development of induced pluripotent stem (iPS) cells from adult somatic tissue [69] may provide major advantages on the development of hepatocyte-like cells. Confirmation that iPS cells have hepatocyte-lineage differentiation capacity comparable to that of existing differentiated embryonic stem cells needs to be studied. Nevertheless, iPS-derived hepatocytes are a very promising population for future therapeutic transplantation.

Hepatocyte Cell Lines

Whether originated from tumors or obtained by oncogenic immortalization, human hepatoma cell lines lack a substantial

set of liver-specific functions, especially many CYP-related enzyme activities. Most hepatocyte cell lines do not express the major metabolizing enzyme activities; they only exhibit some enzyme activities depending on the culture conditions and the source of the cells [16]. HepG2 and other cell lines stably integrated with specific P450 isoforms have been shown to be responsive to CYP inducers, but are of limited value for induction assays due to their lack of liver-specific functions [70]. Reexpression of CYPs has also been obtained by transfection of plasmid constructs expressing liver-specific transcription factors such as c/EBP-alpha, but similarly they do not mimic regulation of gene expression observed in normal hepatocytes [16].

Conclusions and Future Perspectives

Primary hepatocytes continue to be the most relevant in vitro model for advancing our knowledge of liver functions, the mechanisms underlying the regulation and drug induced changes of metabolic enzyme expression, hepatocellular integrity, liver cell-based therapy, and hepatic regeneration.

The value of hepatocytes has been enhanced further in recent years by technical improvements in cell handling conditions (cryo-preservation, cell culture and liver repopulation techniques), in the methods available to explore effects of xenobiotics on critical functions and in our understanding of the key role played by xenosensors and drug discovery.

The use of adenoviruses and transfected cell lines has increased our knowledge of cellular mechanisms, but these cannot yet replace primary hepatocytes for prediction of in vivo drug-drug interactions or hepatotoxicity. Likewise, hepatocyte-like cells are promising, but have a long way to go before they can be considered an alternative to primary hepatocytes. Although there is no complete consensus on the roles of hepatic stem cells in adult mammals, recent findings support a view in which mature-differentiated epithelial liver cells and facultative stem cells mediate liver maintenance and growth. Thus, processes such as normal liver turnover, regeneration after injury, or repopulation following transplantation are mediated by either cell type, depending on the precise circumstances. Oval cells are not a homogeneous well-defined cell population, but represent a complex mixture of different cell types, all of which are activated during progenitor-dependent regeneration. These cells are likely bipotential in that they can produce hepatocytes and bile duct epithelium. Multiple studies support the concept that liver stem cells reside within the biliary tree and are a subset of ductal cells. Extrahepatic cells (ES/iPS cells) also have the potential to become liver epithelium; although their role in liver physiology remains uncertain, they might be developed for use in therapeutic cell transplantation and important drug discovery studies.

For further progress, it will be important to clearly define activities that closely resemble those of primary hepatocytes and, even more importantly, others that are not hepatocyte-like. Recapitulating the above mentioned, gene and function of stem cell-derived hepatocytes must be compared to human fetal or adult liver; mature hepatic characteristics should be demonstrated using drug metabolism detoxification in a gene expression and functional level; additional characterization can be provided by analyzing hepatic transport proteins, mature hepatic transcription factors, and factors related to homeostasis, albumin secretion, production of bile acids, bilirubin conjugation assays, ammonia metabolism with the expression of related enzymes, and finally, the analysis of mature liver gene expression and function in animal models of liver failure after transplantation to the liver or ectopic sites. In addition, a clear definition of non-hepatocyte–like factors is important to identify mechanisms responsible for the lack of activity. Clarification of such mechanisms, for instance, loss of transcription factor expression or modification of signal transducers, is a requirement for further progress. It may be extremely difficult to differentiate stem cells to a cell type that resembles primary hepatocytes in all aspects of drug metabolism. However, promising results have been obtained with extra-hepatic stem cells since some previously silent hepatocyte markers become expressed during differentiation, and metabolic activities start appearing after new protocols have been reported.

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Chapter 4
Biliary Epithelial Cells

Yoshiaki Mizuguchi, Susan Specht, Kumiko Isse, John G. Lunz III, and Anthony J. Demetris

Introduction

Biliary epithelial cells (BEC), or cholangiocytes, line a complex tree-like 3-dimensional network of conduits within the liver that form the biliary tract. The biliary tree can be divided into extrahepatic and intrahepatic components, and receives its blood supply exclusively from hepatic artery branches (Fig. 4.1) [1]. The extrahepatic biliary tract is composed of the gallbladder, common hepatic duct, common bile duct, and cystic duct [2]. The intrahepatic biliary tract contains the bile canaliculi, the canals of Hering (or intrahepatic bile ductules), interlobular bile ducts, intrahepatic bile ducts, and the left and right hepatic bile ducts [1].

BEC comprise only 3–5% of total liver cells (hepatocytes comprise 60% and account for 78% of the liver volume), but they are essential to the formation of bile components in the liver and effective transport of bile into the duodenum. Hepatocytes transport and secrete bile acids and other organic solutes (primary or hepatic bile) into the canalicular space between hepatocytes. The bile, in turn, is transported via the canalicular network to the smallest biliary radicals, or cholangioles, which are the first structures to be lined by typical BEC. After passing through a series of progressively larger BEC-lined channels, the bile is alkalinized and diluted by BEC via a series of secretory and absorptive processes [3]. Despite the comparatively small number of BEC, their secretions account for up to 40% of bile volume in humans [4].

BEC display functional heterogeneity along the biliary tree. For example, those lining large bile ducts participate in mucin secretion and hormone-regulated bile secretion, whereas BEC lining small bile ducts possess proliferative capabilities and a subpopulation display considerable plasticity, being able to assume a "reactive or reparative phenotype" in disease conditions [5, 6]. BEC are quiescent, or reside in the G_0 state of the cell cycle under normal conditions. However, they actively participate in reactive and reparative responses to various pathological stimuli during disease states [7, 8]. BEC lining the large bile ducts respond to direct injury, whereas BEC lining the smallest biliary radicals also participate in repair response involving hepatocytes and after vascular injury.

Cholangiopathies, or diseases that directly involve bile ducts, are categorized according to the underlying cause(s):

1. Immune disorders (discussed in Chap. 49)
   (a) primary biliary cirrhosis (PBC)/autoimmune cholangitis
   (b) primary sclerosing cholangitis (PSC) (discussed in Chap. 50)
   (c) acute and chronic allograft rejection
   (d) graft-versus-host disease (GVHD)
   (e) cholangiolytic adverse drug reactions (see below)
2. Bacterial, fungal, parasitic, and viral infections
3. Vascular/ischemic damage
   (a) intra-arterial chemo-ablative therapy
   (b) post-transplant hepatic artery stenosis/thrombosis
   (c) chronic liver transplant rejection
4. Adverse drug reactions or toxicity
5. Genetic disorders
   (a) Cystic fibrosis
   (b) Alagille syndrome
   (c) (Fibro)polycystic disease
6. Idiopathic diseases
   (a) biliary atresia
   (b) Sarcoidosis, idiopathic adulthood ductopenia
7. Neoplastic diseases: Benign and malignant (e.g. biliary cystadenoma, cholangiocarcinoma, and bile duct cancers [9] (discussed in Chap. 60)).

Cholangiopathies show a clear predilection for specialized regions of the biliary tree probably because of the heterogeneity of the BEC. For example, PBC mainly involves the small

A.J. Demetris(✉)
Department of Pathology, Thomas E. Starzl Transplantation Institute, Pittsburgh, PA, USA
e-mail: demetrisaj@upmc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_4, © Springer Science+Business Media, LLC 2011

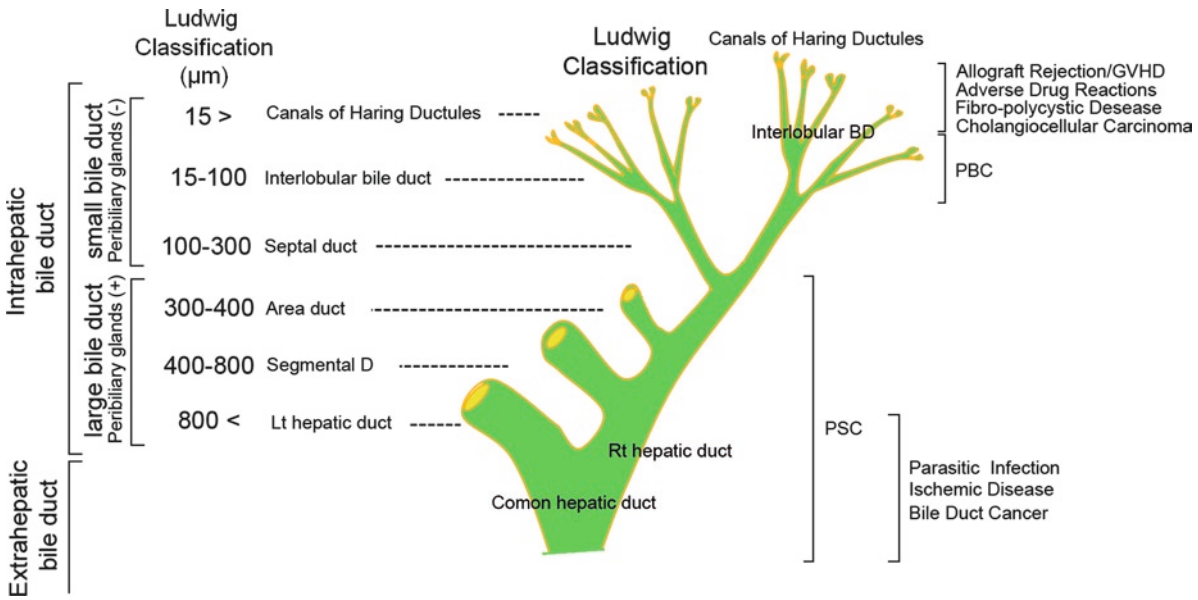

Fig. 4.1 Illustration of the biliary tree and nomenclature according to Ludwig classification, which is primarily based on the size of the bile ducts. The classification has practical utility because cholangiopathies show a clear predilection for specialized sections of the biliary tree

interlobular bile ducts, where secretin-stimulated secretion is more active. In comparison, drug-induced ductopenia is largely restricted to small cholangioles, indicating a difference between major ducts and cholangioles in terms of xenobiotic transport and metabolism [10]. Ischemic injury, parasitic infection, and choledocholithiasis primarily involve the larger bile ducts.

Despite disease heterogeneity, BEC in all cholangiopathies engage in pathophysiologic responses that are common to many studies of injury and repair. Included are the coexistence of BEC death (lytic or apoptotic), proliferation, inflammation and fibrosis, and qualitative/quantitative changes in bile production (cholestasis) [11]. During disease processes, BEC interact with various inflammatory mediators, cytokines and chemokines, products of cell death, and nearby parenchymal and stromal cells, and immune cells. Included are BEC apoptosis and proliferation [12], cell migration, fibrogenesis [13], damage to the peribiliary circulation, histocompatibility antigen expression [14], and alterations in biliary epithelium transport functions.

BEC lining the smallest cholangioles also participate in repair responses that primarily involve hepatocellular injury. This is probably related to the known plasticity of cells lining the smallest cholangioles and periportal hepatocytes: these BEC can trans-differentiate into hepatocytes and periportal hepatocytes can trans-differentiate into BEC [15–17]. In fact, BEC play a crucial role in the development of cirrhosis from most causes via: (1) their increased resistance to injury, compared to hepatocytes; (2) interactions with portal myofibroblasts and classical stellate cells; and (3) participation in "ductular reactions," which distort the hepatic architecture during the development of chronic necro-inflammatory liver disease (reviewed in [18]) (Fig.4.2). In addition, bi-potential progenitor cells or "liver stem cells" are thought to reside in the terminal cholangioles and/or periportal hepatocyte populations [19, 20] (Fig. 4.2). Although control mechanisms for hepatic progenitor cell activation and differentiation are only partially understood, Wnt/β(beta)-catenin [21, 22] and Notch/Jagged pathways [23], and a transcription factor – hepatocyte nuclear factor-6 (HNF-6) [24] have been implicated in hepatic stem/progenitor cell activation. Activation of these cells during disease states is thought to increase the risk of neoplastic transformation through initiation of progenitor cell populations [20, 25, 26] (Fig. 4.2).

There has been a rapid expansion of research aimed at elucidating key signaling pathways that regulate BEC development, differentiation, proliferation, survival, and function, as well as cellular and molecular mechanisms of cholangiocarcinogenesis and progression. This chapter addresses biliary epithelial cell (patho-) physiology by focusing on signaling pathways and mechanisms involved in BEC injury and response to injury.

Anion Transport and pHi Maintenance

Formation of bile and maintenanice of intracellular pH (pHi) are essential BEC functions that contribute to systemic homeostasis, including clearance of xenobiotics, enterohepatic circulation of bile salts, and intestinal absorption of lipids. These processes, in turn, significantly contribute to bile flow.

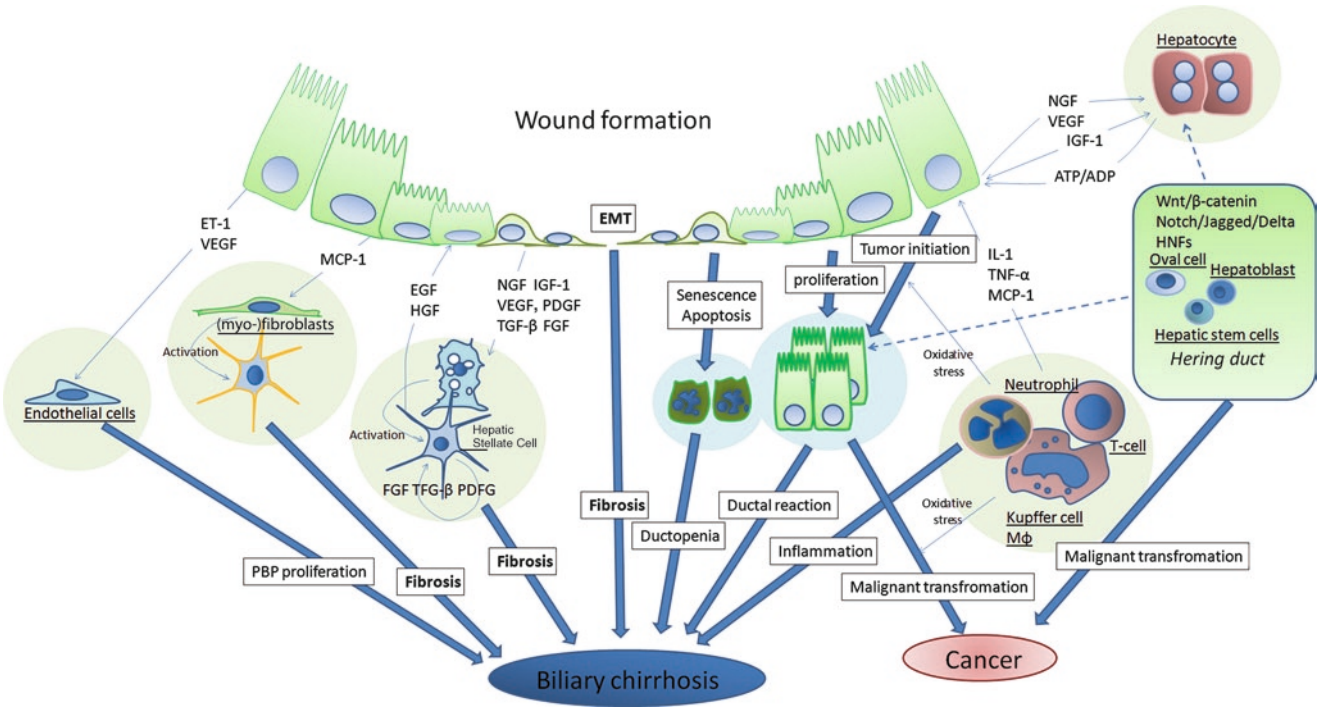

Fig. 4.2 BEC respond to injury by synthesizing and secreting a variety of mediators that enable BEC to interact with other nearby liver cells, such as hepatic stellate cells, portal (myo-) fibroblasts and inflammatory cells during cholangiopathies. Hepatic stem or progenitor cells that are thought to reside in or near terminal cholangioles can be activated during progression of cholangiopathies and differentiate into either hepatocyte or BEC; they also probably play a critical role in hepatic and biliary carcinogenesis

Regulation of pHi is mediated by specific acid/base carriers closely tied to a variety of cellular processes ranging from cell volume regulation to cell mitosis.

Mechanism Involved in Anion Transport and pHi Maintenance

Mechanisms involved in anion transport are depicted schematically in Fig. 4.3. In human BEC, HCO_3^- loading is accomplished by the Na^+/HCO_3^- co-transporter (NBCe2/NBC4/SLC4A5) [27] located on the apical BEC membrane, whereas the Na^+ dependent Cl^-/HCO_3^- exchanger (NDCBE/SLC4A8) [28] is found at the basolateral surface [29–32]. Bicarbonate influx occurs mainly through the exchanger, with the Na^+/HCO_3^- cotransporter being activated only at very low pHi conditions [32, 33]. HCO_3^- is also produced via carbonic anhydrase (CA), which is coupled to a carrier-mediated (NHE) H^+ extrusion [34, 35]. In mammalian liver, CA is mainly expressed in the BEC cytoplasm, but not hepatocytes, and catalyzes the hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ [34]. Acid extrusion is mediated by the Na^+/H^+ exchanger (NHE) in both rat and human BEC (pig BEC have cAMP-activated H^+-ATPase [36]).

Both hepatocytes and BEC appear to have cAMP-responsive intracellular vesicles in which a Cl^-/HCO_3^- exchanger co-localizes with cell-specific Cl^- channels (cystic fibrosis transmembrane conductance regulator, CFTR) and aquaporin-1 [37–39]. cAMP-induced coordinated trafficking of these vesicles to either hepatocanalicular or BEC luminal membranes, and extracellular exocytosis results in increased osmotic forces and passive movement of water with net bicarbonate-rich hydrocholeresis. Bicarbonate secreted via the Cl^-/HCO_3^- exchanger is mediated by AE2/SLC4A2, the main contributor to biliary bicarbonate secretion [30, 40, 41]. This exchange is Na^+-independent and electroneutral. The exchange is facilitated by an outside to inside transmembrane gradient of Cl^- at relatively high intracellular concentrations of HCO_3^-, particularly upon secretin stimulation, and involves chloride efflux through activation of CFTR [30, 42–45].

Secretin, a hormone that interacts with specific G protein-coupled receptors, causes an increase in intracellular cAMP, which in turn, activates PKA and Cl^-/HCO_3^- BEC secretory mechanisms. Besides acid/base transporters, BEC also possess other ion carriers for Cl^-, Na^+ and K^+ that might ultimately lead to biliary excretion of bicarbonate via their exchange with luminal chloride. This exchange is facilitated by relatively low intracellular concentrations of chloride and

high concentrations of bicarbonate. In addition to CFTR, Cl^- also enters the lumen via a dense population of Ca^{2+}/calmodulin dependent Cl^- channels, which are activated through purinergic-2 (P2) receptors and nucleotide (ATP/UTP) binding [39, 46–49]. Cl^- might also enter through a high conductance, G-protein-regulated, Ca^{2+}/cAMP- independent Cl^- channel [50]. The role of these non-CFTR pathways still needs to be clarified, but they are more prominent in single BEC [50]. The basolateral $Na^+/K^+/2Cl^-$ co-transporter NKCC1/SLC12A2 participates in uptake of Cl^- to maintain high intracellular Cl^- concentrations that facilitate apical Cl^- excretion by the Cl^-/HCO_3^- exchanger [46, 51]. BEC possess a basolateral Na^+/K^+ ATPase [52–54] to maintain the cation gradient because NKCC1/ SLC12A2 influxes Cl^- together with Na^+ and K^+.

BEC also express a K^+ channel transporter, SK2/KCNN2, to prevent excess K^+ accumulation [50, 55]. The activity of SK2/KCNN2 is regulated by intracellular Ca^{2+} and cAMP. Activated basolateral K^+ conductance results in hyperpolarization of the BEC [56], facilitating the entrance of Cl^- into the cAMP- and/or Ca^{2+} activated Cl^- channel. This K^+ conductance seems likely to result from another not yet identified K^+ channel [50].

Aquaporins-1 and 4 (AQP1 and 4) are water channels that mediate a bidirectional passive movement of water molecules across BEC in response to osmotic gradients and contribute to ductal bile flow. As mentioned above, AQP-1 is located in intracellular vesicles, and co-localizes with both AE2/SLC4A2 and CFTR, which redistribute to the apical membrane upon secretin/cAMP/PKA stimulation [39].

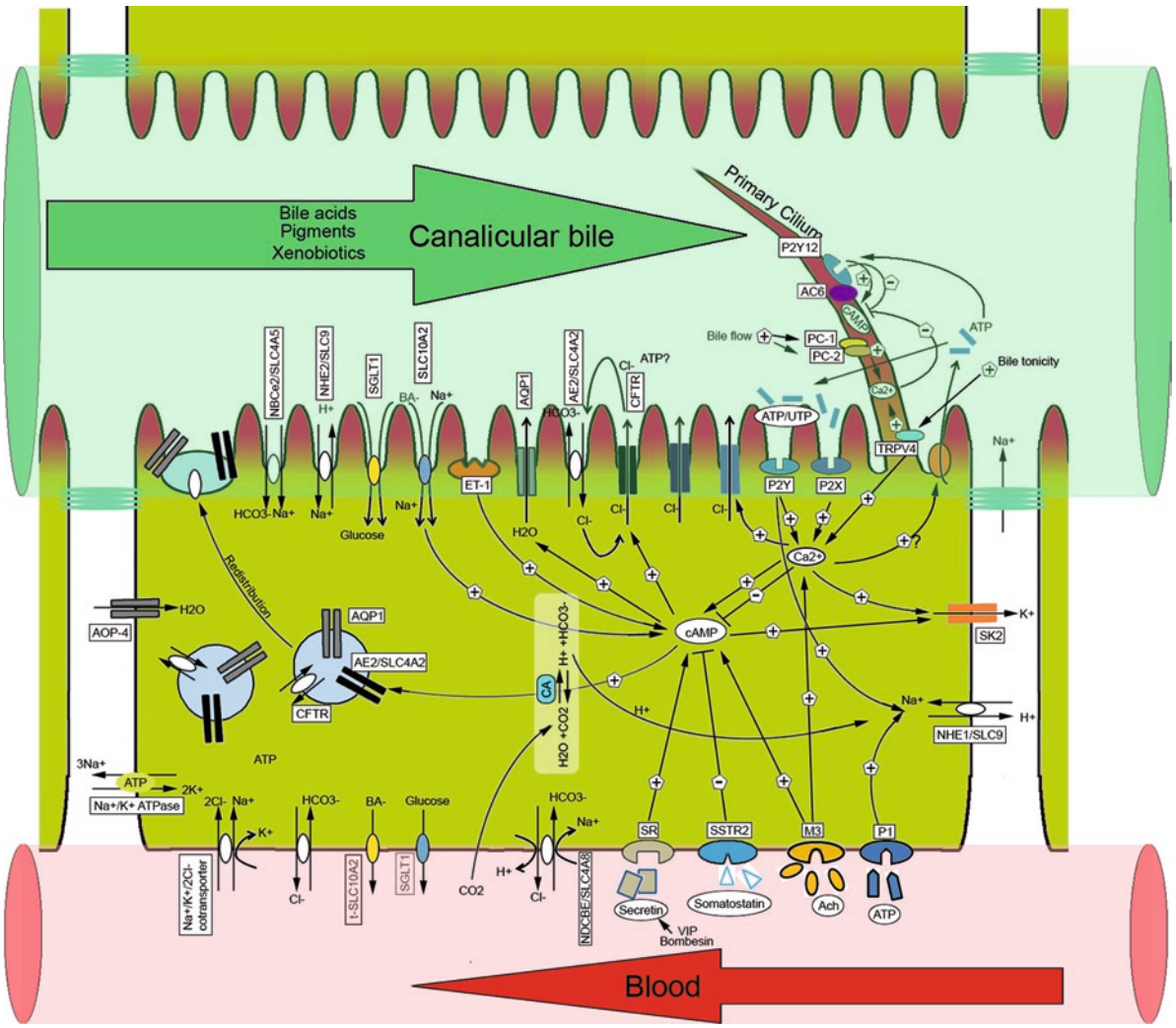

Fig. 4.3 Factors involved in regulation of ion transfer, pHi, and bicarbonate secretion are shown schematically in this figure and described in greater detail in the text.

AQP-4 is another water channel at the basolateral membrane of BEC [57, 58].

Conjugated bile acids [59, 60] are important constituents of bile produced by hepatocytes, but recycled by BEC. They are reabsorbed via an apical sodium-dependent bile acid transporter (SLC10A2, ASBT/ISBT) [59, 61, 62] and are exported through the basolateral membrane into the circulation via a truncated form of the transporter (t-SLC10A2) [63]. SLC10A2 is especially important during cholestasis. In rats, pretreatment of secretin activates SLC10A2 activity and is associated with the cholehepatic shunt pathway.

Regulatory Factors

Hormones

Gastrointestinal hormones and neuropeptides such as secretin, bombesin, vasoactive intestinal peptide (VIP), endothelin-1 (ET1), somatostatin, and gastrin, modulate ductular bile salt-independent flow [64]. These hormones interact with specific G protein-coupled receptors, causing increases in intracellular levels of cAMP and activation of cAMP-dependent Cl^- and HCO_3^- secretory mechanisms.

Secretin is produced mainly by S-cells of the duodenum and jejunum, released into the blood, and plays a pivotal role in the induction of bicarbonate-rich hydrocholeresis in many species [65]. Secretin exerts its physiological actions through its specific receptor SCTR, which is exclusively expressed at the basolateral membrane of BEC [66, 67]. Activation of SCTR stimulates AC/PKA [30, 42, 45, 68, 69] and canalicular 'bile salt-independent flow' [70–72]. Many other hormones contribute to secretin's effects. For example, vasoactive intestinal peptide (VIP), a member of the secretin family of proteins, is produced in neurons of the upper intestine and increases secretin-stimulated bile flow and bicarbonate excretion in humans [65, 73]. The neuropeptide bombesin can increase the release of secretin in dogs [74, 75]. Both these proteins also have pathways that are independent of secretin [76–78].

Somatostatin, produced in pancreatic islet D-cells, and in the stomach and duodenum, binds to its receptor SSTR2 and inhibits the formation of cAMP through AC [79, 80], which in turn, decreases secretin-induced secretion. Gastrin, originating from the stomach and G-cells of the upper intestine, inhibits the effects of secretin by reducing both SCTR expression and cAMP levels [81]. Binding of insulin-like growth factor 1 (IGF1) to its receptor (IGF1-R) was reported to inhibit secretin-induced secretion in bile duct ligated (BDL) rats through activation of PKC and inactivation of secretin-stimulated cAMP/PKA [30, 33].

Other Factors

Acetylcholine transmits parasympathetic signals and stimulates BEC secretion via the M3 muscarinic receptor, which increases cytosolic Ca^{2+} and stimulates cAMP formation via calcineurin [82, 83]. The D2 dopaminergic signal inhibits secretion by activation of PKC-γ(gamma) [84]. And α(alpha)1-adrenergic signals stimulate secretion by activation of AC [85]. There is accumulating evidence that release of ATP into the bile ducts is the final common pathway controlling ductal bile formation, and CFTR is involved in cAMP secretion [86, 87].

The P1 and P2 family receptors might also contribute to control of BEC secretion and anion export [88–93]. The P1 receptor is present on the basolateral membrane and stimulates basolateral NHE activity. The P2Y receptor on the apical membrane binds extracellular ATP found in the bile and activates apical Ca^{2+} dependent Cl^- channels and basolateral NHE that, in the presence of cAMP, stimulates apical AE2/SLC4A2 and bile secretion [91, 94].

BEC Primary Cilium

BEC are the only epithelial cells within the liver that contain primary cilia consisting of a 9+0 pattern microtubule-based axoneme [95]. The cilia extend from the apical plasma membrane into the bile duct lumen enabling detection of changes in bile flow, composition, and osmolality (Fig. 4.3). The physiological implications of BEC cilia are being elucidated.

Bending of BEC cilia by luminal fluid flow induces an influx of extracellular Ca^{2+}, through the functional complex of PC-1 and PC-2, which in turn suppresses cAMP by inhibiting adenylyl cyclase 6 (AC6) [96]. A recent study demonstrated that an osmosensor protein TRPV4 is expressed in rat BEC primary cilia, and is activated with hypotonic changes of bile osmolality [97]. Induction of TRPV4 increases intracellular Ca^{2+}, which in turn, affects ATP released into luminal fluid through unknown mechanisms.

Chemosensation by BEC cilia occurs with the involvement of $P2Y_{12}$, a receptor that is activated by biliary nucleotides (ATP/ADP) causing changes in intracellular cAMP levels [98]. Moreover, mutations in genes encoding ciliary associated proteins causes human congenital cholangiopathies including: (1) Autosomal Dominant Polycystic Kidney Disease (ADPKD) caused by mutations in either *PKD1* or *PKD2*, genes that encode PC-1 and PC-2, respectively [99] and (2) Autosomal Recessive PKD (ARPKD) caused by mutations in a single gene, *PKHD1*, that encodes fibrocystin [100]. This disease association suggests the importance of BEC primary cilia in bile formation and maintenance of BEC homeostasis.

Immune System and Cell Defenses

The immune system plays a critical role in maintaining biliary tract homeostasis and BEC participate in both innate and adaptive immune responses involving the biliary tree. For example, bile is actively involved in the transport of immunoglobulins to the intestine, and BEC's secretion of chemokines, cytokines, and expression of cell adhesion molecules serves to localize and help coordinate various immune responses occurring on, or in the vicinity of BEC. Evidence suggests that BECs may also function as professional antigen-presenting cells (APC) and, in the process, contribute to the modulation of inflammatory reactions. Finally, BECs are also targeted for injury and/or destruction by the immune system in several cholangiopathies (e.g., PBC).

Mechanism Involved in BEC Immune Regulation and Defense

Figure 4.4 shows some of the many possible interactions between BEC and immune cells in the liver. Under normal physiological conditions, the liver contains cells of immune origin and function including dendritic cells, mast cells, natural killer (NK) cells, stellate cells, and T and B lymphocytes, located primarily in the portal space [13]. Liver T lymphocytes contain conventional α(alpha)β(beta) T cell receptor (TCR)$^+$ subpopulations, as well as γ(gamma)δ(delta) TCR$^+$, NKT, and conventional NK lymphocytes (CD41, CD81, and double-negative cells); they altogether account for approximately 25% T cells and 40% NK cells of all hepatic lymphocytes, respectively [101]. Kupffer cells are predominantly distributed on the luminal surface of hepatic sinusoidal endothelial cells. They possess macrophage activities and play an essential role not only in host defense, but also in homeostatic responses [102].

BEC normally are in a quiescent state of the cell cycle [18] and lie on a basement membrane whose matrix is composed of laminin, collagen, and other glycoconjugates. Some basement membrane constituents (e.g. laminin) are produced by the BEC themselves, whereas others are produced by surrounding stromal cells. BEC normally express receptors for anchoring to basement membrane constituents [103]. Alterations of the BEC microenvironment leads to the release of inflammatory mediators, growth factors, and neurotransmitters. Lymphocyte trafficking is typically regulated by membrane glycoprotein adhesion molecules that mediate cell-cell or cell-matrix interactions.

Antigen Presenting Cell Capabilities

So-called "professional" antigen-presenting cells (APCs) present antigens via associations with class I or II major histocompatibility complex proteins (MHC) (or human leukocyte antigen [HLA]) in combination with a variety of co-stimulatory molecules to CD8$^+$ and CD4$^+$ T cells, respectively. In the liver, all cells, including BEC, express at least some level of MHC class I proteins. Class II antigens are found only on endothelial cells, capillary endothelium, and dendritic cells located in the portal, perivenular, and subcapsular areas. BEC under normal conditions are HLA class II negative [14, 104]. Studies on cultured BECs have shown that cytokines like IFN-γ(gamma) and IL-1 can induce the expression of HLA class II, which probably accounts for the upregulation observed in vivo during various cholangiopathies [14, 105, 106]. Intra-peritoneal injection of IL-2 in mice induces HLA class II expression on BEC and lymphocytic infiltration around bile ducts, which appears to be mediated through the induction of endogenous interferon-γ(gamma) [107]. During liver allograft rejection, HLA class II-specific lymphocytes infiltrate the allograft and play a role in the destruction of the biliary epithelium [108]. In addition, PBC patients express increased HLA class II on injured BEC [109].

The role of MHC class II-expressing BEC in inflammatory cholangiopathies is not entirely clear, but evidence suggests that it might enable BEC to function as APCs. However, for sufficient activation and proliferation of naïve T cells by APCs, co-stimulatory molecules such as B7–1(CD80) and B7–2 (CD86) are required along with MHC class II [110]. Leon et al. reported that cultured BEC lacked CD80 or CD86 molecules making them unable to induce effective responses in naïve T cells [111]. In contrast, other groups showed B7-2 expression in damaged BEC of PBC and primary sclerosing cholangitis (PSC) patients, in vivo [109, 112].

In vitro studies showed that cultured BEC expressing co-stimulatory molecules B7-1 and B7-2, were still incompetent at antigen presentation, and could not elicit effective T-cell activation [113]. These paradoxical observations might indicate that BEC present antigens in an inefficient manner to naïve T cells, resulting in specific T-cell anergy or deletion. Alternatively, the antigen presenting machinery of BEC might be effective only for eliciting recall responses in T cells already primed by conventional APC, such as dendritic cells [105, 114, 115].

Adhesion Molecules

BEC express a variety of cell surface adhesion molecules that localize and intensify immune responses (Fig. 4.4). Under in vitro basal conditions, BEC express low to moderate

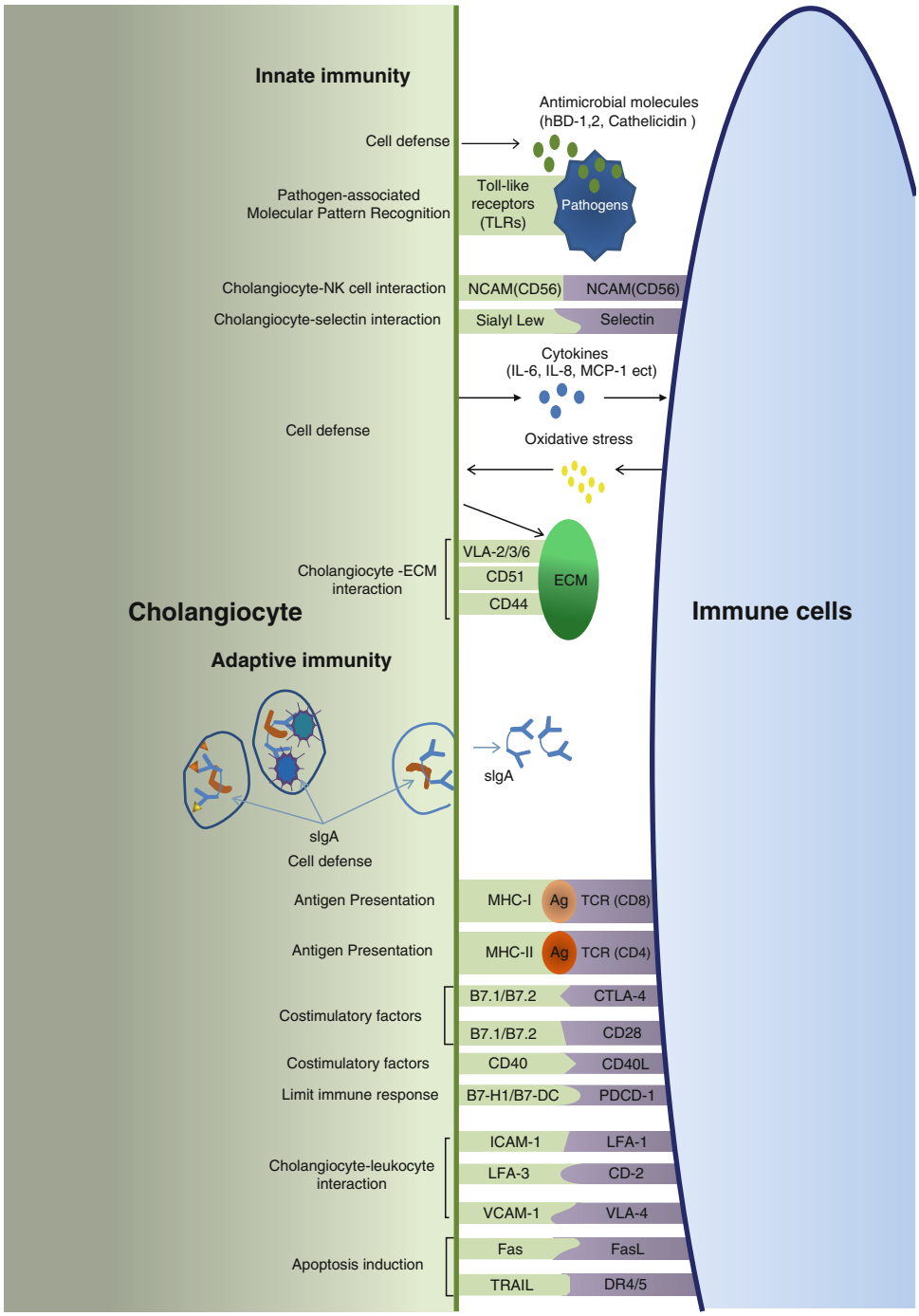

Fig. 4.4 Immune and cell defense of BEC: BEC express adhesion molecules that interact with CD4+ and CD8+ T cells. BEC express MHC class I and class II, and costimulatory factors on their surface, thus BEC can also be targets of cytotoxic injury and/or function as antigen-presenting cells (APCs). BEC also express Fas and TRAIL on their surface, making them susceptible to apoptosis. Moreover, BEC participate in innate immunity by expressing TLR that trigger intra-cellular signaling pathways when confronted with pathogens, and by producing antimicrobial molecules. BEC also produce chemokines and cytokines, which have either autocrine or paracrine effects and modulate immune reactions

levels of intercellular adhesion molecule 1 (ICAM-1), lymphocyte-associated antigen 3 (LFA-3), and MHC class I, but do not express NCAM (neural cell adhesion molecule, CD56), CD51, and MHC class II [14, 103, 116–118]. ICAM induction on endothelial cells is critical for cell-to-cell interactions and leukocyte extravasation at inflammatory sites; ICAM also binds to leukocyte function antigen 1 (LFA-1) present on T cells, neutrophils, and macrophages [119, 120]. On BECs, ICAM-1 expression facilitates binding of cytotoxic lymphocytes and facilitates cell-mediated cytotoxicity

[111]. LFA-3 on the surface of BEC also aids cell-mediated cytotoxicity through interactions with CD2 molecules expressed on cytotoxic T lymphocytes and NK cells [121]. BEC also actively participate in inflammatory reactions and upregulate NCAM (CD56) and CD51 expression in the presence of inflammatory cytokines. NCAM interacts with receptors on NK cells and CD51 assists in binding by recognizing the specific (RGD) sequences in the extracellular matrix (ECM) [103]. A significant increase of ICAM-1, HLA class 1, and HLA class II expression in BEC from normal and PBC liver becomes evident after stimulation with pro-inflammatory cytokines, such as TNF-α(alpha), IFN-γ(gamma), and IL-1 in vitro [14]. Conversely, transforming growth factor β(beta) markedly decreased ICAM expression, whereas it increased the LFA-3 expression [122].

T cells can also be activated by an alternative mechanism that involves CD40 expressed on BEC [122]. Moreover, the CD40/CD40L and the LFA-2/LFA-3 systems trigger production of IL-12, which plays an important role in the cytotoxic response of BEC. CD40 and CD40L expression in BEC are increased, respectively, by stimulation with IFN-γ(gamma) and activation of LFA-2 on T cells [122].

Secretion in Response to Cytokines and Chemokines

Innate immune defenses of the biliary tree are largely coordinated by BECs. By secreting chemokines and cytokines, BECs promote recruitment and activation of circulating leukocytes, resulting in their migration across the portal vein and portal capillary endothelium [12, 112]. Acute cholangitis, biliary obstruction, and other causes of pathological inflammation that target the biliary epithelium, trigger production of these mediators that regulate protective responses against pathogens present in the bile. For example, human BEC constitutively express IL-8 and monocyte chemotactic protein-1 (MCP-1) [123, 124], which are important chemotaxins for neutrophils, monocytes, and T cells. And IL-1 and TNF-α(alpha) increase IL-8 and MCP-1 expression, whereas IFN-γ(gamma) inhibits IL-8 production and upregulates MCP-1 [123]. MCP-1, but not IL-8, is also upregulated by lipopolysaccharide (LPS) stimulation of toll like receptor 4 (TLR-4) and subsequent NF-κ(kappa)B signaling in the absence of inflammatory cytokines [124].

Damaged BEC express TNFα(alpha) and IL-6 and their receptors (TNF-R and IL-6 α(alpha)-chain) [125], and IL-6 expression is upregulated by IL-1 or LPS stimulation [124, 126]. The IL-6 production induced during BEC damage aids in proliferation and biliary wound healing [126, 127]. BEC TNF-α(alpha) and IL-6 production is also involved in recruitment and stimulation of immune cells for the following reasons: IL-6 promotes terminal differentiation in certain T cell subsets and immunoglobulin secretion of B cells; TNF-α(alpha) increases cytotoxic activities of T cells; and TNF-α(alpha) induces the expression of adhesion molecules and HLA antigens on BEC. Thus BEC participate in innate responses as well as antigen specific adaptive immune responses. Furthermore, BEC IL-6 and TNF-α(alpha) production can also contribute to BEC apoptosis and nearby inflammation probably through autocrine mechanisms [128].

Stress, Apoptosis and Senescence-Related Changes

So-called, "vanishing bile duct syndromes" (a reduction in the number of bile ducts, or ductopenia) can be the end result of several immune-mediated liver diseases, such as chronic allograft rejection, cholangitic adverse drug reactions, PBC, PSC, and GVHD. In these disorders, BEC loss prevails over proliferation and apoptosis is the major process by which BEC die [129]. Although BEC are capable of antigen presentation useful for clearance of pathogens and preventing unwarranted infections, BEC are also targeted for injury or death/apoptosis by the immune responses.

Fas receptor/Fas ligand is the most thoroughly characterized BEC apoptotic pathway. BEC are sensitive to Fas-mediated apoptosis, and Fas expression is upregulated by IFN-γ(gamma), TNF-α(alpha), IL-4, and CD40 [122, 125, 130–133]. TNF-α(alpha) produced by inflammatory cells in the portal tract during inflammation can induce BEC apoptosis through TNF-R1 receptor followed by caspase 3 activation [134]. And BEC TNF-α(alpha) receptor expression is upregulated in PBC and PSC patients [125].

CD40 is a member of the TNF receptor superfamily, and CD40 ligand (CD40L) is upregulated on leukocytes from PBC patients [133]. Moreover, CD40 increases BEC expression of Fas/FasL and transcription of NF-κ(kappa)B and activator protein 1, which could result in autocrine and paracrine BEC apoptosis [133]. BEC might also be able to dampen immune responses by binding to PD-1 on leukocytes, which induces leukocyte apoptosis [135]. PD-1 ligands are B7 family members (programmed death-ligand 1 (PD-L1) (B7-H1) and PD-L2 (B7-DC)) normally expressed at low levels on BEC. Expression of B7-H1 is also induced in cultured human BEC following treatment with IFN-γ(gamma) [135]. TNF-related apoptosis-inducing ligand (TRAIL) is a ligand for death receptors 4 and 5 (DR4, DR5), which are expressed on BEC and upregulated by BDL. TRAIL binding and agonistic anti-DR5 antibodies can result in apoptosis of DR5-expressing BEC in certain mouse strains [136]. Although absent in normal conditions, the expression of

TRAIL on BEC is upregulated in patients with PBC and PSC [136]. Apoptosis is also regulated by bcl-2 family members, which are either pro-apoptotic (bcl-xs, bax, bad, or bak) or anti-apoptotic (bcl-2, bcl-xL, mcl-1, or bfl-1) [137]. The expression of bcl-2 in BEC is regulated by glutathione (GSH), a natural antioxidant [138].

Responses to Oxidative Stress and Senescence-Related Changes

Oxidative stress is characterized by the generation of reactive oxygen species (ROS), including the superoxide anion (O_2^-), H_2O_2, –OH, and singlet oxygen [139]. Oxidative stress is a common component of inflammation because of the generation of ROS by inflammatory cells and promotion of radical formation by cytokines [140–142]. ROS is also produced within BECs by nitric oxide synthetase induced by chronic inflammation [143], and is known to induce genotoxic damage [143, 144]. Almost one-half of GSH, the water soluble antioxidant, released by the adult liver, is secreted into bile and reabsorbed by BEC, raising the possibility that BEC GSH is an important BEC defense mechanism [138]. For example, in PBC, tissue GSH levels are markedly reduced. And oxidative stress in PBC BEC is accompanied by cellular GSH depletion [145], decreased Bcl-2 expression [138, 146], and active lipid peroxidation [147], which act together to promote BEC apoptosis. Recently, it has been shown that aberrant expression of genes associated with abnormal oxidative stress, or susceptibility to ROS such as Anion exchange protein 2 ($Ae2_{ab}$) [148], *GSTM1* [149], and *SPRR2A* [150] are upregulated during the progression of various cholangiopathies such as PBC, cystic fibrosis, and cancer.

Cellular senescence is defined as irreversible cell arrest that acts as a safeguard against tumorigenesis [151]. In BEC, cellular senescence results from the aberrant expression of cell cycle regulators like WAF1, p53, p16, and p21 followed by irreversible G1 cell cycle arrest and apoptosis, which deletes genetically damaged cells during carcinogenesis [152, 153]. In mouse BEC, H_2O_2 treatment to promote oxidative stress or exposure to pro-inflammatory cytokines such as INF-β(beta), INF-γ(gamma), and TNF-α(alpha) induce cellular senescence [154]. In PBC livers, cellular senescence progresses in response to genotoxic damage from oxidative stress, which in turn, results in upregulation of WAF1 and p53 in BEC [152]. And during the early phase of chronic liver allograft rejection, cellular senescence changes, including cell enlargement and multi-nucleation, occur in association with TGFβ(beta)1 and p21 expression and both decrease with successful treatment and recovery [153]. Moreover, telomere shortening and an accumulation of DNA damage coincide with markers of BEC senescence, such as increased expression of p16 and p21, which occurs in damaged BEC in PBC. Eventually, these senescent BEC are thought to contribute to progressive bile duct loss typical of this disorder. Recent studies from Nakanuma et al. showed that stemness genes *Bmi1* and *Ezh2* modulate BEC senescence in PBC [155], hepatolithiasis [156], and intrahepatic cholangiocarcinoma [157] through p16 expression.

SPRR2A

Small proline-rich proteins (SPRR) are encoded by a tandemly arranged four-member gene family of the Epidermal Differentiation Complex (EDC). *SPRR* (*SPRR1–4*) genes encode for a series of highly homologous proteins that function primarily as critical cross-linkers with other EDC genes [158–161]. Numerous gene array expression studies show *SPRR2A* to be among the most highly upregulated genes in many non-squamous, stressed and remodeling barrier epithelia (reviewed in [150]). In normal mouse liver, SPRR2A mRNA and protein are not expressed, but are non-coordinately upregulated in BEC after the stress of BDL [162]. Expression levels of SPRR2A after BDL are not related to squamous metaplasia and show strong dependence on IL6/gp130/STAT3 signaling. Deficient BEC *SPRR2A* expression in IL-$6^{-/-}$ mice following BDL is associated with impaired barrier function [162]. IL-6 replacement therapy restores *SPRR2A* expression to levels seen in wild type controls, and reverses the barrier defect in IL-$6^{-/-}$ mice. Moreover, a recent study demonstrated that forced expression of SPRR2A in a bile duct cancer cell line induces epithelial-mesenchymal transition (EMT), and promotes wound restitution by enhancing migration [150]

Other Defense Systems Against Pathogens

Immunoglobulin

Bile protein mainly consists of albumin and immunoglobulin [13]. IgG and IgM, whose origins are either intrahepatic or from plasma, are present in bile and provide immune defense against pathogens [163, 164]. However, secretory immunoglobulin A (sIgA) is the predominant immunoglobulin type of the mucosal immune system and participates in immunological protection at mucous membrane surfaces including the biliary tree. Bile contains approximately twice the concentration of sIgA compared to that found in the upper intestinal luminal fluid [165], and is composed of two IgA molecules, a peptide J chain, and a secretory component [166].

In humans, sIgA is synthesized by plasma cells in proximity to bile ducts, transported to the luminal surface, and secreted

into bile after binding to pIgR (also known as membrane secretory component [SC]) located on the BEC basolateral membrane [167, 168]. Proposed sIgA functions are: (1) binding to and neutralizing pathogens or bacterial toxins to prevent their adhesion to the BEC mucosal surface. Studies in rats demonstrated that bile contains natural IgAs directed against a variety of intestinal bacteria, and that inoculation of various antigens into the intestinal lumen or intestinal lymphoid tissues triggers a secretion of specific IgA antibodies [169]; (2) forming immune complexes with free antigens, facilitating their excretion. This reduces the systemic response caused by pathogens and prevents chronic inflammation derived from antigens [170, 171]; (3) binding to intracellular pathogens and their products during the transcytosis process [172].

The enzymes lactoferrin and lysozyme, which are produced by peribiliary glands, are also part of the defense against bacterial infections in the biliary tree [173].

Toll-Like Receptors

Epithelial immune detection and responses against microbial infection sometimes involve activation of pathogen pattern recognition receptors on BEC, and subsequent intracellular signaling. This signaling cascade triggers expression of a variety of proteins involved in immune responses, such as adhesion molecules, inflammatory mediators, and antimicrobial peptides. Toll-like receptors (TLRs) are the best characterized of these pattern recognition molecules, and BEC express all 10 of the known human TLRs [174]. TLR ligands include bacterial molecules, double or single stranded RNA, CpG, and LPS. The NF-κ(kappa)B and mitogen-activated protein kinase (MAPK) pathways are essential components of this immune defense in BEC [124, 175]. BEC TLR signaling can produce TNF-α(alpha), IL-6, IL-8, and IL-12, which could recruit and activate T-cells, macrophages, and NK cells to prevent or clear various biliary infections.

Antimicrobial Molecules

Defensins and cathelicidins are anti-microbial small cationic peptides belonging to the innate immune system [176]. They protect mucosal barriers by killing pathogens through membrane disruption. Defensins and cathelicidins also participate in adaptive immunity by recruiting $CD4^+$ T cells and immature dendritic cells [177]. Human β(beta)-defensin 1 (hBD-1) is constitutively expressed in the normal intrahepatic BEC, whereas hBD-2 is undetectable in normal bile ducts. However, hBD-2 is upregulated following *Cryptosporidium parvum* infection in a TLR–dependent manner, [174] and after IL-1β(beta) or TNF-α(alpha) treatment [178]. hBD-2 is expressed almost exclusively by BEC in large, diseased bile ducts. Cathelicidin is expressed by normal BEC, as well as hepatocytes. Bile salts and therapeutic bile salts, including chenodeoxycholic acid and ursodeoxycholic acid (UDCA), enhance cathelicidin expression through the farnesoid X and the vitamin D receptors [179].

Key components of anti-RNA viral immunity in the liver are Mx proteins, a class of dynamin-like large guanosine triphosphatases (GTPases), which are induced by IFNs [180]. Recent studies reveal that BEC express Mx proteins under pathological conditions, including chronic and fulminant hepatitis. And, significant increases of MxA proteins are found in patients with biliary atresia and in cultured BEC stimulated with a synthetic analog of viral dsRNA [181–183].

Trefoil Factor Family

The Trefoil factor family (TFF1, TFF2, and TFF3) contribute to biliary protection by increasing mucous viscosity [184–186], and are involved in biliary restitution following injury by promoting epithelial cell spreading and migration [187, 188]. Mouse and human BEC express predominately TFF3, mainly in the large bile ducts and peribiliary glands [189–192]. TFF expression is upregulated or induced in response to injury [192]. For example, expression of all TFFs is augmented markedly in BEC in hepatolithiasis and detected in the hepatic bile of hepatolithiasis patients [191]. BEC TFF3 expression is regulated through IL-6/gp130 signaling, with expression primarily dependent on STAT3 signaling and reciprocal negative regulation through the MAPK signaling pathway [193]. In addition, biliary TFF3 is also regulated by cytokines and growth factors (e.g. HGF and TGF-β(beta)) [189]. TFF3 expression is significantly higher in $IL\text{-}6^{+/+}$ than in $IL\text{-}6^{-/-}$ mouse livers, and in vitro $IL\text{-}6^{+/+}$ BEC show better migration and wound healing than $IL\text{-}6^{-/-}$BEC[189]. Following BDL, $IL\text{-}6^{-/-}$ mice have a chronic deficiency of biliary TFF3 expression and impaired biliary barrier function. Defective BEC migration in the $IL\text{-}6^{-/-}$ can be significantly reversed by treatment with recombinant TFF peptides. [189] In humans, p-STAT3 and TFF3 are co-expressed in BEC that are involved in florid duct lesions in PBC, and at other sites of BEC injury [189, 190]. This likely constitutes a primitive or innate mucosal defense system that guards against injury and stimulates repair.

BEC Proliferation and Wound Healing

The vast majority of BEC in the normal liver resides in G_0 of the cell cycle, but maintains the ability to divide throughout

adult life. BEC proliferation can restore defects and/or subsequently distort the biliary tree architecture in response to injury. An exuberant response of the smallest BEC to injury is thought to play a key role in the initiation and progression of liver fibrosis. Proliferating BEC can acquire mesenchymal, neuroendocrine [194], and progenitor cell characteristics and interact with other liver cells during repair responses (Fig. 4.2). The proliferative response of BEC is regulated through a complex integration of an array of microenvironmental cues, including interactions with matrix, cytokines, growth factors, gastrointestinal and neuroendocrine hormones via autocrine, juxtacrine, and paracrine signaling pathways (Table 4.1). In turn, several other liver cell types are activated by mediators secreted by proliferating BEC [18, 128].

Crosstalk Between Proliferating BEC and Other Liver Cells

Cellular cross talk among BEC and other liver cells exists and are active during development of the cholangiopathies (Fig. 4.2). In many cholangiopathies, small BEC upstream from the site of injury undergo proliferation, which in turn, usually provokes an extensive fibrotic response in the portal and periportal regions (Fig. 4.2). BEC do so by producing a variety of cytokines and growth factors that stimulate nearby stromal cells, such as platelet-derived growth factor (PDGF), transforming growth factor-β(beta) (TGF-β(beta)), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), nerve growth factor (NGF), and nitric oxide (NO) [195–200]. BEC also synthesize basement membrane proteins such as laminin and collagen type IV [201, 202]. Many studies indicate that BEC are the major source of connective-tissue growth factors and consequently play an active role in stimulating the fibrogenic response, possibly by activating quiescent portal (myo-)fibroblasts and hepatic stellate cells (HSC). For example, portal (myo-)fibroblasts express NTPDase2 under normal conditions, which inhibits activation of basolateral P2Y receptors on BEC. When portal fibroblasts lose their NTPDase2 expression, BEC P2Y receptors are activated by nucleotides and downstream events lead to bile duct proliferation [203]. Proliferating BEC trigger portal fibroblast proliferation and myofibroblastic trans-differentiation in a paracrine fashion via release of the cytokine MCP-1 [204].

HSC have a central role in producing connective tissue in the liver, and their activation in cholangiopathies leads to a proliferative myofibroblast phenotype (Fig. 4.2). A recent study showed that during cholestasis, BEC participate in crosstalk with resident portal fibroblasts and injury-activated myofibroblastic HSC through Hedgehog (Hh)-mediated mesenchymal–epithelial interactions [205]. Mesenchymal cells produce Hh ligands that enhance the viability and proliferation of BEC, which in turn produce Hh ligands that promote the growth of myofibroblast cells.

Hepatocytes release purines into the bile that could potentially activate basolateral and apical P2Y receptors on BEC, resulting in Ca^{2+} signaling and activation of anion secretion. Hepatocytes also produce IGF-I and release insulin, which could activate apical receptors on BEC, and induce ERK1/2 and PI3K signaling [196, 206]. Conversely, proliferating BEC produce IGF-1, NGF, and VEGF and secrete them into the peri-biliary vascular plexus (PBP). These mediators subsequently reach the hepatic sinusoids, which in turn, aid hepatocyte survival during cholestasis [9, 207] (Fig. 4.2).

There is also crosstalk between proliferating BEC and endothelial cells of the peribiliary vascular plexus (Fig. 4.2), which is the sole source of their blood supply. BEC secrete the vasoactive substances VEGF-A and VEGF-C, which could stimulate angiogenesis resulting in remodeling of the bile duct vascular supply in response to the enhanced nutritional and functional demands of proliferating cells [197]. This relationship is supported by observations in a rat BDL model, where proliferation of intrahepatic bile ducts was associated with proliferation of the peribiliary plexus [208]. Moreover, VEGF secreted by proliferating BEC stimulate their self proliferation in an autocrine manner [197].

Epithelial-Mesenchymal Transition

Epithelial-Mesenchymal Transition (EMT) refers to the process in which mature epithelial cells lose the cell–cell contacts and protein expression patterns characteristic of epithelia, and acquire the phenotypic characteristics of mesenchymal cells (e.g., loss of E-cadherin). EMT is associated with decreased adhesion and enhanced motility, which have the potential to increase malignant behavior and an unfavorable clinical outcome in several human cancers [209]. Recently, the miR-200 family was shown to suppress EMT through down-regulation of transcription factors ZEB1 and ZEB2 [210]. And there is accumulating evidence that proliferating BEC have a role in the induction of fibrosis, either directly via epithelial-mesenchymal transition (EMT), or indirectly via activation of nearby portal myofibroblasts. For example, recent studies in biliary atresia [211], PBC [212], and chronic liver disease [213] suggested that proliferating BEC directly contribute to fibrogenesis directly via EMT. However, the extent to which this occurs needs to be studied further.

Table 4.1 Factors, receptors, pathways and effects on cholangiocytes proliferation

Hormone/factor	Receptor/ligand	Signal in cholangiocyte	Effect on proliferation	References
Somatostatin	SSTR2	cAMP	↓	[79, 80]
Gastrin	CCKB/gastrin receptor	IP3R/Ca^{2+}/PKC(α(alpha)/β(beta) I/β(beta)II)	↓	[228]
		cAMP		
GLP1 (Exendin-4)	GLP1R	cAMP/PKA	↓	[247]
		PI3K		
		Ca^{2+}/CAMKIIα(alpha)		
VEGF	VEGR2 and VEGR3	IP3R/Ca2+/PKC(α(alpha))	↑	[197]
		Src/ERK1/2		
Estrogen	ERα(alpha)/β(beta)	cAMP/PKA	↑	[250–252]
		Src/ERK1/2		
Progesterone	PR-A/B	Unknown	↑	[253]
NGF	Trk-A (NTRK1)	ERK1/2	↑	[258, 259]
		PI3K/AKT		
Serotonin	5HT1A and 5HT1B	cAMP/PKA/Src/ERK1/2	↓	[218]
		IP3R/Ca^{2+}/PKCα(alpha)		
Acetylcholine	M3	cAMP	↑	[82, 83]
		Ca^{2+}		
EGF	EGFR	Ras/MEK/ERK1/2	↑	[18, 231]
IL-6	gp130	STAT3	↑	[18, 230]
		SHP2/ERK/MAPK		
HGF	met	PI3K	↑	[18, 229]
		Ras/MEK/ERK1/2		
TGF-β(beta)1(Activin-A)	TGFβ(beta)R	Smad	↓	[18, 274]
Ephinephrine, norepinephrine	β(beta)1-AR; β(beta)2-AR	cAMP	↑	[217]
		PKA/ERK1/2		
		AKT		
Histamine (RAMH)	H1R, H2R, H3R, H4R	cAMP/ PKA/ERK1/2/El1(H3)	↓(H3)	[260, 261]
		IP3R/Ca^{2+}/CaMK I/CREB(H1)	↑(H1)	
CGRPα(alpha)/β(beta)	CLR, RAMP1, RCP	cAMP/PKA	↑	[262]
GH, IGF1	GHR, IGF1R	ERK1/2	↑	[196]
		PI3K/AKT		
KGF	KGFR	MAPK	↑	[296]
PGE2, PGF2α(alpha)	–	Unknown	↑	[291]
Prolactin	PRLR	Ca^{2+}/PKCβ(beta)1	↑	[255]
Hyaluronic acid	CD44	Unknown	↑	[234]
FSH	FSHR	cAMP/ERK1/2/Elk-1	↑	[254]
3,3,′5 l-tri-iodothyronine (T3)	TRα(alpha)1, TRβ(beta)1	IP3R/Ca^{2+}/PKCα(alpha)	↓	[256]
		SRC/ERK1/2		
UDCA, TUDCA, GC, GCDC TA,TCA	NTCP2 (transporter)	cAMP, PKCα(alpha)	↓	[137–140]
			↑	

Common Intracellular Signaling Pathways Mediating BEC Proliferation

cAMP/PKA/ERK1/2

Mediators that modulate BEC proliferation (Table 4.1) are associated with changes in intracellular cAMP levels. In mammalian cells, cAMP activates the cAMP-dependent protein kinase PKA, which controls many cellular processes [214]. Acetylcholine, forskolin, dobutamine, and clenbuterol [215–217] increase intracellular cAMP levels, whereas gastrin [79], somatostatin [79], serotonin [218], 6-hydroxydopamine (6-OHDA) [217], and vagotomy [215] decrease intracelluar cAMP levels (Table 4.1).

Mitogen-activated protein kinases (MAPKs) are a widely conserved family of serine/threonine protein kinases involved in many cellular processes. One such MAPK, ERK1/2, can activate signaling in response to a diverse range of extracellular stimuli, including growth factors, and cytokines [219]. Forskolin, an adenylate cyclase (AC) stimulator, supports cAMP dependent BEC proliferation by activating PKA/ERK1/2 and SRC phosphorylation [220]. ERK1/2 is also involved in BEC proliferation through many other stimulators (Table 4.1). There are also multiple AC isoforms that have specific effects on BEC [221, 222]. Although mediators involved with these AC isoforms

are yet to be elucidated, stimulation of AC1 and AC8, and inhibition of AC5 and AC6 resulted in enhanced secretion and proliferation of BEC [221].

PI3K/AKT

Phosphoinositide 3-kinase (PI3K) catalyzes the production of phosphatidylinositol-3,4,5-triphosphate by phosphorylating phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5- bisphosphate (PIP2). In BEC, growth factors and hormones trigger this phosphorylation event, which in turn, promotes cell growth and cell survival [221]. Activation of AKT, which is downstream of PI3K, increases in proliferating BEC of a rat BDL model [223]. In addition, many mediators, including bile acids [224] are involved in PI3K/AKT associated BEC proliferation (Table 4.1).

IP3R/Ca^{2+}/PKC

Inositol 1,4,5-triphosphate receptor (IP3R) is a member of the intracellular calcium release channel family and is located in the endoplasmic reticulum. There are three types of IP3R (IP3R 1, 2 and 3) [225, 226] that have the potential to stimulate the release of intracellular stores of Ca^{2+} [227], although the specific role of IP3R has not been established in BEC. IP3R plays a pivotal role in modulating the effects of different BEC mitogens or mito-inhibitors. For example, a recent study showed that dysregulation of IP3R/Ca^{2+} signaling is involved in cholestasis. The stimulatory or inhibitory effects on BEC proliferation and secretion depend on the PKC isoforms, which are downstream of this signal. PKCα(alpha), is activated by serotonin [218], ursodeoxycholate, and tauroursodeoxycholate [217], whereas gastrin activates PKCα(alpha), PKCβ(beta)I, and PKCβ(beta)II [228], and this results in inhibition of BEC proliferation and secretion. Furthermore, the activation of specific PKC isoforms is important in modulating cAMP dependent BEC proliferation and secretion [85].

Receptor Tyrosine Kinase (RTK)/Ras/MEK/ERK1/2 and STAT3

RTK/Ras GTPase/MAPK and STAT3 signaling pathways are used by BEC to control many different biological processes [18] and HGF, IL-6, and EGF trigger these signaling cascades. However, there are some differences in signal transduction among these cytokines. HGF activates the receptor tyrosine kinase met, resulting in phosphorylation of different intracellular transducers such as: SH2 motifs, such as the p85 subunit of PI3-kinase; Ras Gap; PLC-g; Src-related tyrosine kinases; the Grb-2 adaptor for SOS and Gab-1; and IRS-like multiadaptor protein [18]. Activation of the Ras/ERK1/2 pathway leads to cellular proliferation, whereas the PI3K pathway induces mitogenesis [229].

IL-6 binds to the ligand-specific IL-6 receptor α(alpha) (IL-6Rα(alpha)) or gp80, which leads to phosphorylation of gp130, the common transmembrane receptor that contains the signaling domain, and the associated Janus kinases (JAKs). Phosphorylation of specific tyrosine residues in both gp130 and JAKs induces at least three distinct signal transduction pathways; (1) JAKs; (2) SHP-2/RAS/MAPK; and (3) STAT3 [18]. In BEC, MAPK signaling induces mitogenesis [230], whereas STAT3 signaling is associated with wound healing and ductular reaction [127]. EGF binds to EGFR (ErbB1/Her1), which results in aggregation of EGFR, homo- and heterodimeric interaction within the ErbB family proteins, and trans-phosphorylation of multimeric complexes [231]. A number of intracellular substrates are activated by these trans-phosphorylations including PLC-γ(gamma), the GTPase-activating protein (GAP) of the Ras proto-oncogene and lipocortin I [18].

Factors Regulating BEC Proliferation

The various factors involved in regulating cholangiocyte proliferation are summarized in Table 4.1. Each is discussed in more detail below.

Extracellular Matrix

Growth, differentiation, and maintenance of cultured BECs are greatly affected by the substratum: (a) tissue culture plastic alone; (b) type I collagen in the form of (1) a collagen-coating, (2) a thicker collagen gel or (3) overlaid double collagen gel; (c) fibronectin and; (d) matrigel, which is composed of Type IV collagen and laminin [18]. Plastic alone and collagen coatings usually promote terminal senescence whereas thicker collagen gels trigger signaling pathways that promote extended passes [18]. CD44, a multifunctional adhesion molecule, is responsible for a number of cell–cell and cell–matrix interactions [232, 233] and has been implicated in BEC proliferation. CD44 and hyaluronic acid, the main component of the ECM, are among the pro-proliferative factors for BEC in cholestatic livers [234]. $CD44^+$ BEC are found closely associated with extracellular hyaluronic acid accumulated in the portal tracts of livers after BDL. Furthermore, in vitro BEC proliferation can be stimulated by hyaluronic acid treatment, and blocked by siRNA or antibody directed against CD44 [234]. And high expression levels of hepatic

CD44 have been observed in patients with PSC and cholangiocarcinoma [235, 236].

Bile Acids

The hydrophilic bile acid ursodeoxycholic acid (UDCA) and the UDCA conjugate tauroursodeoxycholic acid (TUDCA) inhibit BEC proliferation after BDL [237–239], whereas other bile salts, specifically the hydrophobic bile acids taurocholic acid (TA) and taurolithocholic acid (TCA), can stimulate BEC proliferation isolated from the larger bile ducts [240] in normal and BDL rats [241]. Other hydrophobic bile acids such as glycocholic acid (GC) and glycochenodeoxycholic acid (GCDC) decrease BEC proliferation at high concentrations [238]. The different effects of various bile acids on BEC proliferation are associated with changes in intracellular cAMP and PKCα(alpha) levels [238, 240], as discussed above.

Hormones

Both somatostatin and gastrin inhibit BEC proliferation [79, 228]. Somatostatin exerts its inhibitory effect through the SSTR2 receptor by decreasing intracellular cAMP levels [79, 80]. Somatostatin and its analog octreotide also decrease BEC proliferation after BDL, and in polycystic kidney diseased rats [85], it also inhibited cholangiocarcinoma growth, suggesting potential clinical usefulness [242–244]. Similar to somatostatin, gastrin inhibited BEC proliferation by decreasing cAMP in BDL rats and reduced cholangiocarcinoma growth through its receptor CCK-B [228, 245, 246]. Glucagon-like peptide 1 (GLP1), which maintains glucose homeostasis, and its receptor agonist extendin-4, both induced BEC proliferation via cAMP/PKA, PI3K, and Ca^{2+}/CAMKIIα(alpha) pathways in cholestatic rats [247].

Based on the female prevalence of PBC [205], and symptomatic autosomal dominant polycystic liver and kidney disease [248], estrogen has been considered for many years to play a role in the development of cholangiopathies. [249] In rats, BEC express both estrogen receptors (ER-α(alpha) and ER-β(beta)), and expression levels increase during cholestasis after BDL [250]. Modulation of BEC proliferation by estrogen was also confirmed by using 17β(beta)-estradiol on cultured BEC, using an anti-estrogen reagent (tamoxifen) or ER antagonist (ICI 182,780) on male BDL rats, and by studying BDL on ovariectomized female rats [250–252]. These experiments linked estrogen to activation of the cAMP/PKA and Src/ERK1/2 pathways.

Progesterone and its receptors (mRPα(alpha), PRGMC1, and PRGMC2) are also involved in proliferating BEC in both males and females. A study in rats showed that progesterone or supernatants from normal and BDL BEC cultures increased BEC proliferation, and this effect was prevented with anti-progesterone antibody or aminoglutethimide treatment [253]. Recently, other studies showed that pituitary hormones, such as follicle stimulating hormone (FSH) and prolactin, regulate BEC proliferation in rats [254, 255]. FSH uses the cAMP/ERK1/2 pathway, whereas prolactin uses the IP3R/Ca^{2}+/PKC pathway.

Thyroid hormone was also shown to influence proliferation. BEC from normal and BDL rats express alpha(1)-, alpha(2)-, beta(1)-, and beta(2)-thyroid hormone receptors (THRs). The thyroid hormone agonist 3,3′ 5 1-tri-iodothyronine (T3) decreased BEC proliferation in vivo in BDL rats through the IP3R/Ca^{2+}/ERK1/2 pathway. Reversal of the T3 effect was shown in vitro, through chemical inhibition of T3 with U-73122 and BAPTA/AM [256]. BEC also respond to growth hormone (GH) with production and release of IGF1 that modulates cell proliferation by transduction through IGF1-R involving both ERK and PI3K pathways. BEC, which normally express IGF1, IGF1-R, and GH-R, overexpress these molecules during BDL-induced BEC proliferation. Moreover, IGF1 and estrogen reciprocally potentiate their proliferative effects on BEC by interacting at both receptor and post-receptor levels [196].

Neuropeptides

Acetylcholine (Ach) normally transmits parasympathetic signals and stimulates secretion through the M3 muscarinic receptor in BEC, resulting in increased cytosolic Ca^{2+} and cAMP formation via calcineurin [82, 83]. The cholinergic effect of ACh on BEC proliferation has been confirmed in a BDL model where rats underwent total vagotomy [215], which downregulated the M3 receptor, decreased intracellular cAMP levels, and impaired BEC proliferation. Administration of forskolin to stimulate acetylcholine release, maintained cAMP levels and prevented the effects of vagotomy on BEC proliferation [215]. Proliferating BEC from BDL rats express the β(beta)1 and β(beta)2 adrenergic receptors [217]. Chemical adrenergic denervation of the liver via the administration of 6-OHDA also inhibited BEC proliferation, increased the number of apoptotic BEC, and decreased intracellular cAMP levels with concomitant impairment of the PKA/ERK1/2 and AKT signaling pathways. The effect of 6-OHDA on proliferation was partially reversed by concurrent treatment with clenbuterol (β(beta)2-adrenergic agonist), dobutamine (β(beta)1-adrenergic agonist), taurocholate, and forskolin [217, 257].

BEC express the serotonin 1A and 1B receptors. Their activation markedly inhibits the growth and choleretic activity of BEC lining the intrahepatic biliary tree during BDL, with downregulation of the IP3R/Ca^{2+}/PKC and cAMP/PKA/Src/ERK1/2 signaling pathways. BEC also secrete serotonin,

and blocking the secretion during the course of cholestasis enhances BEC proliferation. This illustrates the existence of a serotonin-based autocrine loop during the proliferative response of BEC during cholestasis.

Gigliozzi et al. demonstrated the existence of a nerve growth factor (NGF)-based autocrine signaling loop that modulates BEC proliferation via AKT- and ERK1/2-dependent pathways [258]. Inhibition of NGF during BDL in rats resulted in decreased biliary mass attributable to both reduced proliferation and enhanced BEC apoptosis. They proposed that regulation of BEC proliferation by stimulatory and inhibitory autocrine/paracrine loops (such as serotonin and NGF) during cholestasis plays an important role in the pathogenesis of cholestatic liver diseases [259].

Normal BEC after BDL express all of the histamine receptors (H1R-H4R) [260, 261] and the effect of histamine signaling on BEC proliferation is dependent on the specific receptor subtype involved. H3R is significantly increased in proliferating BEC after BDL in rats. Its activation through administration of histamine or the agonist (R)-(-)-α(alpha)-methylhistamine dihydrobromide (RAMH) decreased the growth of the biliary tree without increasing apoptosis and involved the cAMP/PKA/ERK1/2/Elk-1 pathway [260]. In contrast, activation of H1R in small BEC with the agonist histamine trifluoromethyl toluidide (HTMT dimaleate) resulted in increased BEC proliferation through the IP3R/Ca^{2+}/CaMK I/CREB pathway. Lastly, a recent study demonstrated that biliary expression of the α(alpha)-type calcitonin gene-related peptide 1 (α(alpha)-CGRP), which is a potent vasodilator, regulates BEC proliferation during cholestasis through PKA and CREB activation [262].

Cytokines and Growth Factors (Reviewed in [18, 259])

VEGF is a key regulator of biliary proliferation during cholestasis [197, 263]. And as mentioned above, BEC proliferation precedes the expansion of the peribiliary vascular plexus in the intrahepatic biliary tree after BDL [197]. VEGF regulates BEC proliferation during cholestasis in an autocrine manner through upregulation of VEGF secretion and VEGF2/VEGF3 receptor expression. And BEC proliferation can be induced in normal rats by treatment with recombinant VEGF-A and VEGF-C. VEGF-induced BEC proliferation involves the IP3R/Ca^{2+}/ PKCα(alpha) pathway and phosphorylation of Src and ERK1/2 [197].

In normal livers, IL-6 is produced at low levels by the BEC, [264] and secreted into the bile [125]. Virtually any bile duct insult, such as obstruction [265–267], infection [267, 268], or immunologic damage [125, 269, 270] triggers an increase in IL-6 production by BEC and peribiliary hematolymphoid cells [18]. Active IL-6/gp130/STAT3 signaling can be detected in normal IL-$6^{+/+}$, but not IL-$6^{-/-}$ mouse livers. Activated STAT3 (pSTAT3) in the normal biliary tree localizes to occasional BEC lining the large bile ducts and peribiliary glands [189]. pSTAT3 alerts BEC to environmental stimuli and leads to subsequent autocrine, paracrine, and juxtacrine gp130/STAT3 signaling at the site of injury [162, 189, 265, 266]. As in the gastrointestinal tract [193] and skin [271, 272], the absence of IL-6 in IL-$6^{-/-}$ mice leads to impaired wound healing [189] and poor biliary tree integrity [162, 189, 273].

HGF is one of the most well characterized mitogens for BEC [126, 274–276]. In the normal quiescent liver, low levels of HGF are produced primarily by hepatic stellate cells and neutrophils, but not hepatocytes or BEC. It exerts a paracrine effect on BEC, inducing mitogenesis, cell motility, and matrix invasion [265, 277, 278]. HGF/met signaling is likely to be of particular significance in proliferating BEC. Met is a receptor tyrosine kinase, which is expressed in vivo on normal human and mouse BEC, as well as cultured BEC [265, 274, 279]. HGF binding activates the Ras and PI3K pathways [229]. Periductal HGF production is increased after BDL and remains elevated during the BEC proliferative response [274, 280]. When BEC are injured, HGF/met signaling activates BEC proliferation, with eventual disruption of the limiting plate when BEC extend into the periportal hepatic parenchyma [281–283]. Also, adding HGF to BEC cultures containing three dimensional ductal structures causes the BEC to lose polarity, and promotes BEC invasion into the collagen gels, creating an anastomosing network of BEC [283]. In humans, proliferating BEC in hepatolithiasis and PSC have increased expression of met receptor [279, 283].

EGF is one of the EGF family members that binds specifically to EGFR (or Erb1/Her1) [231, 284]. Of all the non-neoplastic cells within the body, hepatocytes express the highest density of EGFR [284]. But EGFR is also expressed on normal BEC in vivo, as well as cultured BEC [285, 286], and BEC are capable of receptor-mediated endocytosis of EGF [286]. In addition to the BEC proliferative response, EGF signaling contributes to biliary ductal morphogenesis. Rat BECs cultured in the presence of EGF form structures that very closely resemble the polarized hyperplastic bile ductules/ducts seen in vivo [287].

There is also strong circumstantial evidence that EGF and its family members can contribute to ductular reactions, in vivo. BDL causes a significant increase in liver EGF [280, 288], which is coincident with BEC/hepatocyte proliferation, and the downregulation and translocation of EGFR to the nucleus [289]. Moreover, monkeys experience BEC hyperplasia following intravenous recombinant EGF infusion [290]. Studies also show that TGF-α(alpha) protein is produced by hepatocytes and released into the bile, where it has the potential to cause biliary proliferation and transformation through EGFR [285]. ErbB2/Her2, one of the EGF family member receptors, also has the potential to induce

BEC proliferation associated with MAPK and cyclooxygenase-2 (COX-2) [18].

The COX enzymes convert arachidonic acid (AA) to prostaglandins. Over-expression of COX-2 is associated with inflammation and neoplasia. COX-2 has been observed in non-neoplastic hyperplastic BEC in patients with hepatolithiasis and PSC [291], and in a variety of human and rat cancers [291, 292]. COX-2 expression has been mechanistically linked to ErbB2 signaling [293]. Consequently, over expression of ErbB2 in the biliary tree of transgenic mice induced COX-2 expression [294]. COX-2 inhibitors suppress the growth of cholangiocarcinoma cell lines, in vitro [291, 295]. In addition, both HGF and IL-6 induce production of arachidonic acid (AA), PGE2, and PGF2 (alpha) in a cholangiocarcinoma cell line [295]. Moreover, COX-2 inhibitors block HGF and IL-6-induced proliferation in these cholangiocarcinoma lines, suggesting that their growth stimulating effects may be at least partially dependent on prostaglandins [295].

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) family, and plays an important role in embryonic development, angiogenesis, and tissue repair [296]. KGF is a potent mitogen in epithelial cells, but has no proliferative effect on mesenchymal cells. KGF stimulates proliferation of hepatocytes, as well as gastrointestinal and urothelial epithelium [296]. KGF binds to a transmembrane tyrosine kinase receptor (KGFR) [296], which initiates a phosphorylation cascade that leads to activation of phospholipase C-γ(gamma) and MAPK. KGF has not been tested on BEC, in vitro. However, the possibility that KGF can induce biliary proliferation is raised by studies in transgenic mice that express hepatic KGF/FGF-7 during late gestation. These transgenic embryos had enlarged livers with prominent biliary and pancreatic ductal epithelial hyperplasia [297].

Transforming growth factor β(beta) (TGFβ(beta)) and activin A inhibit BEC growth, in vitro, in humans [274] and rats [298]. The active form of TGFβ(beta) and Activin A bind to the TGFβ(beta) receptor II (Tβ(beta)R-II) and activin receptor II, respectively. Receptor/ligand binding results in receptor dimerization, and subsequent phosphorylation of TGFβ(beta) receptor I (Tβ(beta)R-I), and activin receptor I, respectively. TGFβ(beta)1 is produced primarily by stellate and inflammatory cells, but also by BEC in diseased livers [299, 300]. TGFβ(beta)2 is produced primarily by BEC in fibrotic livers [301]. Activin A is produced by hepatocytes in normal liver and in stellate cells of diseased livers, particularly at the edge of regenerative nodules [302]. After BDL, the BEC produce TGFβ(beta)1 [300], consistent with the down regulation of BEC proliferation at this time [265]. In addition, the mannose 6-phosphate/insulin-like growth factor II receptor, which activates TGFβ(beta)1, is also up-regulated in hyperplastic BDL BEC. TGFβ(beta)1 may also play a morphogenic role in the biliary tree [303]. TGFβ(beta)1 inhibits cell growth through upregulation of p21 [153, 304], a protein that also has a significant role in cellular senescence.

The immunosuppressive agent cyclosporine, which is excreted in the bile, can induce BEC TGFβ(beta)1 expression, which in turn, can cause upregulation of p21 in BEC cultures [153]. Patients treated with cyclosporine also have a greater frequency of chronic liver allograft rejection [305]. These findings suggest that after BEC injury, medications present in the blood and bile can influence biliary repair responses. BEC in normal livers do not express p21, whereas p27 is universally expressed in BEC. Up to 12 weeks after BDL in mice, BEC express both p21 and p27 [306]. Using the mouse BDL model, BEC p21 expression was induced immediately after BEC proliferation during the post-mitotic period [306], and under conditions of severe stress, where it is thought to have a mito-inhibitory function [306]. In cholangiocarcinomas, p21 expression is directly proportional to Ki67 staining [307], whereas in other non-neoplastic biliary tract diseases such as primary biliary cirrhosis, increased BEC expression of p21 [152] has been linked to BEC stress and apoptosis [152]. In contrast, the ubiquitous expression of p27 in BEC of normal, untreated mice decreases in direct proportion to increases in BEC proliferation.

Future Directions

It is now widely accepted that BEC actively participate in normal liver physiology and in disease pathophysiology; they are not simply the epithelial lining of a passive conduit for delivery of hepatic bile to the intestine. In the last decade, rapid progress has been made in unraveling molecular pathways that contribute to biliary tree and BEC structure, development, differentiation, proliferation, survival, and secretory and absorptive functions. This progress has been based on exploitation of novel experimental techniques, such as knock out and transgenic mice, which are often applied to classical models of biliary disease, such as BDL. The next step will be application of this knowledge to treatment of cholangiopathies, such as molecular targets, including microRNAs for cholangiocarcinoma therapy. And, continued interplay between basic and translational BEC research will provide a variety of choices in the treatment of biliary tract diseases.

Abbreviations

BDL	Bile duct ligation
CAMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CCKB	Cholecystokinin B
CGRP	Calcitonin gene-related peptide

CLR	Calcitonin-receptor-like receptor
CREB	cAMP response element binding
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FSH	Follicle-stimulating hormone
GC	Glycocholic
GCDC	Glycochenodeoxycholic acid
GH	Growth hormone
GLP1	Glucagon-like peptide 1
HGF	Hepatocyte growth factor
IGF1	Insulin-like growth factor 1
IP3R	Inositol 1,4,5-triphosphate receptor
KGF	Keratinocyte growth factor
MAPK	Mitogen-activated protein kinase
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PPARγ(gamma)	Peroxisome proliferatoractivated receptor-γ(gamma)
PR	Progesterone receptor
PRLR	Prolactin receptor
RAMH	α(Alpha)-methyl histamine
RAMP1	Receptor activity-modifying protein 1
RCP	Receptor component protein
SSTR2	Somatostatin receptor subtype
TA	Taurocholic acid
TCA	Taurolithocholic acid
TGF-β(beta)1	Transforming growth factor-beta 1
TUDCA	UDCA conjugate tauroursodeoxycholic acid
Trk-A	Neurotrophic tyrosine kinase receptor type 1
UDCA	Ursodeoxycholic acid
VEGF	Vascular endothelial growth factor

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Chapter 5
Stellate Cells

Chandrashekhar R. Gandhi

History, Location, and Morphological Characteristics of Stellate Cells

Stellate cells were identified by von Kupffer in 1876 [1] using gold chloride staining procedure, but based on their shape, he described them as phagocytes. In 1952, Ito reported cells containing lipid droplets in the perisinusoidal space and called them "fat-storing cells" [2]. Subsequently, Wake employed gold chloride staining, silver impregnation method, and vitamin A autofluorescence, and confirmed that von Kupffer and Ito had described the same cells [3]. The vitamin A- or fat-storing cells were named Ito cells and hepatic macrophages were named Kupffer cells in recognition of von Kupffer's contribution. In addition, stellate cells were also commonly known as lipocytes and perisinusoidal cells. A consensus was reached in 1996 that these cells be termed as hepatic stellate cells (HSCs) [4]. An excellent historical account of HSCs can be found in a review by Geerts [5].

Hepatic stellate cells constitute about 1.5% of the total liver volume and 5–8% of liver cell numbers [6, 7]. They are located in the space of Disse adjacent to hepatocytes, under the fenestrated sinusoidal endothelial layer. The perisinusoidal (also known as subendothelial) cytoplasmic processes of HSCs, which contain filaments and microtubules, can extend over two or three hepatocytes, and thus internucleus distance between two adjacent HSCs can be as much as 40 μm [8]. The cytoplasmic processes of HSCs are also seen to make physical contacts with sinusoidal cells, adjacent HSCs, and autonomous nerve endings. Some of the processes spread over the sinusoids whereas intersinusoidal (or interparenchymal) processes may extend into the adjacent sinusoids by penetrating hepatocyte plates [8] (Fig. 5.1).

Nearly 50–80% of the body's retinoids (retinol plus retinyl esters) are stored in the liver; HSCs accumulate 80–90% of these stores in numerous perinuclear fat droplets [9]. The smaller, type I lipid droplets (less than 2 μm diameter), but not the larger type II lipid droplets (about 2 μm diameter) are electron dense and surrounded by membrane [8, 10]. HSCs with large lipid droplets are found in the pericentral and midzonal areas, and those with smaller droplets in the periportal areas. The lipid droplets consist of retinyl esters (42%), cholesterol (13%), phospholipids (4%), and triglycerides (28%) [11]. Cellular retinol-binding protein-1 (CRBP-1), which mediates esterification of retinol to retinyl esters and its oxidation to retinal and retinoic acid, is highly expressed in the liver, particularly in HSCs. Expression of CRBP-1 in HSCs increases during activation and upon TGF-β(beta) treatment [12].

HSCs can be identified by quickly fading greenish fluorescence due to vitamin A (retinol) upon excitation at 320 nm [8], and by staining the lipid droplets with Sudan black, a neutral diazo dye that stains saturated neutral lipids in black [13]. Rodent HSCs are also identified immunohistochemically for cytoskeletal proteins desmin and vimentin. The number of desmin-positive cells is greater in the periportal area (about 13 cells/mm^2) as compared to the pericentral area (about 9 cells/mm^2) [14]. While rat HSCs express desmin uniformly, it is minimally expressed or even absent in human HSCs [15, 16]. About 70–80% of quiescent rodent HSCs express glial fibrillary acidic protein (GFAP) [17]; quiescent human HSCs do not express GFAP [18].

Physiologically, HSCs are quiescent, long-lived, and contain moderately developed rough endoplasmic reticulum and small number of mitochondria. Accumulation of cells containing fewer lipid droplets of smaller size and abundant endoplasmic reticulum (typical of fibroblasts) in fibrotic areas led to the hypothesis that these cells originate from HSCs [19, 20]. Subsequent work confirmed that HSCs lose retinoids and acquire a highly proliferative myofibroblast-like phenotype containing greater amounts of rough endoplasmic reticulum during liver injury. Similar phenotypical transformation or activation of HSCs also occurs in tissue culture on plastic. Activation of HSCs is associated with expression of platelet-derived growth factor (PDGF) receptors [21, 22], ferritin receptors [23], and decrease in the expression of peroxisome proliferator-activated receptor gamma (PPARγ(gamma)) [24–26]. In contrast to the rodent HSCs, which express α(alpha)-smooth muscle actin (α(alpha)-sma) upon activation, a significant number of human HSCs contain

C.R. Gandhi (✉)
Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: gandhics@upmc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_5, © Springer Science+Business Media, LLC 2011

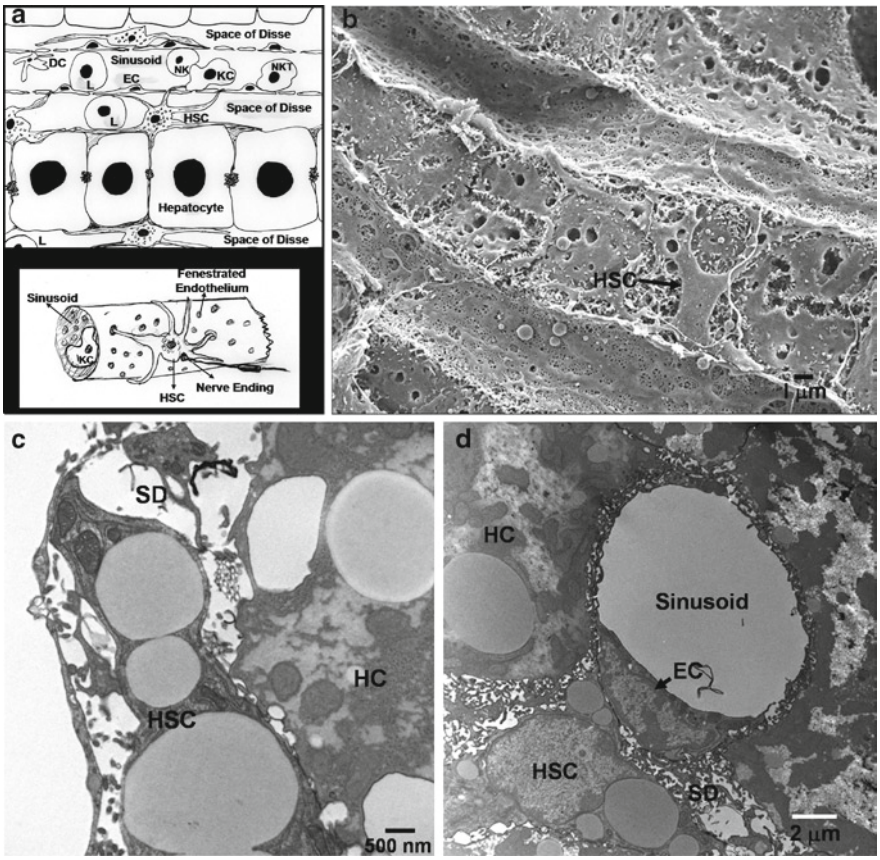

Fig. 5.1 (**a**) Schematic showing localization of HSCs in the normal liver. *DC* dendritic cell, *EC* endothelial cell, *KC* Kupffer cell, *L* lymphocyte, *NKT* natural killer T cell. (**b**) Scanning electron micrograph showing a stellate cell spanning across two sinusoids, which can be differenciated by fenestrated endothelium. (**c**) and (**d**) Transmission electron micrographs showing a hepatic stellate cell (HSC) in the space of Disse (SD). Lipid vesicles are visible. *EC* sinusoidal endothelial cell. Fig. 5.1b–d kindly provided by Dr. Donna Stolz, Center for Biologic Imaging, University of Pittsburg

α(alpha)-sma in physiology [16, 27]. The number of α(alpha)-sma positive cells increase in the cirrhotic human liver exhibiting significant correlation with the volume fraction of fibrosis [28]. Interestingly, activated rodent HSCs have reduced expression of GFAP [17] whereas its expression increases during activation of human HSCs [18]. Activated, but not quiescent, rat HSCs also express neural cell adhesion molecule [29] and nestin [30].

Origin of Stellate Cells

In mouse embryo, desmin-positive stellate-shaped cells appear in the perisinusoidal space from 11.5 days [31]. HSCs were suggested to originate from mesenchymal cells of septum transversum during early stages of organogenesis [32]. The conversion of the subendothelial cells expressing activated leukocyte cell adhesion molecule into myofibroblasts expressing α(alpha)-sma and their uptake of retinol when embedded in collagen gel affirm mesenchymal origin of HSCs [33]. The presence of desmin in HSCs indicates that they are of myogenic rather than fibrogenic lineage. Neural origin of HSCs has also been suggested due to expression by a large percentage of rodent cells of GFAP [17, 34], which is considered to be specific for astroglial cells, and expression of a class VI intermediate filament protein, nestin, by activated rat HSCs [30]. However, in transgenic mice expressing yellow fluorescence protein in neural crest cells, HSCs were found to be negative for this marker [31]. A subpopulation of human fetal liver-derived cytokeratin 7/8 (CK7/8)-expressing $CD34^+$ stem cells differentiate into HSCs in cell culture [35]. Bone marrow-derived stem cells are also shown to differentiate into HSCs; green fluorescence protein (GFP)-positive bone marrow cells injected into the mouse following whole body irradiation appear in the liver, accumulate lipid droplets, and are activated to myofibroblastic α(alpha)-sma-positive cells upon carbon tetrachloride (CCl_4) treatment [36].

Isolation and Culture of HSCs

The principle of isolation and purification of HSCs is based on their distinct density from other hepatic cell types due to the presence of large amounts of stored fat. To increase the

yield of cells, old rats (450–500 g) and mice (30–40 g) are preferred. The initial step in the isolation of HSCs is digestion of the liver with collagenase and pronase. DNase may be added to the digestion buffer. Both anterograde (in via the portal vein and out via the suprahepatic vena cava) and retrograde perfusions are equally effective without compromising the yield of HSCs. In case of inadequate digestion in situ, the liver may be minced and digested in a water bath at 37°C in presence of the enzymes. Nonparenchymal cells are separated from hepatocytes and cell debris by low speed centrifugation ($50 \times g$ x 2 min). The supernatant is centrifuged at $450 \times g$ for 10 min to pellet nonparenchymal cells, following which HSCs are purified by density gradient centrifugation using metrizamide, nycodenz, or percoll [37–42]. HSCs thus obtained are generally quite pure (90–95% purity) with Kupffer cells as major contamination. Kupffer cells can be depleted by magnetic antibody sorting using F4/80 and CD11b antibodies for further purification of mouse HSCs [43].

Purified HSCs are suspended in standard culture medium (e.g., Dulbecco modified Eagle's medium) containing 10–20% fetal bovine serum, or a mixture of 10% fetal bovine serum and 10% horse serum, and plated at a density of 0.05–$0.5 \times 10^6/cm^2$ on plastic culture plates. The cells generally attach within 3–6 h, after which the medium may be renewed to remove contaminating and unattached cells and cell debris. The cells can be easily identified by their classical morphology

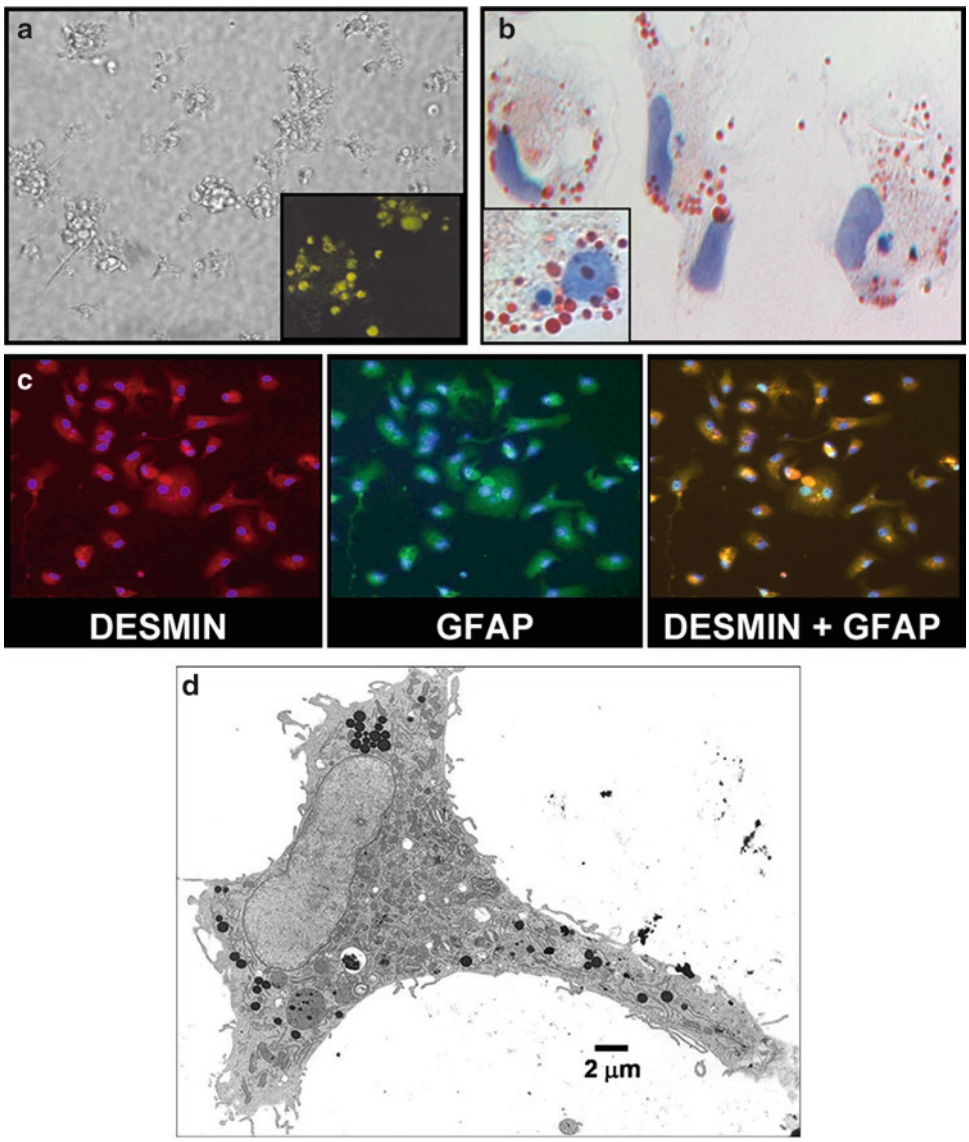

Fig. 5.2 (**a**) Rat HSCs in culture one day after isolation showing appearance of a bunch of grapes. Inset shows vitamin A autofluorescence. (**b**) Oil red staining of rat HSCs on day 3 showing lipid droplets (×60). Inset shows image of an HSC at ×120 magnification. (**c**) Mouse HSCs one day after isolation stained with anti-desmin antibody (*left*; *red*), anti-GFAP antibody (*middle*; *green*), and merged images (*right*). Nucleus is stained blue. (**d**) Transmission electron micrograph of a stellate cell on day 5. Several lipid vesicles are still present. Figure 5.2d was generated in collaboration with Dr. Donna Stolz, Center for Biologic Imaging, University of Pittsburgh

(appearance of clustered grapes) by phase contrast microscopy and vitamin A autofluorescence (Fig. 5.2a), and by staining the lipids with Oil Red (Fig. 5.2b) or Sudan Black [13]. It is a good practice to plate the cells on cover slips in parallel for immunohistochemical determination of specific markers (e.g., desmin, GFAP) to ascertain purity (Fig. 5.2c).

HSCs can be used a day after isolation (putatively quiescent state) [44–46]. However, it should be noted that the process of activation of HSCs begins rapidly after plating in culture media containing high concentration of serum, a rich source of soluble fibronectin and vitronectin, which bind to tissue culture plastic and provide an adhesive layer for the cells [47]. The closest system that can be employed to maintain quiescence of HSCs is culture over basement membrane-like matrix derived from Englebreth-Holm-Sarcoma or matrigel, which resembles the extracellular matrix (ECM) in the space of Disse [48, 49].

Between 7 and 14 days of culture, majority of HSCs express α(alpha)-sma and are considered activated, but they still contain smaller amounts of retinoids [50] (Fig. 5.3). HSCs in passage 3–5 lose their retinoid stores and are considered fully activated closely representing the cells in chronically injured liver. Activated HSCs can be isolated from the fibrotic liver by the same procedure described above, but higher concentrations of the enzymes and longer time of digestion are required. Also, the concentration of the gradient has to be adjusted as these cells are devoid of or contain relatively low levels of stored lipids [43, 51, 52].

The readers should note that discrepancies may exist between the results from various laboratories with regard to the responses of HSCs to specific stimuli. It is, therefore, important to carefully consider the phenotype of HSCs (early cultures between 2 and 10 days, early and late passages, and cells isolated from the fibrotic liver) in planning experiments to investigate mechanisms of the functions of HSCs and their relevance to hepatic physiology or pathology.

Activation and Proliferation of Stellate Cells

Activation of HSCs is a primary tissue repair response to hepatic injury of various types. The process of activation or transdifferentiation of HSCs into myofibroblast-like phenotype includes progressive loss of retinoids, proliferation, expression of α(alpha)-sma and increased contractility, and enhanced production of abnormal ECM. Activated HSCs are considered to be the major cell type to deposit ECM and cause fibrosis of the liver. A large body of evidence with regard to the mediators and mechanisms of activation and

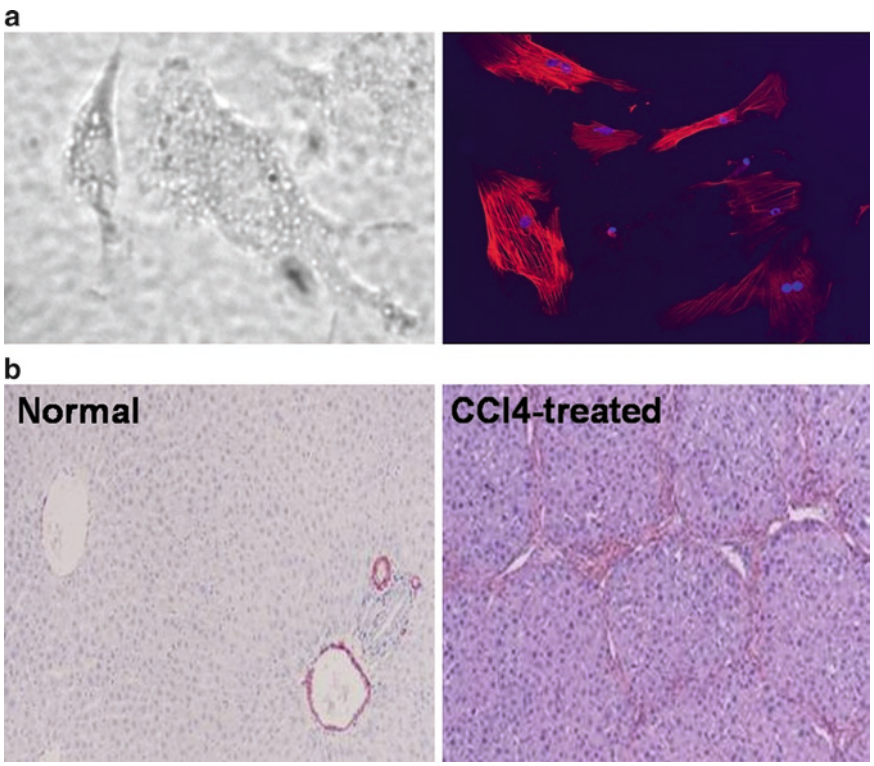

Fig. 5.3 (**a**) Rat HSCs on day 7 of culture show myofibroblast-like morphology, but still contain significant number of lipid droplets (*Left*). Staining with anti α(alpha)-sma antibody shows that most cells express this established marker of HSC activation (*Right*). (**b**) Sections of the liver from normal and CCl4-treated rats (6 weeks treatment) stained for α(alpha)-sma. While α(alpha)-sma is expressed only by smooth muscle cells of the blood vessels in normal liver, in the CCl4-treated liver cells, in the fibrotic area (activated HSCs) intensely staine for α(alpha)-sma

proliferation of HSCs is obtained from in vitro experiments exploiting their property to undergo phenotypical transformation in culture. However, differences might exist between the properties of cells activated in vitro and in vivo as indicated by significant difference in the gene expression pattern between HSCs activated in vivo (bile duct ligation or CCl_4 treatment) and in vitro [43].

Retinoids

Since activated HSCs found in chronic liver diseases are depleted of the retinoid stores [53–55], it is apparent that their proliferation is dependent upon the loss of retinoids. Active proliferation of cultured HSCs begins following an initial rapid and progressive loss of retinoids in the first 3 days followed by a lag period of 4–5 days [50, 56–58]. Uptake of retinoids by activated HSCs reduces their proliferation rate [50]. PDGF-induced proliferation of activated HSCs is also ameliorated upon treatment with retinoic acid without affecting the PDGF receptor density or ligand binding, cell morphology or viability, and collagen type I expression [59]. However, late passages of HSCs are impervious to the inhibitory effect of retinoids, which is attributed to the markedly reduced levels of cellular retinol-binding protein [57]. Transfer of activated HSCs onto matrigel leads to uptake of retinoids and deactivation as evidenced by reduced expression of α(alpha)-sma, procollagen I, tissue inhibitor of metalloproteinase-1 (TIMP-1), and reduced serum induced proliferation [60]. Furthermore, presence of retinoic acid during culture slows the activation process as characterized by morphology, and lower expression levels of α(alpha)-sma, and collagen type I than in cells incubated without retinoic acid [61].

Soluble Mediators

The accumulation of activated HSCs in the areas of necroinflammation indicates that mediators produced by inflammatory cells and macrophages as well as necrotic hepatocytes cause chemotaxis and activation/proliferation of HSCs. Experiments to identify soluble mediators responsible for activation of HSCs led to the finding that medium conditioned by rat Kupffer cells enhances activation and proliferation of HSCs. The process of activation is accelerated when Kupffer cells from CCl_4-injured rat liver [62], D-galactosamine- or thioacetamide-injured rat liver, or Kupffer cells stimulated with zymozan or phorbol esters [58] are used. Similarly, coculture with hepatocytes and rat hepatoma cells [63], as well as medium conditioned by tumoral rat hepatocytes [64] induce activation/proliferation of rat HSCs. A principal mediator released by hepatocytes that causes proliferation of HSCs was identified to be insulin-like growth factor I (IGF-I) [63]. IGF-I is also a potent chemoattractant for HSCs [65]. Additionally, oxidative stress, PDGF, epidermal growth factor (EGF), TGF-α(alpha), b-fibroblast growth factor (bFGF), and endothelin-1 (ET-1) are the mediators that can cause activation of HSCs [21, 63, 66–69]. Table 5.1 lists the mediators that affect activation, proliferation, survival, and other properties of HSCs.

Table 5.1 Various mediators that influence activation/proliferation of HSCs

Mediator	Source	Effect on HSCs
ROS	KCs, ECs, neutrophils, monocytes, hepatocytes	Activation/proliferation; fibrosis
TNF-α(alpha)	KCs, HSCs, lymphocytes	Promotes survival
PDGF	Platelets, monocytes, ECs	Survival; proliferation; chemotaxis
Thrombin	Platelets	Proliferation; contraction
TGF-α(alpha)	KCs, platelets, hepatocytes, HSCs	Proliferation
EGF	Brunner's gland, circulation	Proliferation
TGF-β(beta)	KCs, platelets, HSCs, ECs, monocytes	Survival; promotes proliferation
IGF-I	Platelets, ECs, hepatocytes	Proliferation; chemotaxis
ET-1(ETA receptor)	ECs, HSCs, hepatocytes,	Proliferation; contraction
Ang II	Circulation, HSCs	Proliferation; contraction
ROS	KCs, ECs, neutrophils, monocytes, hepatocytes	Apoptosis of activated HSCs
IFN-γ(gamma)	T-lymphocytes	Inhibits activation
TGF-β(beta)	KCs, platelets, HSCs, ECs, monocytes	Inhibits proliferation
Nitric oxide	HSCs, ECs, hepatocytes,	Inhibits proliferation; relaxation
ET-1 (ETB receptor)	ECs, HSCs, hepatocytes,	Inhibits proliferation; contraction
PGE2	HSCs, KCs, ECs	Inhibits proliferation; relaxation
Retinoic acid	HSCs, hepatocytes, circulation	Inhibits activation and proliferation

Ang angiotensin, *ECs* endothelial cells, *EGF* epidermal growth factor, *ET* endothelin, *IFN* interferon, *IGF* insulin-like growth factor, *KCs* Kupffer cells, *PDGF* platelet derived growth factor, *PG* prostaglandin, *ROS* reactive oxygen species, *TGF* transforming growth factor, *TNF* tumor necrosis factor

Reactive Oxygen Species

An early event during organ injury is the generation of reactive oxygen species (ROS) by infiltrating blood cells and resident macrophages (Kupffer cells), which persists with continued inflammation [70–75]. ROS are also produced intracellularly by cells exposed to certain types of stimulation (e.g., by inflammatory mediators). Generation of ROS exceeding the capacity of antioxidant defense results in peroxidation of lipids and LDL, and protein modification, which play an important role in hepatic fibrogenesis similar to fibrotic diseases of other organs [76–81]. Thus, oxidative stress represents a common link between chronic liver injury caused by toxins, iron overload, alcoholic hepatitis, and viral hepatitis [82–88], and involves ROS-induced activation of quiescent HSCs and fibrogenic activity of activated HSCs [89, 90]. The relationship between oxidative stress and activation/proliferation and fibrogenic activity of HSCs has been investigated in animal models. Oxidative stress and lipid peroxidation products are demonstrated to cause activation of HSCs during CCl_4-induced liver injury in rodents [91–94]. Following CCl_4 administration, the thymidine labeling index and the number of desmin-positive cells increase in the liver; the migration and increase in the number of HSCs occur at a much greater rate in the pericentral than in periportal areas [14, 95]. Amelioration of CCl_4-induced tissue damage, expression of TGF-β(beta), and collagen deposition by treatment with antioxidants such as vitamin E and butylated hydroxytoluene confirm the role of oxidative stress in activation of HSCs and fibrosis [92, 96, 97].

Activation of signaling via nuclear factor kappa B (NFκ(kappa)B)/Rel family of proteins is an important pathway that regulates an array of genes including those involved in inflammatory and immune modulating reactions [98]. TGF-α(alpha) and collagen type I both induce oxidative stress, nuclear translocation of NFκ(kappa)B, and activation of HSCs plated on matrigel to maintain quiescence [92]. However, unabated activation of HSCs expressing Iκ(kappa)B dominant negative protein, which blocks nuclear translocation of NFκ(kappa)B [99], suggests that NFκ(kappa)B might have a different function. Indeed, tumor necrosis factor (TNF)-α(alpha) induces nuclear translocation of NFκ(kappa)B in activated but not in quiescent HSCs [100–102], and activated HSCs expressing Iκ(kappa)B dominant negative protein undergo apoptosis upon stimulation with TNF-α(alpha) [99]. TGF-α(alpha) also stimulates activation of an oncogenic protein c-Myb in HSCs [92]. The Myb family of proteins are known to regulate cell growth and differentiation. Transfection of quiescent HSCs with c-Myb antisense oligonucleotide prevents their activation by TGF-α(alpha) [92] whereas transfection with c-Myb stimulates activation [103]. On the other hand, transfection with c-Myb antisense reduces α(alpha)-sma expression, a marker of activation, in activated HSCs that express c-Myb constitutively [103]. Thus, c-Myb causes activation of HSCs whereas NFκ(kappa)B is not essential for activation, but is required for the survival of activated HSCs. TGF-α(alpha) and oxidative stress-induced HSC activation are summarized in Fig. 5.4.

Platelet-Derived Growth Factor and Endothelin-1

PDGF is the most potent of all the growth factors for HSCs. The importance of the effects of PDGF on HSCs in hepatic injury is exemplified by expression of PDGF receptors by activated HSCs [21], positive correlation between increased PDGF/PDGF receptor levels, the severity of histologic lesions and collagen deposition [104, 105]. PDGF is produced by platelets, monocytes, and macrophages as a dimer of two polypeptide chains A and B in combinations as PDGF-AA, PDGF-BB, and PDGF-AB. While rat HSCs express only PDGF-β(beta) receptor, human HSCs express both PDGF-α(alpha) and PDGF-β(beta) receptors in abundance [106]. PDGF causes proliferation of activated HSCs via phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3-kinase)/protein kinase B (c-Akt) [107], and phosphatidic acid mediated sustained activation (phosphorylation) of extracellular signal-regulated kinase (ERK) [108]. Blockade of PDGF-induced PI3-kinase partly inhibits ERK activity [107] and inhibition of PDGF-induced ERK phosphorylation inhibits c-fos activation, AP-1 binding activity and STAT-1 activation, but causes partial inhibition of cell proliferation [109]. These results suggest involvement of other signaling pathways in PDGF-induced proliferation of HSCs. In this regard, PDGF increases intracellular Ca^{2+} [110], stimulates Na^+/H^+ exchange and peroxisome proliferator-activated receptors [111–114], increases intracellular oxidative stress via NADPH oxidase and causes p38 MAPK activation [113, 115] all of which are coupled to proliferation of HSCs. PDGF-induced PI3-kinase activation is upstream of stimulation of NADPH oxidase activity, ROS production, and proliferation of HSCs [115]. PI3-kinase/c-Akt signaling also induces antiapoptotic pathway and promotes survival of activated HSCs [116]. Furthermore, association between stimulation of PI3-kinase, ERK-MAPK, Rac and Rho signaling pathways in activated HSCs and recruitment of nonactivated HSCs has been suggested to be a critical mechanism of perpetuation of fibrogenic activity in the injured liver [65, 117]. PDGF is also profibrogenic in conditions where inflammation is less evident, such as experimental cholestatic liver injury [118, 119]. Thus, PDGF clearly plays multifaceted roles in modulating HSC biology during liver injury.

Progressive increase in the number of activated HSCs during fibrogenesis is accompanied by their apoptosis [120–122].

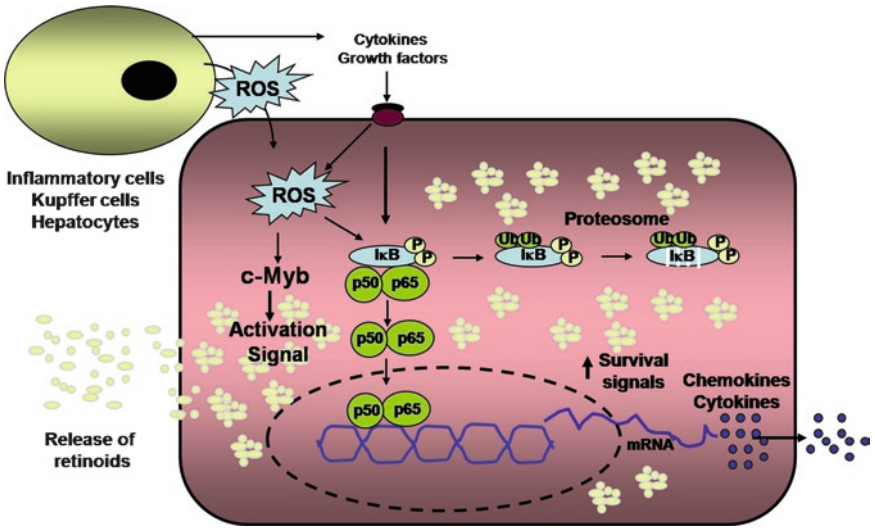

Fig. 5.4 Mediators (including reactive oxygen species, ROS) released by Kupffer cells, inflammatory cells and hepatocytes upon liver injury stimulate activation of cytosolic NFκ(kappa)B in HSCs. Cytokine-induced intracellular ROS also induce NFκ(kappa)B activation. NFκ(kappa)B is present as hetero- or homodimer of Rel family of proteins, the most common being p65:p50 heterodimer. The heterodimers are associated with inhibitory proteins Iκ(kappa)Bα(alpha) or Iκ(kappa)Bβ(beta). Its stimulation involves specific kinase-dependent phosphorylation of Iκ(kappa)B resulting in its dissociation from the NFκ(kappa)B complex, ubiquitination and degradation by proteosome. This reaction allows translocation of the NFκ(kappa)B homo- or heterodimers into nucleus, their binding to specific sites on the DNA and stimulation of transcription of genes involved in survival of HSCs (e.g., increased expression of antiapoptotic proteins such as Bcl2) and synthesis of cytokines and chemokines. Concomitant ROS-induced activation of c-Myb is responsible for transdifferentiation of HSCs into myofibroblastic phenotype

These observations suggest that while HSCs respond to the mitogenic effects of growth factors such as PDGF, simultaneous counter-regulatory signaling pathways limit their proliferation. A major mechanism of this effect involves increased levels of a second messenger cyclic adenosine monophosphate (cAMP), which is formed by the action of adenylate cyclase on adenosine triphophate (ATP). PDGF and another mitogen for activated HSCs ET-1 both stimulate synthesis of prostaglandin E2 (PGE2) and consequently cAMP, which inhibits proliferation of HSCs [123–125]. Interestingly, ET-1 exerts dual effect on HSCs; its binding to ETA and ETB receptors respectively stimulates pro- [69] and anti-proliferation [125] signaling pathways. The mechanisms of negative effect of cAMP on proliferation of HSCs appear to involve: (a) inhibition of Raf kinase, an upstream activator of ERK [126, 127], and/or (b) inhibition of STAT1 activation [128]. The PDGF- and ET-1-induced signaling pathways responsible for proliferation of HSCs are summarized in Fig. 5.5.

The other known mechanism of limiting HSC proliferation is contrasting effects of ROS. Although ROS induce activation of HSCs and stimulate synthesis of ECM in them, a subpopulation of culture-activated HSCs was found to undergo caspase-3-mediated apoptosis upon stimulation with exogenous ROS [122, 129]. Increased intracellular oxidative stress also induces death of activated HSCs [130, 131]. Resistance of quiescent HSCs to the proapoptotic effect of ROS suggests that retinoids, which possess potent antioxidant property, provide protection against the injury. ROS-induced apoptosis of activated HSCs is ameliorated upon pretreatment with retinoic acid [129]. Moreover, concentration of another antioxidant glutathione (GSH) is also lower in activated than in quiescent HSCs.

HSCs retain their proliferative activity through passages but become progressively less responsive to mitogenic effects of PDGF and EGF [50]. These results suggest that passaged HSCs either produce adequate amounts of autocrine growth factors or have fully functional constitutively activated signaling pathways responsible for proliferation. It also remains to be determined whether the reduced response of late passage HSCs to exogenous growth factors is due to decreased number of receptors or blunted post-receptor signaling.

Transforming Growth Factor-β(beta)

In addition to the mitogens that influence proliferation of HSCs, platelets as well as activated macrophages are also the source of a potent fibrogenic and cell growth-regulatory cytokine TGF-β(beta) during inflammation and tissue repair [132]. Among the peptides belonging to the TGF-β(beta) family, specifically the isoforms TGF-β(beta)1, TGF-β(beta)2, and TGF-β(beta)3, TGF-β(beta)1 is most abundant in both normal and fibrotic liver [133]. While TGF-β(beta)1 exerts growth-inhibitory and pro-apoptotic

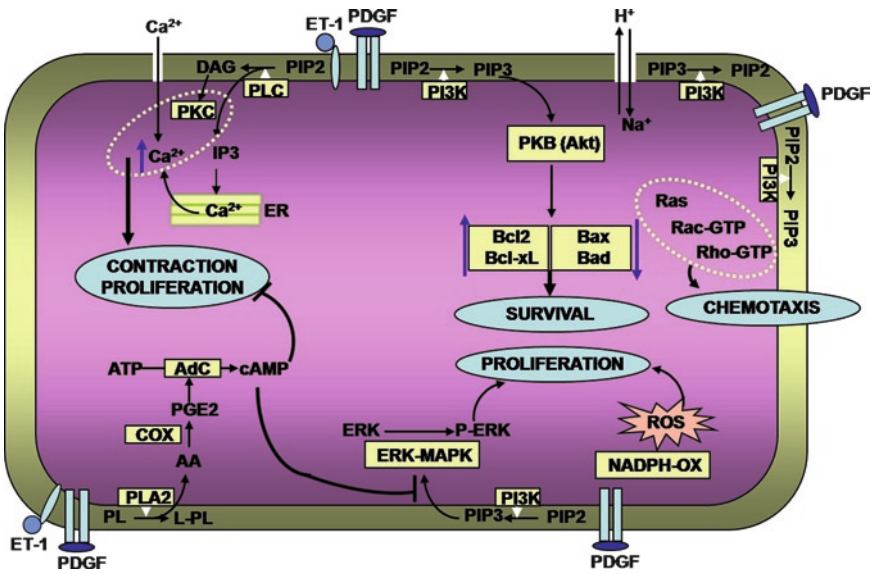

Fig. 5.5 Growth factors such as PDGF cause autophosphorylation of their receptors and recruit phosphatidylinositol-4,5-bisphosphate-3-kinase (PIP2–3-kinase or PI3-kinase) for intracellular signaling to elicit cellular responses. The recruitment PI3-kinase, which consists of an 85 kDa regulatory and a 110 kDa catalytic subunit, is achieved by binding of the Src-homology 2 (SH2) domain of the 85 kDa subunit to phosphotyrosine residue of the receptor (not shown). The binding relieves inhibition of the PI3-kinase catalytic subunit allowing the enzyme to catalyze phosphorylation of PIP2 to PIP3. PIP3 causes colocalization of downstream signaling proteins such as Akt leading to their survival. PIP3 also stimulates phosphorylation of ERK, Na^+/H^+ exchanger as well as reactive oxygen species (ROS) generation via NADPH oxidase (NADPH-OX), which then stimulates proliferation of HSCs. Another pathway of PDGF or endothelin-1 (ET-1)-induced proliferation is phospholipase C-dependent hydrolysis of PIP2 into two second messengers inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which release Ca^{2+} from intracellular stores and activate protein kinase C (PKC) respectively. This effect is followed by uptake of extracellular Ca^{2+} further raising its cytosolic concentration. This (PIP2 IP3 + DAG) pathway is also responsible for ET-1-induced contraction of HSCs. A negative feed-back regulation of contraction and proliferation is achieved via phospholipase A2 (PLA2)-induced release of arachidonic acid (AA) from phospholipids (PL) with formation of lysophospholipids (L-PL). AA is converted to prostaglandins (PGE2 in this case) by cyclooxygenase (COX). PGE2 stimulates synthesis of inhibitory second messenger cyclic AMP (cAMP) from ATP via adenylate cyclase (AdC). PDGF also stimulates Ras, Rac, and Rho pathways via PI3 kinase activation. This pathway then induces chemotaxis of HSCs and perpetuation of the overall responses during liver injury

effects on hepatocytes [134, 135], conflicting results are observed with regard to its role in activation and proliferation of HSCs. TGF-β(beta)1 was reported to accelerate transdifferentiation of quiescent HSCs to the activated phenotype in cell culture [136], to prevent spontaneous apoptosis of activated HSCs [45] and to potentiate their mitogenic response to PDGF and EGF [21]. However, another study observed that TGF-β(beta) inhibits the mitogenic effect of PDGF on HSCs by down-regulating its receptors [59]. Since activated HSCs express IGF-II/mannose-6-phosphate receptor that facilitates conversion of latent TGF-β(beta)1 into active form [137], HSCs appear to develop an interesting mechanism that regulates their own growth and fibrogenic response to TGF-β(beta)1. TGF-β(beta)1, per se, may not be required for the activation of HSCs as demonstrated by normal in vitro activation pattern of the cells isolated from TGF-β(beta)1 knockout mouse [138]. However, reduced synthesis of collagen type I that correlates with the extent of α(alpha)-sma expression in CCl_4-(single dose) treated TGF-β(beta)1 knockout mouse [138] indicates that TGF-β(beta)1 plays an indirect, but critical role in the activation of HSCs in vivo.

Hepatic Fibrosis and Stellate Cells

While the molecular basis of hepatic fibrosis is presented in Chap. 30, here we present a stellate cell perspective in this event. The postulate that HSCs may be the precursors of the cells responsible for excessive synthesis of ECM and hepatic fibrosis [139] was confirmed in a number of studies. An initial response to hepatic injury is infiltration of inflammatory cells (lymphocytes, granulocytes, and monocytes/macrophages), their attachment to the endothelial layer, and transmigration through the sinusoidal lining into the parenchyma [140–142]. These inflammatory cells participate in initiation and progression of liver fibrosis by releasing mediators that stimulate ECM synthesis in HSCs [143–146]. Although this chapter is devoted to HSCs, it is important to note that portal myofibroblasts are also a critical cell type contributing to hepatic fibrosis in chronic liver disease. Portal myofibroblasts are present in a large number in the fibrous tissue, and are differentiated from activated HSCs by their expression of fibulin-2, and absence of vimentin and an extracellular matrix protein reelin. These cells and their functions are reviewed in Chap. 31 and elsewhere [147, 148].

Recent evidence indicates that biliary epithelial cells can make transition into fibroblastic cells and contribute to hepatic fibrosis. The nature of this epithelial-mesenchymal transition could be reversible [Reviewed in 149, 150].

Extracellular Matrix

A fine balance between the synthesis and degradation of ECM components determines their steady state levels in physiology. The enzymes that regulate ECM levels are matrix metalloproteinases (MMPs) that degrade the ECM components, and tissue inhibitors of metalloproteinases (TIMPs), which inhibit activities of MMPs. An increase in the TIMP activity in association with decreased MMP activity is a primary mechanism of the pathogenesis of fibrosis. HSCs are at the center of fibrogneic activity, and in addition to synthesizing the components of ECM, they also produce MMPs and TIMPs [121, 151–153].

About 25 members of Ca^{2+}-dependent enzymes of the MMP family and 4 TIMPs (TIMP-1, -2, -3, and -4) have been identified. The MMPs are classified into five subclasses namely interstitial collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromolysins (MMP-3, MMP-7, MMP-10, and MMP-11), membrane type (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and metalloelastases (MMP-12). This classification is not rigid as MMPs belonging to different classes exhibit overlapping activities toward their substrates. MMPs are secreted as inactive proenzymes, which are activated by variable mechanisms. During activation of HSCs in vitro, the expression of MMPs is down-regulated, while that of TIMPs is up-regulated [154]. The expression of the MMPs and TIMPs is regulated by cytokines such as TGF-β(beta), and TNF-α(alpha) [154], as well as by retinoids [155, 156]. For a detailed review of hepatic MMPs and TIMPs, see Benyon and Arthur [153].

Disturbance in the balance between the expression and activities of MMPs and TIMPs during liver injury causes remodeling of the ECM. In addition, HSCs also increase deposition of abnormal ECM components. For example, Type I and type III collagens, levels of which are relatively low in the normal liver, are major constituents of the ECM deposited in fibrotic liver [157]. Extensive investigations have been performed to identify components of the ECM produced by HSCs, and mechanisms underlying their synthesis and degradation. While freshly isolated HSCs express mRNA for collagen type IV, followed by collagen type III, and very small amounts of collagen type I, activation of HSCs is associated with increased mRNA expression of collagen type I, type III, and type IV, appearance of fibronectin mRNA and decrease in the expression of chondroitin sulfate proteoglycan core protein [158]. Similarly, HSCs isolated from fibrotic livers have increased mRNA expression of procollagen type I, type III, and type IV compared to the cells from normal liver [159]. HSCs in primary culture are also found to synthesize laminin [37, 56], tenascin [160, 161], undulin [162], and glycosaminoglycan hyaluronic acid [163].

ECM is not only responsible for maintenance of the organ's architecture (in physiology) and its distortion (in pathology), but its components also exert profound influence on proliferation, survival and synthetic capabilities of the cells [164]. The pericellular ECM potentiates signals elicited by soluble growth and survival factors via receptors of α(alpha)/β(beta) integrin family [165, 166]. The ECM components also cause perpetuation of the transdifferentiation and proliferation of HSCs, and their fibrogenic activity by storing mediators such as TGF-β(beta)1, TNF-α(alpha), and PDGF.

Reactive Oxygen Species

In addition to causing activation of HSCs, ROS and lipid peroxidation products also act as pro-fibrogenic stimuli for HSCs [84, 86, 167]. Oxidative stress induced by ascorbate/$FeSO_4$ increases procollagen I mRNA expression in cultured human HSCs [96]. Reactive aldehydes such as 4-hydroxy-2,3-alkenals (HAK) exert direct profibrogenic effects on HSCs via activation and nuclear translocation of c-Jun NH2-terminal kinases (JNKs), upregulation of c-jun, and increased AP-1 binding [168]. However, 4-hydroxy-2,3-nonenal (HNE), an HAK, inhibits PDGF receptor tyrosine phosphorylation and attendant signaling associated with cell proliferation [169] indicating negative feed-back loop limiting the wound healing or fibrogenic response. During oxidation of LDL, oxidatively modified lipids such as PPARγ(gamma) ligands, aldehydes, and lipid hydroperoxides are formed that might be liberated from oxidized LDL [76, 170]. These substances induce activation of HSCs and elicit fibrogenic response [24, 81, 168, 171]. ROS scavengers and blockers of PPARs inhibit fibrogenic activity of HSCs due to oxidized LDL [172].

Transforming Growth Factor-β(beta)

TGF-β(beta)l is perhaps the most extensively studied molecule as a fibrogenic agent due to its stimulatory effect on the synthesis of ECM components including collagens type I and type III in activated HSCs [173–175]. Expression of TGF-β(beta)l is increased markedly in experimental models of hepatic fibrosis [98, 133, 174–179], as well as in human cirrhosis [136, 180], and there is strong correlation between the levels of hepatic

TGF-β(beta)l mRNA and collagen α(alpha)l(I) in experimental and human liver fibrosis. Further, transgenic mice overexpressing TGF-β(beta)1 develop hepatic fibrosis spontaneously [181, 182] and neutralizing antibodies against TGF-β(beta)1 ameliorate fibrosis in the animal models [183].

TGF-β(beta)1 is synthesized both by Kupffer cells [67] and activated HSCs [117], and as mentioned above, activated HSCs develop mechanism to convert latent TGF-β(beta)1 into the active form. Additionally, TGF-β(beta)1 increases its own mRNA expression in an autocrine manner in activated HSCs [158]. Targeted deletion of Bcl-xL (an anti-apoptotic molecule) from hepatocytes in hepatocyte-specific conditional Bcl-xL-knockout mice leads to intralobular fibrosis that follows persistent apoptosis of hepatocytes and increased synthesis of TGF-β(beta)1 [184]. These authors also reported that apoptotic hepatocytes induce TGF-β(beta)1 synthesis in cultured hepatocytes and macrophages. These observations provide a link between apoptotic loss of hepatocytes and development of fibrosis [185, 186]. Phagocytosis of apoptotic hepatocytes during liver injury is a function of Kupffer cells, which produce ROS and cytokines that promote activation and fibrogenic response of HSCs. However, HSCs can also engulf apoptotic bodies that results in fibrogenic response in association with further activation, and enhanced synthesis of TGF-β(beta)1 [187].

In activated HSCs, p38 MAPK-dependent Smad3 phosphorylation was found to cause TGF-β(beta)l-induced ECM production both in vitro and in vivo [188]. However, the magnitude of TGF-β(beta)l receptor binding and TGF-β(beta)l-induced Smad phosphorylation is greater in quiescent than in fully activated HSCs [189]. Further, while TGF-β(beta)l inhibits DNA synthesis and stimulates collagen α(alpha)2(I) mRNA expression in quiescent HSCs, the myofibroblastic HSC phenotype is impervious to these effects [189]. Since both HSC phenotypes express TGF-β(beta)1 receptors I and II, expressions of which are increased in activated HSCs [190], it is likely that the receptor density on the membrane of activated HSCs is reduced due to internalization. Interestingly, constitutively increased nuclear expression of Smad3 and Smad4 is observed in HSC cell line developed from cirrhotic liver, and exogenous treatment with TGF-β(beta)1 does not affect the increased collagen α(alpha)2(I) expression in them [191]. Since binding of TGF-β(beta)1 to its receptors on HSCs isolated from normal liver and CCl_4-treated rat livers at 48 or 72 h is similar [190], receptor down-regulation in fully activated HSCs might be an explanation for blunted response to exogenous TGF-β(beta)1.

Another mechanism by which TGF-β(beta)l promotes fibrosis may be by its influence on the expressions of MMPs and TIMPs. TGF-β(beta)l inhibits ECM degradation in fibroblasts by (a) inhibiting the production of MMPs such as collagenase and stromolysin and (b) by stimulating synthesis of plasminogen activator inhibitor (PAI-1) and TIMPs [192]. A similar mechanism might exist in HSCs. However, although TGF-β(beta)l causes an initial rapid decline in steady-state levels of MMP-13 mRNA at the time of induction of collagen α(alpha)1(I) mRNA expression in HSCs, expression of MMP-13 mRNA increases at later times [193]. Clearly, additional investigations are necessary to delineate precise mechanisms of TGF-β(beta)l-mediated fibrosis from the initiation of liver injury through the stages that lead to cirrhosis.

Endothelin

A vasoactive peptide endothelin-1 (ET-1) has also been shown to exert profibrogenic effect on HSCs. Progressive increase in hepatic ET-1 and its receptors during CCl_4-induced liver injury leading to cirrhosis in rats [194, 195], and increased hepatic ET-1 and its receptors in human cirrhosis [69, 196] suggested that endothelin system may have a critical role in the pathogenesis and complications of chronic liver disease. The increased levels of ET-1 in the cirrhotic liver is a combined effect of its enhanced synthesis and reduced metabolism [194, 196, 197]. The connection between ET-1 and tissue fibrosis was demonstrated by development of renal fibrosis in ET-1-overexpressing transgenic mice [198]. ET-1 was also shown to increase collagen synthesis in cardiac fibroblasts and vascular smooth muscle cells [199, 200], and up-regulate type I collagen gene expression in HSCs [51]. ET-1 and TGF-β(beta)1 demonstrate an interesting relationship in hepatic pathophysiology. TGF-β(beta)1 stimulates the synthesis of ET-1 in hepatic endothelial cells [201] as well as in quiescent and activated HSCs [52, 69, 202]. In contrast, ET-1 stimulates TGF-β(beta)1 and collagen synthesis in quiescent, but not in HSCs activated in vivo, probably because of the high level of basal synthesis of both substances [52], and TGF-β(beta)1-induced down-regulation of ET-1 receptors in activated HSCs [202].

Renin-Angiotensin System

The renin-angiotensin system expressed by the activated HSCs has been implicated in hepatic fibrosis with a major role of NADPH oxidase-derived ROS [203]. Isolated quiescent HSCs express components of renin-angiotensin system (angiotensinogen, renin, and angiotensin-converting enzyme) at barely detectable levels and do not secrete angiotensin II, but both in vivo- and culture-activated HSCs express high levels of active renin and angiotensin-converting enzyme, and secrete angiotensin II [204]. PDGF, EGF, ET-1 and thrombin, but not TGF-β(beta)1 and proinflammatory cytokines, stimulate synthesis of angiotensin II in HSCs [204].

Angiotensin II, TGF-β(beta), and PDGF-AB all stimulate synthesis of ET-1 in human activated HSCs [69]. Thus, autocrine loop of activation-dependent synthesis and actions of TGF-β(beta)1, ET-1 and angiotensin-II on contraction, proliferation and fibrogenic activity of HSCs contribute in a major way to the pathology of chronic liver disease.

Other Mediators

Other cytokines that are reported to modulate fibrogenic activity of HSCs are TNF-α(alpha) and IL-1β(beta). While both TNF-α(alpha) and IL-1β(beta) inhibit synthesis of collagen type I in passaged HSCs [176], TNF-α(alpha) does not elicit a similar response in primary culture of HSCs [158]. However, reduced hepatic collagen type I gene expression and collagen synthesis are observed in nude mice in which steady state level of TNF-α(alpha) is increased by gene transfection [205].

Adipocytokines

Higher incidence of cirrhosis in obese than in nonobese subjects [206], and accelerated progression of liver injury strongly correlating with visceral obesity due to steatosis in hepatitis virus C (HCV)-infected patients [207] indicate that obesity related factors positively influence hepatic fibrosis. Thus, the observations that serum levels of leptin are elevated in patients with alcoholic liver cirrhosis [208], and activated but not quiescent HSCs express leptin [209] stimulated interest in understanding the role of adipocytokines (leptin, adiponectin, resistin, and plasminogen activator inhibitor-1), produced in the adipose tissue, in hepatic fibrosis [recently reviewed in ref. 210]. Subsequent work showed that toxin-induced hepatic fibrosis in animals is augmented by recombinant leptin [211]. Very poor fibrogenic response of leptin-deficient *Ob/Ob* mice and leptin receptor-deficient Zucker rats to CCl_4, thioacetamide, or schistosoma infection [212–217] further strengthened the hypothesis that the effect of leptin on HSCs is an important mechanism of hepatic fibrosis. The mechanisms of leptin-induced fibrosis involve synthesis of TGF-β(beta)1 in hepatic endothelial and Kupffer cells [213], and leptin's direct promitogenic and profibrogenic effects on HSCs [215, 218, 219]. In contrast to leptin, adiponectin has been shown to exert antifibrogenic effect. Adiponectin knockout mice are more susceptible than wild type mice to CCl_4-induced fibrosis [220], and adiponectin is shown to reduce fibrogenic effect of leptin on activated HSCs [221]. Adiponectin inhibits AKT pathway through adenosine monophosphate-activated protein kinase and suppresses proliferation of activated HSCs [222, 223].

Hepatitis Virus C

Accelerated recurrence of fibrosis in a significant number of patients receiving transplantation for HCV-induced liver cirrhosis is a major clinical challenge. It is apparent that the subjects vulnerable to this life-threatening pathology that may necessitate retransplantation develop mechanisms of HSC activation. Cultured human HSCs have been found to express CCR7; activation of CCR7 with CCL21, levels of which are elevated in HCV-cirrhosis, induces signaling pathways associated with fibrogenic activity [224]. Human activated HSCs express mRNA for HCV receptors CD81, LDL receptor, and C1q receptor, and their incubation with HCV core and nonstructural proteins induce proliferative and fibrogenic response [225]. Moreover, the magnitude of HSC activation positively correlates with activation of peripheral $CD4^+$ and $CD8^+$ T cells, and is associated with enhanced IL-15 gene expression in HIV/HCV-coinfected and HCV-monoinfected subjects [226]. These results suggest a pathogenic role for IL-15-driven immuno-mediated hepatic fibrosis. Thus, it seems that $CD4^+$ and $CD8^+$ activation-dependent release of IL-15 (and perhaps other cytokines) induce activation of HSCs and their subsequent proliferation and fibrogenic activity is mediated by HCV core protein. Additional work is required to address this critically important issue for development of strategies to prevent HSC activation, fibrosis, and rejection of the graft.

Regulation of Sinusoidal Blood Flow by Stellate Cells

Although vascular smooth muscle cells (VSMC) of the pre- and post-sinusoidal vasculature play major role in hepatic blood flow regulation, substantial experimental evidence indicates an important contribution of sinusoids in this process. About 40–50% of hepatic vascular resistance has been attributed to sinusoids despite the lack of smooth muscle cells [227]. With their ability to contract and relax in response to vasoactive mediators, HSCs have emerged as an important cell type in sinusoidal blood flow regulation. Ultrastructural analysis has revealed the contact of HSCs with autonomous nerve endings [228], and presence of actin-like filaments in the cytosolic extensions [8] that spread over the hepatocytes and around the sinusoidal walls (see Fig. 5.1). Thus, the neurotransmitters released in the vicinity of HSCs might induce contraction or relaxation of HSCs [229].

Perhaps, two of the most important mediators that regulate sinusoidal blood flow are endothelin-1 (ET-1; a powerful vasoconstrictor) and nitric oxide (NO; a vasodilator). Vascular endothelial cells constitutively produce both ET-1 and NO; balance between their synthesis, actions, and

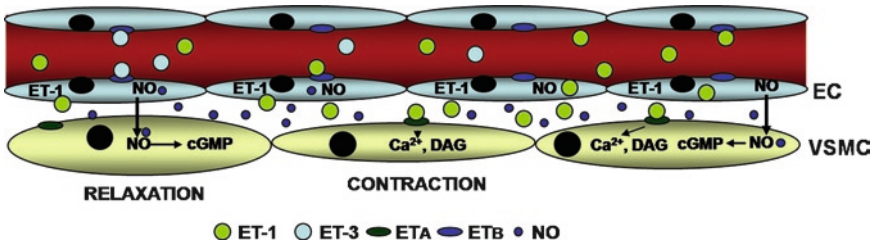

Fig. 5.6 ET-1 belongs to a family of small peptides, three mammalian isoforms (ET-1, ET-2 and ET-3) of which have been identified. ET-1 followed by ET-3, are the predominant forms found in mammalian systems. Originally, ET-1 was discovered to be produced by vascular endothelial cells, but various other cell types are now known to synthesize ET-1. ET-1 is produced as an inactive precursor protein named preproET-1 (about 200 amino acids) that is converted by two step hydrolysis, first to pro or big ET-1 (39 amino acids) and then to active ET-1 (21 amino acids) by endothelin-converting enzyme. Almost all mammalian cell types express ET-1 receptors, which belong to the 7-transmembrane G-protein-coupled superfamily of receptors. There are three subtypes of ET-1 receptors: ETA (ETA1 and ETA2), ETB, and ETC. Activation of ETA on vascular smooth muscle cells (VSMC) elicits vasoconstriction via Ca^{2+} and diacylglycerol (DAG; stimulates protein kinase C)-dependent mechanism. Physiologically, constitutive release of ET-1 and NO by EC, and their effects on VSMC maintain vascular tone. ET-1 exhibits equal affinity for ETA and ETB, whereas ET-3 has much higher affinity for ETB and thus has greater vasodilatory property. Function of ETC, the nonETA and nonETB receptor, are not defined. In addition to the vasoactive effects, ETs have been shown to elicit a variety of biological effects on vascular and nonvascular cell types. [Reviewed in refs. 230–233]

metabolism regulates vascular tone and blood flow (Fig. 5.6) [230–233]. In the liver, ET-1 is synthesized by vascular and sinusoidal endothelial cells [201], biliary epithelial cells [234], HSCs [202, 235, 236] and hepatocytes [197], and metabolized by hepatocytes [237]. ETA and ETB receptor subtypes constitute, respectively, 20% and 80% of ET-1 receptors in rat HSCs [202, 235, 236, 238]. The human HSCs, on the other hand, have predominance of ETA over ETB, but this pattern reverses upon activation with increasing predominance of ETB over ETA receptors during subcultures [69, 239].

Bolus portal introduction of both ET-1 and ET-3 at very low concentration induce powerful and long-lasting constriction of the hepatic vasculature [240]. Although vascular smooth muscle cells of the hepatic blood vessels may be responsible for a major part of this effect, intravital microscopic studies demonstrated ET-1-induced decrease in sinusoidal diameter due to contraction of HSCs [241]. Moreover, ET-1 was also shown to induce contraction of cultured quiescent and activated HSCs [235, 242–244]. Stimulation of both ETA and ETB receptors causes similar contraction of transitionally activated rat HSCs [245]. Since ETA is primarily responsible for contraction of VSMCs, ETB-mediated contraction of HSCs may have important pathophysiologic significance. In this regard, as mentioned above, density of ETB receptors is much greater than that of ETA receptors in rat HSCs and activated human HSCs [69, 202, 236]. Further, ET-3 and sarafotoxin S6c, which bind ETB receptor preferentially, cause potent contraction of hepatic vasculature [51, 241, 246]. In addition to several inflammatory mediators, fibronectin synthesized by activated HSCs may stimulate ET-1 synthesis in an autocrine manner [247]. These data and activation-dependent increased contraction of HSCs [248] indicate that up-regulation of ET-1 system in chronic liver disease [69, 196] has critical implications in the contractile component of portal hypertension. Amelioration of increased portal pressure in various experimental models of liver disease by ET-1 receptor blockade or inhibition of ET-1-induced signaling supports this notion [195, 249–252].

ET-1 stimulates phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate into two second messengers – inositol-1,4,5-trisphosphate and diacylglycerol [253]. The former causes release of Ca^{2+} from the intracellular (endoplasmic reticulum) stores while the latter stimulates activity of protein kinase C. Both Ca^{2+}-dependent and -independent pathways are responsible for the actomyosin-based contraction of HSCs [254] (see Fig. 5.5). Increase in intracellular Ca^{2+} [242], activation of protein kinase C [253], high conductance Ca^{2+}-activated K^+ channels [255], and stimulation of rho-associated kinase [249, 256] are mechanisms by which ET-1 mediates contraction of HSCs. Evidence indicates that increased Ca^{2+} causes the initial transient contraction while activation of protein kinase C is responsible for the sustained phase [257–259]. Thrombin, angiotensin-II, and norepinephrine are also found to induce contraction of human activated HSCs via increase in cytosolic Ca^{2+} [242, 260]. However, thrombin and angiotensin-II are unable to elicit contraction of rat HSCs [248] indicating species-specific differences in HSC biology.

Interestingly, superoxide causes increase in the functional ETB receptors and enhances the release of ET-1 from HSCs [236]. Constriction of the hepatic vasculature due to increased levels of endogenous ET-1 is a major mechanism of decreased hepatic blood flow during ischemia/reperfusion as indicated by amelioration of liver injury by ET-1 antagonists [261, 262]. ET-1-induced contraction of quiescent (nontransformed) HSCs in vivo [241] and in cell culture [244] suggests that sinusoidal narrowing due to the action of ET-1 on HSCs may contribute to ischemia/reperfusion injury.

The contractile effect of ET-1 on HSCs can be accompanied by parallel counter-regulatory relaxation. ETB receptor activation of human activated HSCs stimulates synthesis of

PGE_2 and PGI_2 [123], which cause their relaxation [243]. The liver endothelial cells express constitutive NO synthase (cNOS or eNOS), and Kupffer cells and HSCs express inducible NOS (iNOS) upon stimulation with inflammatory mediators and/or endotoxin. Inhibition of ET-1-induced contraction of HSCs by NO donors has been demonstrated [243]. While ET-1 stimulates NO synthesis in hepatic endothelial cells [263], gram-negative bacterial endotoxin (lipopolysaccharide, LPS) stimulates ET-1 [264] as well as NO synthesis in HSCs [264, 265]. Furthermore, soluble mediators released by endotoxin-challenged HSCs induce iNOS expression and stimulate NO synthesis in hepatocytes [266, 267]. However, NO synthesis in endothelial cells is reduced in cirrhotic rat liver [268], and LPS inhibits ET-1-induced NO synthesis in hepatic endothelial cells [263]. Thus, complex interactions between HSCs, ET-1 and NO regulate hepatic hemodynamics in physiology and pathology. In chronic liver disease and upon reperfusion following ischemic conditions, vasoconstrictor effect of ET-1 appears to predominate in contributing to portal hypertension and ischemia/reperfusion injury respectively.

Carbon monoxide (CO) is also investigated as a mediator of many important biological events including hemodynamic regulation in the liver [269–271]. Along with biliverdin, CO is generated as a by-product of the reaction catalyzed by heme-oxidizing enzymes heme oxygenase-1 (HO-1) and HO-2. Constitutive HO-2 is expressed by hepatocytes and to a smaller extent in Kupffer cells, while inducible HO-1 is expressed by Kupffer cells and HSCs upon appropriate stimulation [269, 270]. The role of CO in sinusoidal blood flow regulation via HSCs is indicated by (a) hepatic venous CO efflux; (b) elevation in vascular resistance concurrent with discrete patterns of constriction in sinusoids at the sites of HSCs and reduction of the sinusoidal perfusion velocity by heme oxygenase inhibitor zinc protoporphyrin IX; and (c) reversal of this effect by CO and cGMP [269].

Role in Hepatic Growth, Inflammation, and Immune Regulation

The liver receives nearly 75% of its blood from the abdominal organs, and thus is constantly exposed to gut-derived toxic substances, including endotoxin and antigens. Under pathological conditions, the liver receives greater amounts of microbial products, including LPS, as well as microbes, viruses, and viral-derived products. But the liver has the ability to process and clear these harmful substances with high efficiency. In addition to harboring the largest resident macrophage population (Kupffer cells) that plays a major role in clearance of toxins and microbes, the liver also contains immune competent cells, including dendritic cells (DCs), natural killer (NK), natural killer T (NKT), and extrathymic T cells [272, 273]. Moreover, the liver cells synthesize complement and acute phase proteins [274]. Thus, the liver provides the first line of host immune defense against antigens and aberrant cells by removing them from the portal circulation, and also contributes toward induction of peripheral immunotolerance and surveillance against circulating pathogens.

The blood cells move slowly through the liver's vasculature due to sluggish flow in the narrow sinusoids allowing lengthy interactions between the lymphocytes and liver-resident antigen-presenting cells (APCs). Such interactions are further lengthened when resistance to blood flow increases due to narrowing of the sinusoids (as during reperfusion following transplantation and resection). This causes extravasation of lymphocytes through endothelial fenestrations into the subendothelial space, allowing their direct contact with HSCs and hepatocytes. Professional APCs such as hepatic DCs and Kupffer cells express molecules required for antigen presentation (mainly HLA class II) and exhibit immune properties under basal conditions. The nonprofessional APCs such as sinusoidal endothelial cells gain this function primarily upon stimulation with cytokines [275–278]. Both types of cells express class I MHC and the B7 (CD80, CD86) co-stimulatory molecules, and can prime T cells in an antigen-specific and MHC-restricted manner [272, 278]. Nonprofessional APCs thus play a supporting role to the immune system in inflamed tissues.

Due to their central "privileged" location, ability to produce a number of cytokines, chemokines and growth mediators, and expression of antigen-presenting and co-stimulatory molecules, it was realized that HSCs could be a critical cell type to participate in hepatic inflammation and immunity. Table 5.2 shows the inflammatory and immune-regulatory molecules expressed and produced by HSCs. HSCs secrete chemokines such as monocyte chemoattractant protein-1 (MCP-1) [279–281], platelet-activating factor (PAF) [282], and IL-8 or its rat homolog cytokine-induced neutrophil chemoattractant [46, 283–285]. In quiescent HSCs, TNF-α(alpha) and not LPS induces MCP-1 expression, but LPS stimulates MCP-1 and macrophage inflammatory protein-2 (MIP-2) expression in activated HSCs independent of endogenous production of TNF-α(alpha) [286, 287]. Exogenous TNF-α(alpha) as well as other proinflammatory cytokines such as IL-1α(alpha) and IFN-γ(gamma) potently stimulate MCP-1 expression in activated HSCs [279, 287]. HSCs enhance neutrophil and monocyte transmigration from hepatic sinusoids into the parenchyma and induce their activation during hepatic injury, thus playing a major role in hepatic inflammation. These data have important clinical implications. For example, expression of MCP-1 in HSCs is elevated in patients with chronic viral hepatitis [279, 280], serum and hepatic levels of IL-8 correlate with severity of chronic viral hepatitis and cirrhosis [283], and marked neutrophil infiltration is associated with a rapid progression of alcohol-induced hepatitis to cirrhosis and poor prognosis [288].

Table 5.2 Inflammatory and immune-regulatory molecules expressed by HSCs, and their functions

Mediators	Functions
TNF-α(alpha)	Induction of surface ICAM-1 expression and chemokines
MCP-1	Chemotaxis of mononuclear leukocytes
MIP-2	Chemotaxis of neutrophils
PAF	Chemotaxis of neutrophils
CINC/IL-8	Chemotaxis of neutrophils
ICAM-1	Adhesion of inflammatory and immune regulatory cells
VCAM-1	Adhesion of inflammatory and immune regulatory cells
IL-10	Inhibition of macrophages and Th1 lymphocytes
TGF-β(beta)	Downregulation of Th1 lymphocytes; induction of Treg
Osteopontin	Chemotaxis for Kupffer cells and macrophages
IFN-β(beta)	Antiviral activity
Nitric oxide	Suppression of T cell activation
MHC I and MHC II	Antigen presentation
CD80, CD86, CD40	Costimulation of T cells
CD1d	Lipid antigen presentation to NKT cells

CINC cytokine-induced neutrophil chemoattractant, *ICAM-1* intracellular adhesion molecule 1, *IL* interleukin, *MCP-1* monocyte chemoattractant protein 1, *MIP-2* macrophage-inhibitory protein 2, *PAF* platelet-activating factor, *TGF* transforming growth factor, *TNF* tumor necrosis factor, *VCAM-1* vascular cell adhesion molecule 1

ICAM-1, expressed on the cell surface, contributes to cellular adhesion and transmigration of leukocytes through the vascular wall via interactions with β(beta)2 integrins, LFA-1 (CD11a/CD18) and myelin-associated glycoprotein-1 (CD11b/CD18) located on the leukocyte cell membrane [289, 290]. Although quiescent HSCs express mRNA transcript of ICAM-1 and also of VCAM-1, the protein expression is very low, mostly localized in the cytosol, activation of HSCs is associated with increased expression of these molecules at the cell surface [291, 292]. LPS and TNF-α(alpha) both upregulate the expression of ICAM-1 and VCAM-1 in activated HSCs and stimulate transmigration and attachment of lymphocytes [46, 291]. These properties of HSCs present an additional mechanism that contributes to hepatic inflammation.

The physiologic concentration of LPS in the portal blood is 1–10 ng/ml. LPS is endocytosed predominantly by Kupffer cells, which respond to its actions via specific receptors [293, 294]. Activation of LPS-responsive cells, such as monocytes and macrophages, occurs after binding of LPS to CD14 [295, 296]. The effects of LPS are enhanced in the presence of lipopolysaccharide binding protein (LBP) produced by hepatocytes and secreted in serum [294, 297]. TNF-α(alpha) is rapidly induced in cells of the mononuclear phagocyte system after stimulation with LPS [298, 299]. Production of TNF-α(alpha) by macrophages in response to microbial stimuli is critically dependent on activation of Toll-like receptor-4 (TLR-4) [300–302], which belongs to the family comprising of more than ten Toll-like receptors (TLRs). Mammalian TLRs are transmembrane proteins named after Toll, originally identified as a molecule essential for embryonic development of Drosophila. The cytoplasmic portion of mammalian TLRs exhibit similarity to IL-1 receptors, but the extracellular domains are quite different. TLRs function as pattern-recognition receptors and instigate cell's response by recognizing pathogen-associated molecular patterns. LPS acts as a ligand for TLR-4 whereas TLR-2 recognizes bacterial lipoproteins and glycolipids. For detailed review on basic characteristics of TLRs see Akira et al. [302].

Both quiescent and activated HSCs express CD14 [267, 303], and while the expression of TLR-4 is low in quiescent HSCs, it increases upon their activation [46, 267, 303]. TLR-4 elicits inflammatory signals such as synthesis of IL-8 and MCP-1, and expression of ICAM-1 and VCAM-1 in activated HSCs [46]. LPS stimulates synthesis of proinflammatory cytokines TNF-α(alpha) and IL-6, and of NO (induction of iNOS) via NFκ(kappa)B- and p38-MAPK-dependent signaling in HSCs [266, 267, 303]. LPS-induced synthesis of these molecules in quiescent rat HSCs that express very low levels of TLR-4, in the absence of lipopolysaccharide-binding protein [267], indicates an interesting possibility of non-TLR-4, non-CD14-mediated mechanism. Using ligands specific for various TLRs, antiviral cytokine (primarily IFN-β(beta)) production was found to occur upon stimulation of TLR-3 and -4 in murine HSCs and of TLR-3 in human HSCs [304].

Whereas TNF-α(alpha), IL-6 and NO produced by HSCs and other hepatic cell types are known to support hepatic regeneration, HSCs are a primary cell type to produce the most potent hepatocyte mitogen, hepatocyte growth factor, as well as another mitogen TGF-α(alpha) [134, 135]. HSCs can also support liver regeneration after fulminant liver failure by influencing oval cells. Oval cells are seen to proliferate and differentiate in close proximity to HSCs in both human and rodent livers [305–308]. In rats, HSCs penetrate continuous basement membrane surrounding the ductules formed by oval cells, and establish direct contact with them [306]. Further, activated HSCs express keratinocyte growth factor, which stimulates growth of hepatocytes via FGFR2-IIIb receptor exclusively expressed by the latter [308]. In contrast to the pro-mitogenic effects, HSCs can also prevent proliferation of hepatocytes. As yet, unidentified soluble mediators released by LPS-stimulated HSCs cause induction of iNOS, endoplasmic reticulum stress, inhibition of DNA synthesis, and promote apoptosis of hepatocytes [266, 267, 309]. Further, NO produced by LPS-stimulated HSCs and in turn by hepatocytes may be an important mechanism of the suppression of T cell activation [310]. Thus, multiple responses provoked by LPS in HSCs can contribute significantly to the pathophysiological changes associated with acute and chronic liver diseases, liver resection, and liver transplantation.

Recent work from several laboratories has shown that HSCs can act as antigen presenting cells by expressing MHC-I, MHC-II, CD80, CD86, CD40, and CD1d [311, 312]. Coculture of cytokine (IL-1β(beta)+IFN-γ(gamma))-stimulated

HSCs induce proliferation of allogeneic whole lymphocyte preparation in an HLA-II-dependent manner [311]. With this machinery and mechanism, HSCs can induce T cell activation and proliferation, and contribute to allogeneic graft rejection. However, activated mouse HSCs were found not to cause activation of $CD8^+$ T cells but to induce apoptosis of allogeneic activated $CD8^+$ T cells through the expression of inhibitory B7 molecule, B7H1 [312]. This apoptotic effect is partly inhibited by anti B7H1 antibody suggesting existence of additional mechanism. A major mechanism of T cells death in the liver involves Fas (expressed by T cells)/FasL interaction [273]. HSCs express and release FasL (Fig. 5.7), and can induce tolerance by promoting apoptosis of T cells. In this regard, apoptotic $CD4^+$ and $CD8^+$ T cells are observed close to HSCs in concanavalin A-treated fibrotic liver in vivo, and activated HSCs cause apoptosis of concanavalin A-activated T cells in vitro [313]. On the other hand, HSCs are also shown to present lipid antigens to CD1-restricted hepatic natural killer T (NKT) cells and promote their homeostatic proliferation through interleukin-15 [314]. Further, adaptive transfer of HSCs primed with bacterial peptides, mediate protection against bacterial infection by activation of T cells in antigen-specific manner [314]. Moreover, accumulation of $CD8^+$ T cells and NK cells in the proximity of HSCs in periportal area and along fibrous septa of CCl_4-treated rats suggests that this might be a mechanism of antigen presentation by the CD11c/MHC-II-expressing HSCs [315]. Thus, HSCs exhibit capacity to perform dual function of antigen-presentation and activation of T cells, and of eliciting apoptosis of activated T cells.

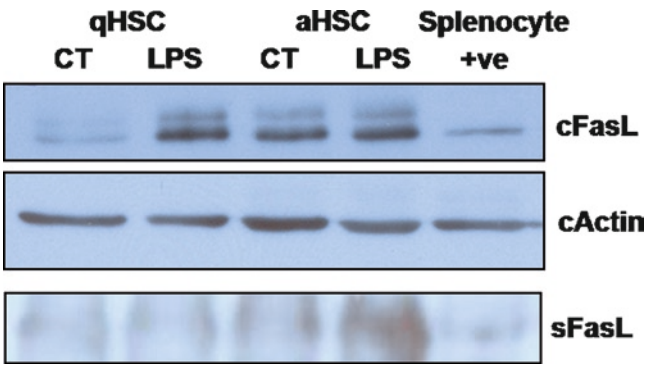

Fig. 5.7 Quiescent HSCs (day 2 of culture) or activated HSCs (day 8 of culture) were challenged with 100 ng/ml LPS and at 24 h, the medium was aspirated. FasL in the medium was immunoprecipitated. Western analysis was then performed to determine FasL expression in the cells and its release in the medium. Splenocytes were used as a positive control.

Recent investigations have provided strong evidence for the role of the transcription factor FoxP3-expressing $CD4^+$ regulatory T cells (Tregs) in immunological tolerance. The importance of retinoic acid in modulating immune cell responses was postulated over three decades ago [316], but only recently, mechanisms of immunoregulation by retinoic acid are being uncovered. An important mechanism of retinoic acid-induced suppression of immune responses involves induction of $FoxP3^+$ Treg. The generation of Treg from naive $CD4^+$ T cells is dependent on TGF-β(beta) and enhanced by retinoic acid [317, 318]. Retinoic acid also causes tropism of Treg [319–321]. As HSCs store large amounts of retinoic acid and also produce TGF-β(beta), they have the ability to promote graft tolerance via Treg induction and expansion. However, retinoic acid inhibits the generation of inflammatory IL-17 secreting $CD4^+$ T cells [318]. Thus, generation of Treg and Th17 cells follows reciprocal developmental pathways with TGF-β(beta) and retinoic acid inducing Treg, and TGF-β(beta) combined with IL-6 (both produced by HSCs [266, 267, 303]) inducing Th17 cells [322]. Co-transplanted activated-mouse HSCs were shown to protect islet allografts from rejection by reducing allograft immunocyte infiltration and enhancing their apoptosis [323]. The mechanisms of such protection involve expansion of $CD4^+CD25^+FoxP3^+$ Tregs, in addition to B7H1-mediated death of T cells [324, 325]. Figure 5.8 depicts possible mechanisms by which HSCs may provide protection, or contribute to rejection of the transplanted liver graft.

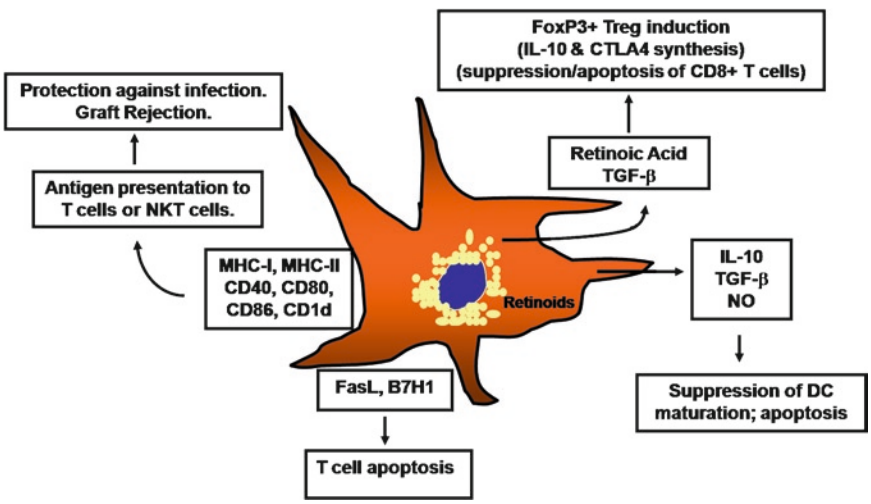

Fig. 5.8 Various currently known mechanisms by which HSCs can regulate hepatic immune responses. See text for description

Selective Elimination of HSCs as Therapeutic Strategy

The first clue that hepatic fibrosis can be reversed was presented nearly 30 years ago [326]. Subsequently, spontaneous recovery of fibrosis was demonstrated in rat models of bile duct ligation-induced [327] and CCl_4-induced [120] liver fibrosis upon termination of the causal factor. Several clinical trials have shown improved liver functions upon treating human cirrhosis of various etiologies with antiviral agents, steroids, immunosuppression, and surgical manipulations [328–335]. Treatment with antioxidant S-adenosine methionine (SAMe) improved liver functions in cholestatic liver diseases and alcoholic cirrhosis [336, 337], and some Chinese herbal medicines have demonstrated improvement in HBV-induced liver cirrhosis [338].

These findings demonstrate promising results in animal models and significant success in clinical trials in treating chronic liver diseases. It is beyond the scope of this chapter to describe the details of these investigations and all relevant publications (reviewed in 121, 339–341). Apart from anti-viral drugs and abstinence from alcohol or surgical correction for cholestatic liver injury, prevention of proliferation with induction of apoptosis of activated HSCs or their reversal to quiescent phenotype is a rational approach in treating hepatic fibrosis. Strategies employed to arrest and/or reverse cirrhosis by direct or indirect effects on HSCs are described below.

Inhibitors of Endogenously Produced Substances

Reducing the hepatic levels of TGF-β(beta) or antagonism of its actions is a logical approach in limiting fibrosis because of its multiple effects on HSCs. These effects include (a) potentiation of activation, survival and proliferation induced by agents such as PDGF and TNF-α(alpha), (b) stimulation of the synthesis of ECM components and TIMPs and inhibition of the synthesis of MMPs, and (c) stimulation of the synthesis of vasoconstrictor ET-1. Another advantage of anti-TGF-β(beta)1 treatment is that the cytokine has anti-growth and pro-apoptotic activities towards epithelial cells including hepatocytes [134, 135]. TGF-β(beta)1 neutralizing antibodies were found to reverse bile duct ligation-induced liver injury in rats [183].

As described above, ET-1 system is up-regulated in human cirrhosis of various etiologies [69, 196]. Because ET-1 is profibrogenic and mitogenic for HSCs [51, 69], and also a potent constrictor of vascular smooth muscle cells and activated HSCs, ET-1 antagonists can be promising therapy to reduce fibrosis and ameliorate contractile component of portal hypertension. The spontaneous resolution of CCl_4-induced liver cirrhosis is accelerated when rats received endothelin antagonist treatment [342], and arrest of fibrosis progression and reversal of the so called irreversible cirrhosis was demonstrated with ET-1 receptor antagonist treatment while continuing CCl_4 administration [343]. ET-1 antagonists were also found to be effective in reversing bile duct ligation-induced fibrosis [183, 344, 345]. It should, however, be noted that although ET-1 antagonists have shown benefits in treatment of chronic heart disease and pulmonary hypertension, in some of these trials there was evidence of mild liver damage [346–349]. On the other hand, one year treatment of Child A and Child B cirrhotic patients with angiotensin type 1 receptor blocker Candesartan cilexetil was found to cause mild reduction in portal pressure [350]. The effect was suggested to be due to reduced fibrogenic activity.

ECM stimulates focal adhesion kinase and in turn tyrosine phosphorylation in HSCs via integrins expressed on the cell surface [351]. Such interactions cause cell contraction, and potentiate PDGF-induced cell proliferation, cytoskeletal organization, and cell motility [352, 353]. Inhibition of the actions of integrins by specific neutralizing antibodies or antisense mRNAs was found to inhibit activation of HSCs and cause apoptosis of activated HSCs [351, 354].

Polyenylphosphatidylcholine, a mixture of polyunsaturated phosphatidylcholines, was also found to inhibit activation of HSCs and to ameliorate PDGF-induced proliferation of activated HSCs [355]. Polyenylphosphatidylcholine also prevents lipid peroxidation [356] and inhibits LPS-induced TNF-α(alpha) synthesis in Kupffer cells [357]. Moreover, treatment with polyenylphosphatidylcholine reduced the number of HSCs in patients with alcoholic liver disease [358]. SAMe, a precursor of glutathione, was found to inhibit HSC activation in both in vitro and in vivo models of liver fibrosis [359–361]. Similarly, vitamin E and agents such as silymarin, colchicine, and milotilate that stimulate antioxidant mechanisms have been shown to inhibit hepatic fibrosis in animal models [339, 362].

An endogenous cannabinoid anandamide (N-arachidonylethanolamine) causes necrotic death of both rat and human activated HSCs. However, anandamide also induced apoptosis of hepatocytes [363]. This observation, and inflammation and tissue damage associated with necrotic death of HSCs induced by anandamide as well as its vasodilatory property [364, 365] necessitate substantial work and development of its derivatives that target activated HSCs for therapeutic application. Another endogenously produced molecule that causes apoptosis of activated HSCs is nerve growth factor (NGF), receptor, which is expressed by activated but not quiescent HSCs [366]. Interestingly, NGF is expressed by hepatocytes during liver injury suggesting a paracrine action of the mediator on HSC apoptosis [367].

Inhibitors of Signaling Pathways Associated with Activation and Fibrogenic Activity of HSCs

An essential role of NFκ(kappa)B activation in survival and proliferation of HSCs generated the hypothesis that prevention of its activation will block proliferation and induce apoptosis of activated HSCs. Indeed, the compound sulfasalazine selectively blocked NFκ(kappa)B-dependent gene transcription and promoted apoptosis via activation of JNK [368]. Another substance pentoxifylline also inhibited NFκ(kappa)B activation and proliferation of activated HSCs in vitro and in vivo [369, 370].

Targeting signaling associated with PDGF-induced proliferation of HSCs (tyrosine phosphorylation of the receptor and PI3-kinase activation) is another strategy tested in cultured HSCs and in models of liver fibrosis in vivo. Treatment with tyrosine phosphorylation blocker SU-5874 led to significant decrease in CCl_4-induced hepatic fibrosis in rats; SU-5874 also markedly inhibited the proliferation of activated HSCs [339]. Transfection of HSCs with an adenovirus expressing a dominant negative form of PI3-kinase under control of α(alpha)-sma promoter was found not only to reduce proliferation, migration, and several profibrogenic genes, but also induced cell death [371]. Although this adenoviral transfection in vivo caused reduction in pro-fibrogenic mechanisms in a short-term bile duct ligation-induced injury in rats, it did not prevent liver cell damage [371]. It remains to be determined whether HSC-targeted PI3-kinase inhibition in animal models of advanced liver disease has any benefits.

Summary and Perspective

Since their discovery more than a century ago, and initial identification of their lipid-storing property over 50 years ago, the realization that HSCs could be the main cell type to cause liver fibrosis stimulated extensive research to elucidate the mechanisms of this function. Over the years, researchers unfolded many other interesting and important properties of HSCs ranging from storage and release of retinoids, to the synthesis of a number of cytokines/chemokines and growth mediators, contractility, and more recently hepatic inflammation and immune regulation. With their central privileged location, HSCs are uniquely versatile in their interactions with and influence the characteristics not only of the other hepatic cell types, but also of the blood cells that traffic through the liver. The Dr. Jekyll and Mr. Hyde personalities of HSCs have become a subject of intense interest for basic as well as clinical scientists, particularly to target specific elimination of these cells as a therapy for chronic liver diseases. Although considerable success has been achieved in inhibiting fibrogenesis by activated HSCs in animal models and human clinical trials, these cells have been successful in hiding the secret of that magic bullet that would remove them from the chronically injured liver. Undoubtedly, research in coming years will achieve this important goal. The ongoing and future work will also provide precise understanding of the role HSCs play in hepatic inflammation and immunity and its application to acute and chronic liver diseases, as well as liver transplantation.

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Chapter 6
Kupffer Cells

Chandrashekhar R. Gandhi

Origin, Location, and Life Span of Kupffer Cells

Kupffer cells, the resident hepatic macrophages, form a major part of the reticuloendothelial or mononuclear phagocyte system. In 1876, German anatomist Karl Wilhelm von Kupffer observed cells that stained with gold chloride in the liver. He designated them as "sternzellen" based on their star shape and proposed that they were specialized endothelial cells that function as phagocytes. In 1898, a Polish pathologist Tadeusz Browicz proposed that the cells described by von Kupffer were macrophages [1]. In 1974, Wisse used electron microscopy and peroxidase staining, and correctly identified resident sinusoidal macrophages that are named Kupffer cells [2, 3]. Of the main nonparenchymal cell types (Kupffer cells, satellite cells, endothelial cells, and Pit cells or natural killer cells), Kupffer cells account for about 30–35% by volume and about 20% by number of the nonparenchymal cells [4, 5].

Bone marrow stem cells that circulate as monocytes differentiate and mature into Kupffer cells in the liver through the actions of mediators such as macrophage colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, and interleukin-3 (IL-3) [6, 7]. Kupffer cells are irregular shaped and are located in the lumen of sinusoids (capillaries in the liver) adhering to the endothelial cells (Fig. 6.1). Their cytoplasmic processes (pseudopodia), which indicate phagocytic function, protrude into the sinusoidal lumen and may also penetrate into Disse's space through larger endothelial fenestrations.

Kupffer cells can be identified by immunostaining with antibodies against macrophage surface ED2 antigen CD163 (rat) (Fig. 6.2) [8, 9], a glycoprotein that binds low density lipoprotein CD68 (human) [10, 11], or a cell surface glycoprotein F4/80 (mice) related to the seven transmembrane-spanning family of hormone receptors [12, 13]. Kupffer cells in zone 1 (periportal), zone 2 (midzonal), and zone 3 (pericentral) of the liver acinus exhibit a ratio of 4:3:2 [14]. The size and phagocytic activity (lysosomal content and enzyme activity) of Kupffer cells are greatest in the periportal and least in the pericentral area [14, 15]. Functional differences in the periportal and pericentral Kupffer cells are also illustrated by relatively greater level of tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), and IL-1 synthesis by larger Kupffer cells, while reverse scenario is observed in regard to their nitric oxide (NO) synthetic capacity [16].

Based upon the distribution of the cells labeled with latex particles and [^{3}H]thymidine labeling index, the life span of rat Kupffer cells was estimated to be several months [15]. However, cells of donor origin repopulate the liver within 14–21 days after bone marrow transplantation in mice [17]. Depletion of Kupffer cells in rodents by liposome-encapsulated clodronate causes a robust proliferative response from the residual cells and recruitment of monocytes [18], resulting in complete repopulation between 14 to 16 days [8, 19]. In monocytopenic mice, Kupffer cells were found to be maintained for over 40 days with strong mitogenic activity [20]. While Kupffer cells of recipient origin completely replace the donor cells within 15–30 days after liver transplantation in rats, the cells of the donor liver persist for up to 1 year in humans [21].

Isolation and Culture of Kupffer Cells

Kupffer cells are isolated by collagenase and protease digestion of the liver via the portal vein. After separating the cells from the digested liver, the parenchymal cells are removed by low speed centrifugation, and the nonparenchymal cells are pelleted from the supernatant by centrifugation at higher speed. Kupffer cells can then be purified by isopycnic sedimentation in two-step Percoll gradient (25 and 50% Percoll) [22] with a yield of about $40–60 \times 10^6$ cells/rat liver. For additional purification, nonparenchymal cells are first obtained by density gradient centrifugation in 17.5% metrizamide; Kupffer cells and endothelial cells are then separated by centrifugal

C.R. Gandhi (✉)
University of Pittsburgh, No. 1542, 200 Lothrop Street, Pittsburgh, PA 15213, USA
e-mail: gandhics@upmc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_6, © Springer Science+Business Media, LLC 2011

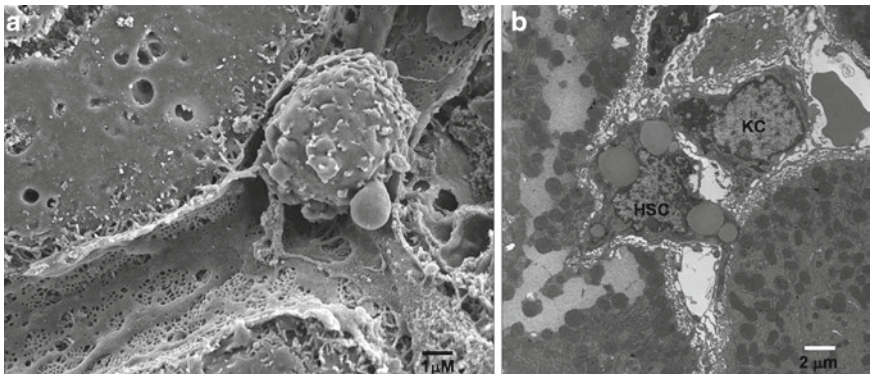

Fig. 6.1 (**a**) Scanning electron micrograph showing a Kupffer cell in the sinusoid of the liver. (**b**) Transmission electron micrograph showing a Kupffer cell and a stellate cell in the sinusoid, and in the space of Disse respectively. The images were kindly provided by Dr. Donna Stolz of the Center for Biologic Imaging, University of Pittsburgh

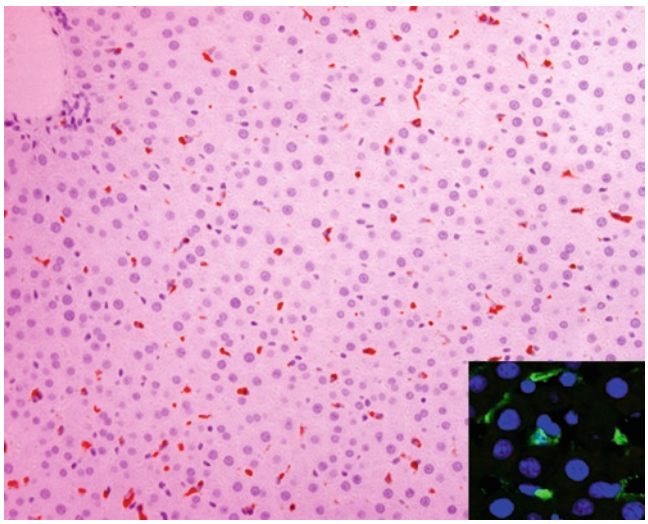

Fig. 6.2 ED2-positive Kupffer cells can be seen in a liver section (×20). *Inset* shows ED2-positive cells in the liver section by confocal microscopy (×40)

elutriation [23, 24]. The cells can be plated in standard culture medium (e.g., William's medium E, or RPMI medium) supplemented with 10% fetal calf serum, and used after overnight culture or within 3 days following isolation. The primary contaminants in Kupffer cell preparation are hepatic endothelial cells (2–5%), but they do not attach to the plates under these culture conditions, and thus removed during renewal of the medium. The purity of the cells is determined by immunostaining with ED2 (Fig. 6.3) or F4/80 in conjunction with immunostaining for stellate cells (desmin or GFAP), endothelial cells (SE1 or factor VIII related antigen) and epithelial cells (clone AE1/AE3) [25, 26].

Mouse Kupffer cells can be purified via selection with CD11b or F4/80 antibodies. The nonparenchymal cells obtained as described above are treated with anti-CD11b or anti-F4/80 antibodies followed by purification of Kupffer cells by magnetic microbead cell sorting [27, 28].

Phagocytosis and Clearance Functions of Kupffer Cells

The liver provides the first line of the body's defense against noxious substances including gut- and environment-derived toxins, microbial and viral products, and xenobiotics to which it is constantly exposed via the portal blood. Kupffer cells play a major role in fulfilling this obligation, which is aided by sluggish blood flow through the sinusoids thus allowing their lengthy interactions with the components of the portal blood. Kupffer cells also remove microorganisms, senescent and damaged red blood cells, and circulating neoplastic cells. Phagocytosis of a variety of particulate matter including cell debris and bacteria or bacterial fragments is facilitated by their binding to plasma fibronectin, which is recognized by specific trypsin-sensitive receptors on Kupffer cells. The particulate material of size greater than 10 nm is phagocytosed by Kupffer cells, following binding to the surface receptors. Smaller particles are phagocytosed after aggregation to increase the size. The phagocytic activity of Kupffer cells can be measured by the ability to ingest latex particles [29]. Phagocytosis by Kupffer cells triggers synthesis of ROS, arachidonate metabolites (prostaglandins, thromboxanes, and leukotrienes), cytokines and chemokines, which play important role in defense as well as inflammatory responses [30].

In addition to the uptake of defective and senescent red blood cells [31, 32], Kupffer cells also remove efficiently vesicles containing hemoglobin shed by the red blood cells [33, 34] and hemoglobin–haptoglobin complexes [35]. Scavenger receptors class A type I and II, and CD163 mediate this clearance thus providing protection from free hemoglobin-mediated oxidative injury. Hemoglobin is degraded by heme oxygenase (HO)-1 (inducible isoform) and HO-2 (constitutive isoform) with the formation of iron, biliverdin, and carbon monoxide (CO). These three molecules play critically important protective roles against tissue

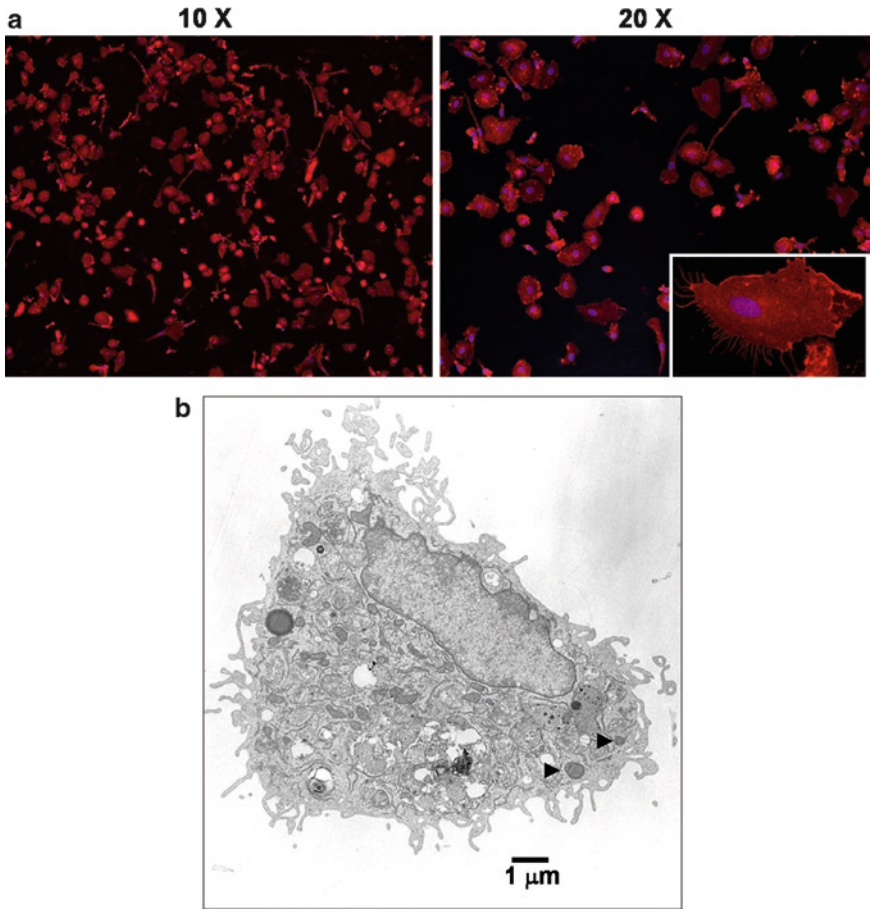

Fig. 6.3 (**a**) Kupffer cells in primary culture on day 2 stained with ED2. *Inset* shows a Kupffer cell with pseudopodia (×100). (**b**) A transmission electron micrograph of a cultured Kupffer cell on day 2. Pseudopodia are visible and lysozomes are indicated by *arrowheads*

injury: low levels of iron are cytoprotective and antiapoptotic [36]; bilirubin formed from biliverdin has antioxidant properties [37]; and CO imparts cytoprotection and prevents liver damage caused by stress conditions such as ischemia/reperfusion [38–40]. Apart from being a vasodilator, CO also exhibits antiapoptotic, anti-inflammatory, and antiproliferative properties [41]. Anti-inflammatory agents such as IL-10 and glucocorticoids increase expression of CD163 and HO-1 in Kupffer cells [39, 42].

As mentioned above, Kupffer cells phagocytose neoplastic cells, and therefore provide a critical mechanism of preventing hepatic metastasis of gastro-intestinal malignancies. The malignant cells detached from the primary tumor enter into the portal circulation [43], with Kupffer cells as the primary cell type to encounter them. The aggressive increase in the development of tumor in Kupffer cell-depleted liver exemplifies their importance in causing effective elimination of the metastatic cells [44–46]. The antitumor activity of Kupffer cells was shown to increase upon stimulation with IFNγ, granulocyte–macrophage colony stimulating factor, and muramyl dipeptides [47, 48].

Activation of Kupffer Cells

Kupffer cell responses are subject to alterations depending upon the concentrations and nature of substances present in the portal blood. Substances such as complement factors C3a and C5a [49], bacteria- and fungi-derived b-glucans [50], and gram-negative bacterial endotoxin (lipopolysaccharide, LPS) [19, 30, 51, 52] all cause activation of Kupffer cells. The effects of LPS on Kupffer cells have been studied extensively. LPS-induced activation of Kupffer cells occurs following its binding to CD14, a glycosylphosphatidylinositol-anchored membrane protein. The concentration of LPS required to cause activation of Kupffer cells is significantly reduced and the effects enhanced by its association with circulating LPS-binding protein (LBP) [53, 54], a 60-kDa acute-phase protein secreted by hepatocytes. The LPS-LBP interactions may have physiological importance considering chronic exposure of Kupffer cells to low levels of LPS. LPS-LBP complexes are shown to be more potent than LPS alone in stimulating synthesis of proinflammatory mediators such as TNF-α [55, 56]. However, the basal expression of CD14 in Kupffer cells is relatively much lower than in

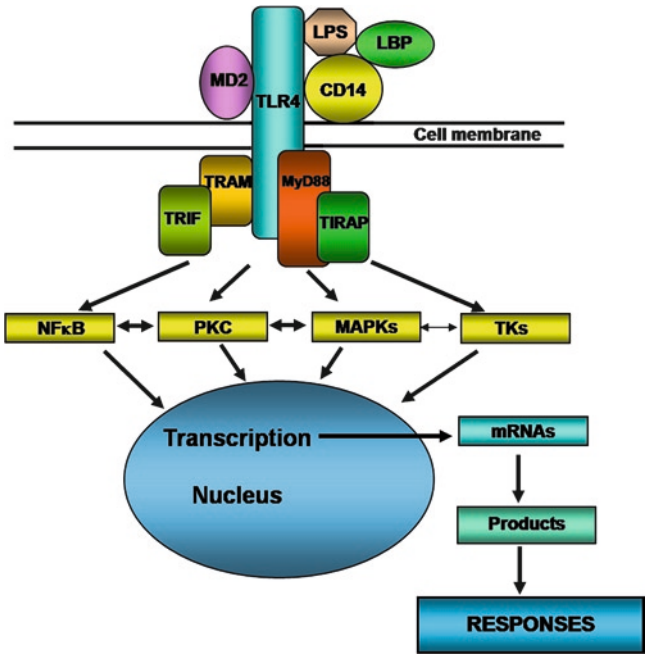

Fig. 6.4 The established TLR4/CD14 assembly of LPS receptor complex and transmembrane signaling molecules that mediate the intracellular effects of LPS. Please see text for detailed description

monocytes [57], and is up-regulated by a several mediators including LPS itself [58], and in liver diseases of various types [52]. The LPS/LBP/CD14 ternary complex stimulates multiple signaling pathways, including activation of nuclear factor κ-B (NFκ-B), nonreceptor tyrosine kinases (TK), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) (Fig. 6.4) [59–65].

Since CD14 is a membrane anchored protein without transmembrane domain, it requires additional mechanisms to elicit cellular effects. These are provided by membrane-spanning Toll-like receptors (TLRs) [66], the most notable and extensively studied among them being TLR4 [67–69] (Fig. 6.4). MD-2, a secreted protein, closely associates with the extracellular domain of TLR4 imparting LPS responsiveness to the cells [68, 70]. TLR4 signals via four intracellular adaptor proteins, which operate in functional pairs: MyD88 (the myeloid differentiation factor 88)/TIRAP (toll-interleukin 1 receptor-containing adapter protein), and TRAM (TLR adapter molecule 2)/TRIF (TIR domain-containing adaptor-inducing IFN-β) [70–73]. Downstream of TLR4, signaling occurs via MyD88, which associates with IL-1 receptor associated kinase (IRAK) and TNF-activated factor-6 (TRAF-6). TRAF-6-mediated signaling pathways activate NFκ-B, which results in the production of proinflammatory cytokines [66].

TLR2 has also been suggested to cause LPS-mediated signaling by binding to LPS in the presence of LBP and CD14 [74, 75]. In Kupffer cells, TLR2 activation by zymosan was found to stimulate chemokine production that was mediated via activation of p38 and c-Jun N-terminal kinase (JNK) MAPK [76]. Further, upregulation of TLR2 in Kupffer cells during endotoxemia in mice suggests its role in hepatic innate immune responses via these macrophages [77].

LPS activates p38-, JNK-, and extracellular signal-regulated kinase 1/2 (ERK1/2)-MAPK in Kupffer cells. However, blockade of p38 activation was found to inhibit LPS-induced synthesis of both TNF-α and IL-10 [78]. These authors also reported that blockade of ERK1/2 activation reduced LPS-stimulated TNF-α production without having any effect on IL-10 synthesis. ERK1/2-dependent LPS-induced synthesis of TNF-α was also reported by other authors [79]. Interestingly, Kupffer cells isolated from ethanol-fed mice produce much greater amounts of TNFα upon LPS stimulation in the absence of NFκ-B activation indicating an alternate mechanism of the cytokine production [79]. Thus, LPS-induced TNF-α synthesis coupled to stimulation of early growth response protein-1 (Egr-1) was observed in Kupffer cells (Fig. 6.5). Greater nuclear binding of Egr-1 in LPS-stimulated Kupffer cells from ethanol-fed rats in comparison to cells from pair-fed rats was suggested to be a mechanism of increased TNF-α synthesis [79].

While NFκ-B activation has been implicated in the synthesis of inflammatory cytokines, receptor-mediated phopholipase C-induced phosphoinositide signaling pathway also plays an important role in Kupffer cell responses. Most G-protein coupled receptors, upon ligand binding, stimulate phospholipase C-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol (DAG) and inositol-1,4,5-trisphophate (IP3). DAG stimulates PKC and IP3 causes release of calcium from intracellular stores (specifically endoplasmic reticulum), thus increasing concentration of cytosolic calcium (Fig. 6.5). In several instances, this initial reaction is followed by influx of extracellular calcium through specific membrane channels. Increased cytosolic calcium stimulates phospholipase A2 which catalyzes hydrolysis of membrane phospholipids with the release of arachidonic acid from sn-2 position. Arachidonic acid is then converted into eicosanoids including prostaglandins, thromboxanes, and leukotrienes by the enzymes cyclooxygenases (COX-I or COX-II), or lipoxygenase (Fig. 6.5). While PGE2 [80] and PGD2 [81, 82] stimulate glycogenolysis in hepatocytes [83], thromboxane A2 and leukotiene C4 cause hepatic vasoconstriction [84, 85]. Kupffer cell-derived PGE2 has also been implicated in hepatoprotection [86], putatively by inhibiting synthesis of TNF-α and prostaglandins [87, 88].

Kupffer cells are the primary hepatic site of the synthesis of a potent lipid mediator platelet-activating factor (PAF) in response to particulate matter [89] or LPS stimulation [83, 90, 91] (Fig. 6.5). Experimental findings that administration of PAF causes effects similar to those of LPS, such as hypotension, cardiac failure, vasoconstriction, and vascular leakage indicate that this lipid mediator plays a critical role

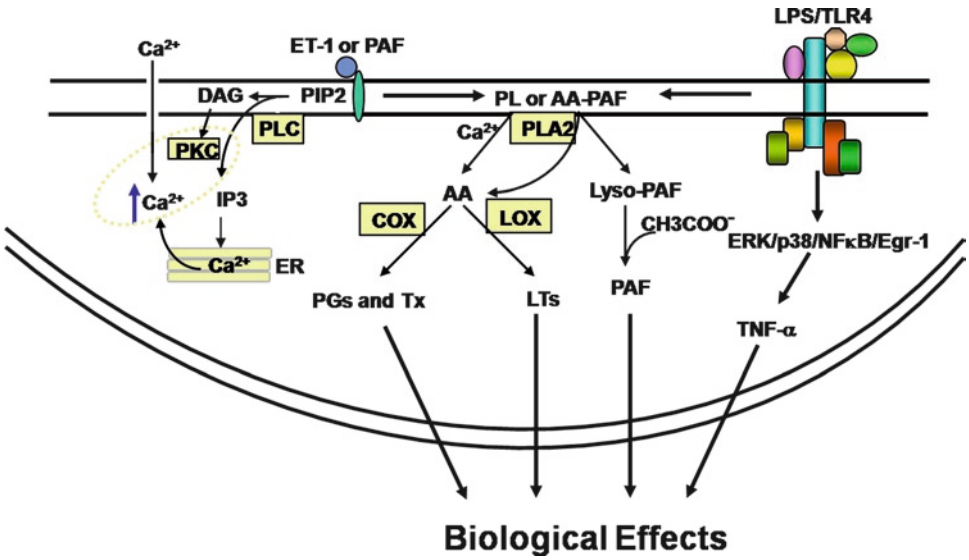

Fig. 6.5 Mechanisms of the effects of endothelin-1, PAF, and LPS on Kupffer cells. Endothelin-1 (ET-1) or platelet activating factor (PAF) bind to the G-protein coupled receptor and stimulate phospholipase C (PLC)-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into DAG and inositol-1,4,5-trisphosphate. IP3 causes release of calcium from intracellular stores (endoplasmic reticulum: ER), and DAG stimulates protein kinase C (PKC). The binding of ET-1 or PAF also facilitates entry of extracellular calcium. Increased cytosolic calcium stimulates phospholipase A2 that causes hydrolysis of phospholipids or 1-alkyl,2-arachidonyl-3-phosphocholine with the release of arachidonic acid, and formation of lysophospholipids or lyso-PAF. Lyso-PAF is converted to PAF with addition of acetyl moiety at sn2 position. Arachidonic acid is metabolized to prostaglandins (PGs) + thromboxanes (TXs) via the action of cyclooxygenase (COX), or to leukotrienes via the action of lipoxygenase. PAF and the arachidonic acid metabolites are released, and exert actions on the other cells of the liver. LPS also stimulates phospholipase A2 and subsequent reactions, in addition to stimulating the synthesis of inflammatory mediators via activation of ERK1/2 or p38 MAPK, NFκ-B or Egr-1

in LPS-induced biological responses *in vivo* [92]. PAF binds to a G-protein coupled receptor on Kupffer cells and stimulates synthesis of arachidonic acid metabolites [83, 93–95] (Fig. 6.5). LPS also stimulates synthesis of a potent vasoconstrictor endothelin-1 (ET-1) in rat liver [96], in hepatic stellate cells [97] and endothelial cells [98]. ET-1 instigates phospholipase C/phosphoinositide signaling cascade in Kupffer cells coupled to synthesis of prostaglandins and PAF [94, 95, 99] (Fig. 6.5). In isolated perfused rat liver, portal infusion of PAF as well as ET-1 causes vascular resistance and glucose output [100–102]. PAF administered via portal vein in situ causes hepatic vasoconstriction and systemic hypotension [103]. Such intrinsic mechanism of the synthesis of PAF, ET-1, and prostanoids, involving Kupffer cells, can be an integral part of metabolic changes and hemodynamic alterations acutely (ischemia/reperfusion) and in chronic liver diseases (portal hypertension). PAF has also been shown to be a mediator of LPS-induced liver injury in partially hepatectomized rats [104].

The binding of PAF to its receptor causes activation of NFκ-B [105, 106] and up-regulation of the TNF-α gene [107]. While PAF on its own was found to cause modest stimulation of TNF-α and superoxide generation, much greater level of synthesis of both TNF-α and superoxide induced by LPS (receptor-mediated), or zymosan (phagocytosis) are abrogated by PAF receptor antagonism [108]. The authors concluded that the PAF antagonists elicited such effect by interfering with LPS- and zymosan-induced signal transduction. On the other hand, LPS and PAF were reported to exert additive effect on the synthesis of nitric oxide in Kupffer cells [109].

At high concentrations, LPS can activate Kupffer cells indirectly by triggering complement activation either in the portal or in the systemic circulation [110]. The complement factors C3a and C5a stimulate phospholipase C cascade of signaling with the synthesis of ROS including superoxide (via NADPH oxidase), eicosanoids, and cytokines [111–113]. While ROS activate signaling pathways responsible for physiological processes and killing of the invading microorganisms [114], they are also implicated in the initiation and progression of liver pathologies [115, 116]. ROS and their lipid peroxidation products have been shown to damage macromolecules including DNA and to cause death of several cell types including hepatocytes [117–121]. Thus activated Kupffer cells, by producing an array of biologically active mediators, and by interacting with factors released in the hepatic microenvironment by other cell types, play critical roles in a variety of pathophysiological conditions. These conditions are described in the following sections.

Role in Liver Regeneration

Molecular basis of liver regeneration is discussed independently in Chap. 18.

The liver responds to many types of injuries (toxin-, chemical-, or virus-induced; partial resection to excise tumors; and transplantation) that cause cellular loss by initiating robust regenerative response. Most of the factors identified to influence regeneration process following injury are produced by the liver itself, but the contribution of humoral factors is also of significant importance. While powerful mitogens such as epidermal growth factor (EGF), transforming growth factor (TGF)-α, and hepatocyte growth factor (HGF); co-mitogens such as insulin, glucagon, insulin-like growth factor (IGF), and priming agents TNF-α and IL-6 appear to facilitate this process, the role of growth inhibitors, TGF-β and possibly IL-1β that are upregulated contemporaneously, is considered equally important. [122–124]. Many of these factors (HGF, TGF-α, TNF-α, IL-6, IL-1β, and TGF-β) are produced by Kupffer cells.

Substantial information has been obtained in regard to the role of Kupffer cells in hepatic regeneration and liver injury by inducing blockade of their functions by gadolinium chloride (GdCl3), or their elimination by liposome-encapsulated clodronate. Mice treated with clodronate show much lower level of HO-1 and eNOS expression and blunted regenerative response than control mice following resection [125]. Kupffer cells also promote liver regeneration by increased expression of ICAM-1 that facilitates recruitment of leukocytes. Liver regeneration after partial hepatectomy has been reported to be impaired in ICAM-1 knockout mice [126].

Stimulation of hepatic DNA synthesis by TNF-α administration [127, 128], increase in its concentration before activation of transcription factors STAT-1, c-jun, c-fos, AP-1, and NFκ-B [129–131], and its ability to cause fourfold increase in the mitogenic response of hepatocytes to HGF and TGF-α [132], indicate the importance of TNF-α in hepatic regeneration. Pretreatment of rats with neutralizing antibodies against TNF-α before partial hepatectomy was also found to inhibit hepatocyte proliferation for up to 72 h after the surgery [51, 133]. Because serum concentrations of TNF-α are not elevated after partial hepatectomy, it was suggested that liver injury probably results in local release of TNF-α [51]. Indeed, Kupffer cells are found to be a major source of intrahepatic TNF-α as indicated by lower levels of TNF-α and slow regeneration in Kupffer cell-depleted mice as compared to the control mice [126]. Comparison of early molecular responses to partial hepatectomy in anti-TNF-α-treated and control animals indicated that the trophic actions of TNF-α involve activation of JNK [134], several growth-related transcription factors (c-Jun [130], CCAAT enhancer binding protein-p (C/EBP+), and C/EBP-8 [135]) within minutes of liver injury. There is evidence that TNF-α elicits its actions in hepatic regeneration via IL-6 [136]. It has been shown that TNF-α stimulates IL-6 synthesis in Kupffer cells [137] and the defect in hepatic regeneration in animals lacking TNF receptors is restored by administration of IL-6 [138].

In contrast to the findings described above, rodents in which functions of Kupffer cells were blocked by pretreatment with GdCl3 still demonstrated increased TNF-α, IL-6 mRNA expression, and enhanced liver regeneration in association with enhanced signaling coupled to mitogenic responses [139]. The alternate or additional source of these cytokines remains to be determined. Although stellate cells produce both TNF-α and IL-6 in response to LPS [25, 140, 141], whether they synthesize these cytokines in partially hepatectomized animals is unclear. Kupffer cells were also found to exert detrimental effect on regeneration in mouse partial liver transplantation model, in which GdCl3 pretreatment improved portal blood flow and sinusoidal perfusion with superior survival outcome [142]. Similar outcome of partial liver transplantation in TNF receptor I knockout mouse led the authors to conclude that signaling events involving Kupffer cell-derived TNF-α is responsible for poorer results in control mice [142].

Role in Ischemia/Reperfusion Injury

The liver undergoes injury during procurement and cold preservation prior to transplantation, and during surgical resection when the blood supply is interrupted. The injury is escalated upon reperfusion of the graft, or of the residual post-resection liver due to accumulated, and newly formed ROS [120, 121]. The reperfusion injury is of even greater concern in case of marginal donor- and small-for-size (living-related donor) liver transplantation [143–145]. Amelioration of portal venous clamping-induced [146, 147] as well as transplantation-related [40] ischemia/reperfusion injury in rats by GdCl3 blockade of Kupffer cells, indicates their role in the pathological developments. Experimental evidence from several laboratories demonstrated that activation of Kupffer cells is an important multifactorial cause of ischemia/reperfusion injury. Kupffer cells have been found to be activated during cold preservation of the graft [86]. Furthermore, LPS, levels of which are generally high in patients with liver diseases and remain elevated for several days following liver transplantation [148, 149], can induce activation of Kupffer cells. Activation of Kupffer cells is associated with morphological alterations that cause reduction in sinusoidal blood flow [147, 150]. As described before, activated Kupffer cells also produce increased amounts of ROS, several proinflammatory cytokines such as TNF-α, IL-6, IL-1, arachidonic acid metabolites, PAF, and chemokines [30, 52, 151, 152]. In addition to causing damage to hepatocytes directly, Kupffer cell-derived

cytokines and chemokines also recruit inflammatory polymorphonuclear leukocytes and neutrophils, which escalate and perpetuate the liver injury [153–158].

The role of TNF-α and IL-6 in ischemia/reperfusion injury is of high significance. CO inhalation by recipient animals results in inhibition of hepatic TNF-α and IL-6 expression, and reduced ischemia/reperfusion injury to the transplanted graft [40]. Treatment with neutralizing TNF-α antibody also ameliorates ischemia/reperfusion injury [157, 158]. Reduced LPS-induced release of TNF-α and IL-6 by CO-treated, cultured Kupffer cells [40] not only supports the role of these macrophages in ischemia/reperfusion injury, but also suggests a therapeutic avenue. While there is overwhelming evidence for significant contribution of Kupffer cells to ischemia/reperfusion injury, activated Kupffer cells also produce anti-inflammatory mediators such as IL-10 and IL-13 [151, 152] suggesting an internal mechanism of limiting the injury caused by proinflammatory cytokines and ROS.

Another mechanism by which Kupffer cells cause and perpetuate ischemia/reperfusion injury is by producing PAF [83], or by releasing TNF-α, or TGF-β that stimulate synthesis of ET-1 in endothelial cells and stellate cells [159–163]. Both ET-1 and PAF are potent constrictors of the hepatic vasculature [102, 164]. Blockade of endothelin actions has been shown to ameliorate portal venous ligation-induced as well as transplantation-related ischemia/reperfusion injury [165–168]. Reduced endothelin secretion and improved liver function after warm ischemia/reperfusion in rats pretreated with GdCl3 [169], confirm the role of Kupffer cell in this pathology by stimulating ET-1 synthesis. PAF antagonism has also been shown to ameliorate ischemia/reperfusion injury induced by portal vein occlusion and liver transplantation [170–172].

Role in Alcohol-Induced Liver Injury

Alcohol consumption causes increased gut permeability and increase in the circulating levels of endotoxin (LPS) [173, 174]. Increased LPS elicits hepatic inflammatory response, which is primarily due to inflammatory cytokines produced by Kupffer cells. Work from several laboratories has shown that Kupffer cell activation and inflammatory mediators produced by them including TNF-α, IL-6, IL-1α, and IL-1β play a critical role in the onset and progression of ethanol-induced liver injury. While steatosis is the most common manifestation of alcohol-induced hepatic pathology in almost all of laboratory animals fed with alcohol, they do not develop hepatic fibrosis that is seen in advanced alcoholic liver disease in humans [131, 175]. Kupffer cell-derived inflammatory mediators and ROS have been proposed to play an important role in fibrosis development by inducing activation of hepatic stellate cells to the fibrogenic phenotype [176–178] (see also Chap. 5). The early events of the pathologic progression (fatty liver and inflammation) were found to be prevented upon depletion of Kupffer cells in rats fed with alcohol, intragastrically [179].

Kupffer cell-derived TNF-α plays an important role in alcohol-induced hepatic injury. Its concentration is found to increase in the blood of alcoholics [180, 181] and rats fed alcohol via gastric infusion [174]. Alcohol-induced liver injury is ameliorated in rats administered with anti-TNF-α antibodies [182] in TNF receptor 1-knockout mice [183], and in rats pretreated with antisense oligonucleotide targeted against TNF-α mRNA [184]. Since repeated exposure to LPS both in vivo and in vitro causes endotoxin tolerance, it is expected that Kupffer cell responses to LPS should be desensitized during ethanol feeding [185]. Interestingly, however, long-term ethanol consumption increases the susceptibility of rats to endotoxin-induced liver injury [186, 187]. Furthermore, alcohol feeding hypersensitizes Kupffer cells to LPS actions as illustrated by fourfold greater increase in TNF-α release by cells isolated from rats fed with alcohol in response to LPS compared to pair-fed rats [79]. This effect was attributed to enhanced activation of ERK1/2 and increased binding of early growth response-1 (Egr-1) to the TNF-α promoter upon LPS stimulation [79]. Prevention of LPS-induced NFκ-B and ERK1/2 activation, and TNF-α production in Kupffer cells from rats ingesting alcohol chronically by antioxidant dilinoleoylphosphatidylcholine [188] suggests that these effects are dependent upon generation of reactive oxygen species. In LPS-stimulated Kupffer cells, ROS are generated by membrane NADPH oxidase since its specific inhibitor diphenyliodonium abrogated ERK1/2 phosphorylation, and ROS, and TNF-α production [189, 190].

The above-mentioned intracellular signaling events coupled to LPS-induced increased TNF-α synthesis by Kupffer cells isolated from alcohol-fed animals may be explained by increased CD14 (receptors for LPS) expression in Kupffer cells, and elevated hepatic LBP concentration [191]. Up-regulation of CD14 in Kupffer cells by LPS [58], and reduced alcohol-induced liver injury in CD14-knockout mice [192], as well as LBP-knockout mice [193] strongly support LPS-Kupffer cell interactions in the pathology of alcoholic liver disease. Although unaltered [194] as well as increased [195] expressions of TLRs (particularly TLR4 and TLR2) have been reported in alcohol-fed animals, TLR4-knockout mice were shown to be resistant to alcohol-induced injury [196].

The other pathway by which Kupffer cell-derived TNF-α contributes to alcoholic liver disease is by increasing the generation of Th1 cytokines IL-6, IFN-γ, and IL-12 [197]. These authors also showed much greater levels of bioactive TNF-α and the Th1 cytokines by endotoxin treatment of mice on high fat, and alcohol diet relative to those on high fat diet alone.

Role in Non-alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease in Western countries [198–200], with insulin resistance as the hallmark of its pathophysiologic progression [201]. NAFLD encompasses a wide range of pathologies, which include development of simple steatosis found in obese people that can progress to nonalcoholic steatohepatitis (NASH), decompensated cirrhosis and hepatocellular carcinoma. Many features of NAFLD are very similar to alcoholic liver disease with steatosis and inflammation driving each other's progression. TNF-α produced by activated Kupffer cells plays an important role in the development of steatohepatitis [202] and hepatic fibrosis associated with NASH [203]. The importance of TNF-α in NAFLD is supported by observations that its increased release is associated with development of insulin resistance and fibrosis [204, 205], and its deletion protects animals from high fat diet-induced insulin resistance [206]. TNF-α-induced JNK activation has been shown to inhibit tyrosine kinase activity of the insulin receptor and is suggested to be a critical mechanism of obesity-induced insulin resistance [207].

The low level subacute activation of NFκ-B in transgenic mice expressing IKK-β was found to mimic the inflammatory pathways leading to insulin resistance and fat deposition seen in mice fed high fat diet [208]. This effect, also observed systemically, was attributed to synthesis of proinflammatory cytokines including TNF-α, IL-6, and IL-1β in Kupffer cells, and could be alleviated by IL-6 neutralization [208]. Hepatic and circulating IL-6 levels have been shown to be increased in NAFLD and exhibit correlation with the severity of inflammation, fibrosis, and insulin resistance [209]. Based on the evidence that TNF-α-induced hepatic regeneration is mediated by IL-6 [136–138] and protection of high fat-induced insulin resistance by blockade of TNF-α [206], it is likely that IL-6 is a mediator of the effects of TNF-α in these conditions.

In addition to their direct involvement, activated Kupffer cells also produce CXC (e.g., IL-8) and CC chemokines (e.g., MCP-1) that induce recruitment and activation of neutrophils and polymorphonuclear leukocytes, and subsequent fibrogenic activity via transdifferentiation of stellate cells [210]. Activation of Kupffer cells in NAFLD, similar to alcoholic liver disease, appears to involve LPS as indicated by a high frequency of small intestinal bacterial overgrowth, and endotoxemia in patients with NASH [211]. Although activation of Kupffer cells appears to be critical in the development of NAFLD, mice with increased constitutive activation of NFκ-B in myeloid cells due to lack of Iκ-B kinase also develop insulin resistance [212].

Recent work has indicated decreased negative regulation of NAFLD progression due to reduced anti-inflammatory cytokine production by Kupffer cells. Such negative regulation is provided by IL-10, the synthesis of which is stimulated by adiponectin, an anti-inflammatory adipokine [213]. Furthermore, adiponectin also inhibits LPS-stimulated TNF-α synthesis in Kupffer cells [214]. Circulating adiponectin levels were found to be reduced in alcoholic liver disease [214, 215] and NASH [216]. Interestingly, adiponectin receptors were found to be underexpressed in visceral fat, but overexpressed in the liver in NASH [216]. The importance of this adipokine as an anti-inflammatory agent and antisteatotic agent is underscored by the observations that hepatic triglycerides, inflammation, oxidative stress, and insulin resistance are increased upon deletion of adiponectin receptors, and steatosis is reduced in genetically obese mice overexpressing adiponectin [217].

Role in Regulation of Immune System

Being exposed continuously to a variety of noxious substances, including antigens and bacterial products, the liver has an obligation to respond to them rapidly and effectively. The liver eliminates circulating $CD8^+$ T cells specific for systemically disseminated antigens, and thus plays a critical role in preventing generalized inflammation [14, 218–220]. Hepatic natural killer cells, dendritic cells, and Kupffer cells form an integral and a major part of the hepatic innate immune system to provide such response and clear the potentially dangerous external attack [221]. Expression of a number of TLRs including TLR2, TLR4, and TLR9 by Kupffer cells and their response to stimulation of these receptors [76, 77, 195], attests to the seminal importance of Kupffer cells in hepatic innate immune system. Kupffer cells express scavenger receptors that recognize Fc component of the immunoglobulins and activated complement factor C3b, thus eliminating IgA- and IgM-coated particles and immune complexes [222–225]. Kupffer cells also phagocytose neutrophils and thereby contribute profoundly to the adaptive immune functions of the liver.

The liver is also naturally tolerant as evident from the persistent viral (hepatitis virus B and hepatitis virus C) and parasitic (malarial parasite) infections [226–228]. Such immunological privilege is also evident from the rare incidence of accelerated liver allograft rejection across the ABO barriers and is caused by the generation of antidonor antibodies seen frequently in kidney and heart transplantation [229–231]. Chronic rejection of the liver allograft is also infrequent as compared to the other organs [232, 233]. Kupffer cells can act as liver-specific professional antigen-presenting cells by expressing class I and class II MHC and co-stimulatory molecules (CD40, CD80, and CD86), and thus activate infiltrating and resident T cells, and NKT cells in an antigen-specific and MHC-restricted manner. However, in vitro experiments

demonstrated that despite the repertoire of antigen presentation machinery, the efficiency of Kupffer cells to present antigen and activate $CD4^+$ T cells is inferior to the macrophages derived from spleen, or bone marrow [234].

Abrogation of allograft survival induced by portal vein administration of allogeneic donor cells upon blockade of Kupffer cell functions [235] indicated their role in providing tolerance. Kupffer cells isolated from chronically accepted liver allografts were found to cause apoptosis of alloreactive T cells, and their infusion was found to prolong the survival of hepatic allografts in an acute rejection model [236]. A mechanism of such tolerance could be via generation of immunosuppressive cytokines IL-10 and TGF-β by Kupffer cells probably in response to LPS in the portal circulation [234]. Hepatic sinusoidal endothelial cell- as well as Kupffer cell-induced activation of CD4+ T cells was shown to be suppressed by IL-10 through down-regulation of receptor-mediated antigen uptake, and inhibition of cell surface expression of class II MHC and co-stimulatory molecules [237]. Moreover, IFNγ, and LPS [238], as well as phagocytosis [239] induce overexpression of FasL in Kupffer cells. Thus FasL-induced death of T cells [219, 221, 227, 239, 240] can be an additional important mechanism of liver tolerance.

Summary and Perspectives

The seminal role of Kupffer cells in protecting the liver and other organs from harmful substances and microorganisms is unquestionable. In addition to performing these functions by their phagocytic and endocytic activities, Kupffer cells also produce a variety of cytokines and ROS that destroy the microorganisms. By producing cytokines and growth factors, Kupffer cells also play an important role in hepatic regeneration when required. Furthermore, they have the ability to recruit white blood cells in performing these functions. However, Kupffer cells also contribute to pathological developments by persistently producing higher levels of the very same cytokines, chemokines, and growth mediators as well as ROS. These pathological conditions include, but are not limited to, reperfusion injury, alcoholic liver disease, NASH, and failure of transplanted liver graft. Depletion of Kupffer cells or blocking their functions to understand their role in physiology and pathology has provided critical information. However, evidence for both beneficial and detrimental roles of Kupffer cells in hepatic pathology has emerged from these studies. A more elaborate examination of Kupffer cell functions, as well as their interactions with other hepatic cell types, especially endothelial cells to which they are attached, stellate cells in the perisinusoidal space of Disse, and cells of the immune system (both resident and recruited via circulating blood) will be required to exploit Kupffer cells in liver health and disease.

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Chapter 7
Sinusoidal Endothelial Cells

Donna Beer Stolz

The Liver Lobule, Microcirculation, and the Sinusoidal Endothelial Cell

In all organs in the body, the vasculature is lined by simple, squamous epithelium, properly termed endothelium. However, the endothelia represent an extremely heterogeneous population of cells, and those of each organ, or more appropriately, each specific functional part of an organ, maintain characteristic features that enable the vasculature to perform particular roles at the blood–tissue interface [1, 2]. Regardless of the organ in question, the endothelia in normal tissues maintain a nonthrombogenically lined conduit through which a variety of blood cells and vehicle (plasma) assist in delivering oxygen, nutrients, and maintenance factors to, while removing debris, waste, and breakdown products from the underlying tissue. While the liver sinusoidal endothelium undeniably perform these duties, they possess additional phenotypes with a wide variety of unique capabilities essential to maintaining liver function.

The capillaries of the liver are more properly called liver sinusoids since they are lined with discontinuous endothelium that do not possess a continuous basal lamina, similar in structure, but not function, to those of lymphoid tissue and some endocrine organs [3] (Fig. 7.1). This vasculature is distinctive among the endothelial population, in that, liver sinusoidal endothelial cells (LSEC) in normal, adult liver have fenestrations without semipermeable proteinaceous diaphragms (Fig. 7.2). Fenestrations (Latin fenestrae: "window") are transcellular pores with an average diameter of approximately 160 nm (in rats) that represent a porosity of about 6–10% along the hepatic microvascular surface [3]. First described in great detail by Dr. Eddie Wisse in the early 1970s [3, 4], these pores are concentrated in attenuated areas of the LSEC and arranged in groups of 20–50 fenestrations called sieve plates. There are reported differences in porosity, fenestration diameter, and basement membrane deposition among different mammalian and nonmammalian species, but the general architectural phenotype in the hepatic sinusoids is surprising well retained [5–8].

In contrast, the larger vessels of the liver lobule (i.e., portal and central veins and venules, and hepatic arteries and arterioles) are not fenestrated and possess the characteristic continuous basal lamina found in microvascular endothelium of other organs as well as larger vessels [9]. The endothelia of central venules are very smooth and often contain numerous attached leukocytes [9] (Fig. 7.3). However, the endothelia of the portal venules display a rippling of membrane at areas of cell–cell contact and rarely show attachment of leukocytes to the vessel wall under normal situations (Fig. 7.1c). Little is known about the function of this morphology, but it likely represents a potentially reactive surface by which circulating leukocytes may gain access to the underlying space of Mall, the fluid, and extracellular matrix rich area situated around the portal triad [10, 11]. There is a very abrupt morphological change in the endothelium when the portal venule transitions into the sinusoid. Here, the continuous endothelia of the portal venule become fenestrated immediately at the opening of the sinusoid (Fig. 7.1c). Likewise, the LSEC lose their fenestrations as the vessel merges with the central venules (Fig. 7.3a).

In a healthy individual at rest, hepatic blood volume represents approximately 25% of the total cardiac output and the liver itself is 25–30% blood by volume [10]. The total volume of blood circuits through the liver over 300 times in one 24-h period. Given this expansive reservoir of blood, it follows that the liver is a highly vascularized organ and a large percentage of the total body blood volume is in contact with the hepatic microvasculature at any given time. Approximately 75% of the hepatic blood enters the liver through the portal vein via the hepatic portal circulation directly from the intestines, spleen, and pancreas. This blood is nutrient rich, but oxygen poor. Additionally, under physiologic conditions, endotoxin concentrations in portal venous blood are usually between 100 pg/mL and 1 ng/mL [12]. The remaining 25% of the blood originates from the hepatic artery, a branch of the celiac artery, and brings oxygenated blood to the liver. These two sources of

D.B. Stolz (✉)
Department of Cell Biology and Physiology,
University of Pittsburgh, Pittsburgh, PA, USA
e-mail: dstolz@pitt.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_7, © Springer Science+Business Media, LLC 2011

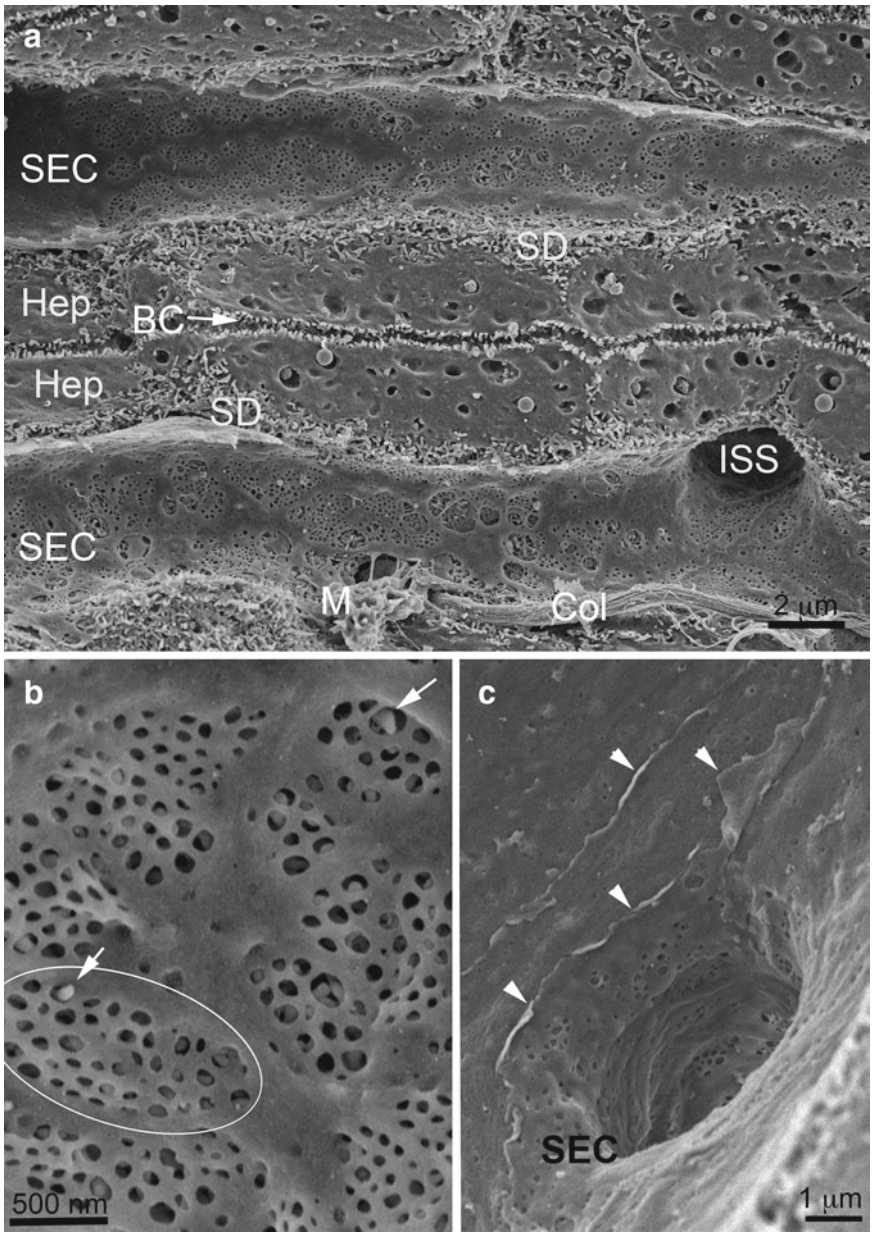

Fig. 7.1 Scanning electron micrographs of mouse liver sinusoidal endothelial cells. (**a**) A low magnification image accentuating the anatomy of the hepatic plate bounded by sinusoids. Fenestrated sinusoidal endothelial cells (SEC) line the sinusoids on either side of a hepatic plate comprised of two rows of hepatocytes (Hep) separated via their apical membrane by the bile canaliculus (BC). An intersinusoidal sinusoid (ISS) is observed entering one of the sinusoids. The ISS link parallel microvascular tracts in the liver. Also, a macrophage (M) is observed on the surface of a sinusoid, and a collagen bundle (Col) is observed emerging from a space of Disse (SD). (**b**) A high magnification micrograph of a sinusoid highlighting sieve plates (collections of fenestrations, *circled*) with the occasional hepatocyte basolateral microvillus poking through a fenestration into the sinusoid lumen (*arrows*). (**c**) Transition of the portal venule into the sinusoid (SEC). Endothelium in the portal venule have areas of raised membrane at cell–cell junctions (*arrowheads*). There is an abrupt change of nonfenestrated to fenestrated endothelium from the portal venule to the sinusoid

blood mix directly in the sinusoid as they exit the portal-triad area. The blood then continues through the length of the lobule via the sinusoids, to exit at a centrilobular hepatic venule where it empties into the hepatic vein to rejoin the systemic circulation via the inferior vena cava. This configuration results in metabolic zonation along the lobule that is driven primarily by an oxygen-tension gradient [13]. Blood entering periportal sinusoids have a pO_2 (partial pressure) of about 60–65 mmHg (~8% O_2), while for blood exiting the perivenous sinusoids, the pO_2 is only about 30–35 mmHg (~4% O_2). Heterogeneity in the LSEC along this gradient has been reported and reflects their

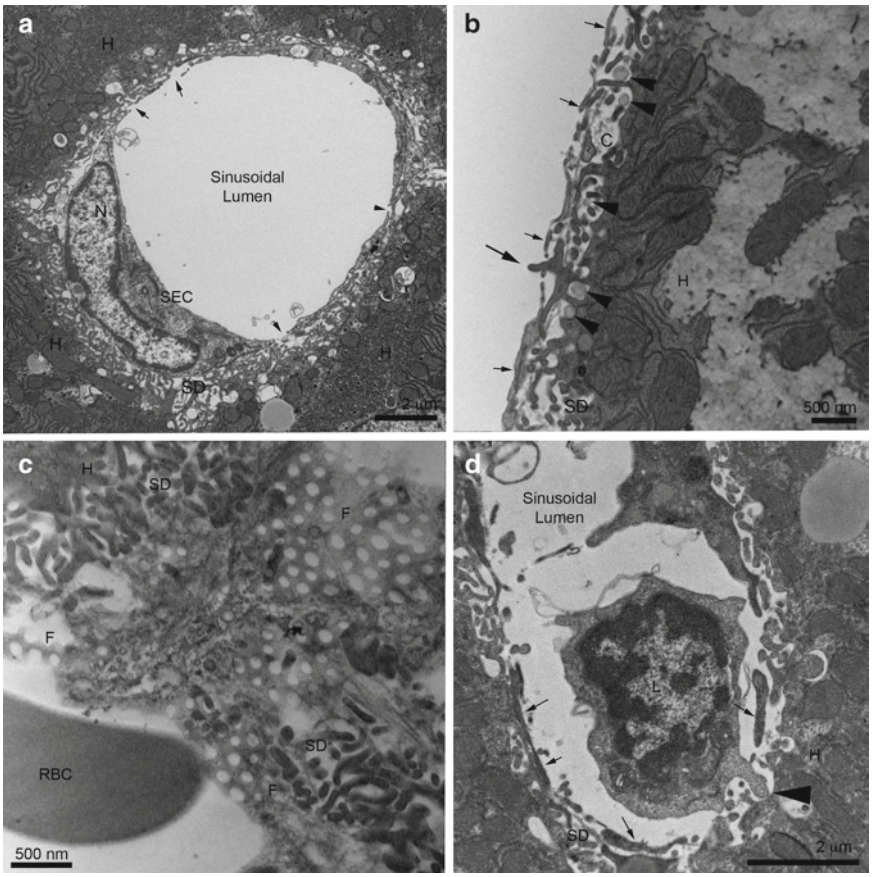

Fig. 7.2 Transmission electron micrographs of the hepatic sinusoid of a mouse. (**a**) Cross section through a typical sinusoid in the liver. The sinusoidal endothelial cell (SEC) outlines the sinusoidal lumen. Areas of fenestrations and/or gaps in the SEC are indicated by *arrows*. The SEC nucleus (N) is observed in this section. Separated by the space of Disse (SD), hepatocytes (H) surround the sinusoid. (**b**) High magnification of the space of Disse (SD) which is bounded by the SEC (*small arrows*) and the hepatocyte (H). A hepatocyte microvillus is observed penetrating through a fenestration in this field (*large arrow*). Chylomicron remnants are seen within the space of Disse (*arrowheads*). There is no basement membrane observed in the space of Disse. (**c**) Tangential section through a sinusoidal endothelial cell, accentuating the fenestrations (F) and the hepatocyte (H) microvilli within the space of Disse (SD). A red blood cell (RBC) is observed in the sinusoidal lumen. (**d**) A lymphocyte coursing through the sinusoid pokes a pseudopod through a larger fenestration of the sinusoidal endothelium (*arrows*) to make contact with the underlying hepatocyte (*arrowhead*)

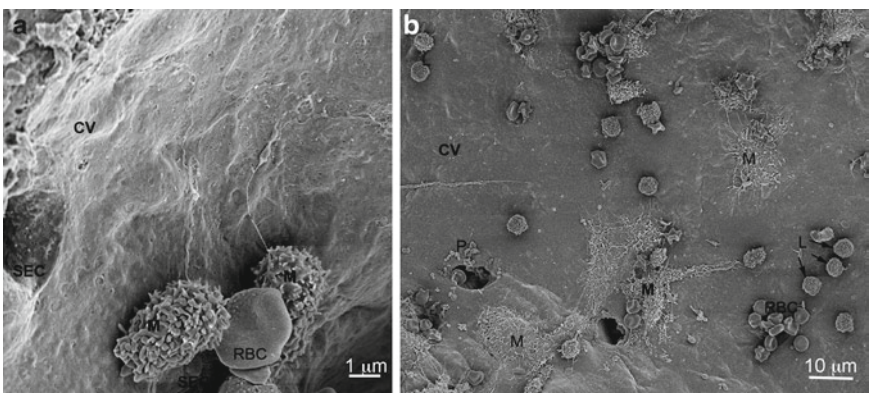

Fig. 7.3 Scanning electron microscopy of the central venules of a mouse liver. (**a**) Fenestrated endothelium (SEC) abruptly lose their fenestrations when merging with the smooth surface of the central venule (CV). Attached to the central venule surface are two macrophages (M) as well as a red blood cell (RBC) at an area exiting the sinusoid. (**b**) A lower magnification view of the central venule showing many flattened macrophages (M) and attached leukocytes (L), as well as red blood cells (RBC), and platelet deposition (P) on the CV surface

function in the lobule including changes in porosity, specific lectin binding, and endocytic capability.

While the blood courses through the sinusoids, the role of the liver in metabolism, detoxification, endocrine, and immune function come into play. Given its peculiar confluence of blood supplies, it has become especially apparent that the liver is truly an immunological organ [14, 15]. Environment sensing is an important function in the liver since, the portal supply transporting blood primarily from the intestines can contain many food and bacterial antigens that have navigated through the intestinal barrier. Liver nonparenchymal cells, specifically Kupffer cells, a variety of leukocytes, and the sinusoidal endothelium, are all poised to react to changes, either in content or concentration, of these antigens. In cases of inflammation, the endothelia serve as a gatekeeper to the infiltration of inflammatory cells into tissue, by modifying their surface markers to allow for cell–cell interactions with circulating immune cells, assisting in their retention or homing to their correct destination.

Organization of Cells Relative to the Liver Sinusoidal Endothelial Cell

Hepatocytes are the parenchymal cells of the liver and constitute the largest number (60%) and volume (80%) of cells in the organ [14, 16]. Hepatocytes are arranged in anatomizing plates that are one cell thick and are bounded on either side by the sinusoids (Fig. 7.4). Lined by the sinusoidal endothelium, several other nonparenchymal cells are present and have specific roles within this environment. LSEC make up the second most abundant cell type in the liver, (about 50% of the nonparenchymal cells) and are positioned between the blood and the hepatocytes [14] (Figs. 7.1, 7.2, 7.4, and 7.5). As the lining of the specialized liver capillary, they occupy a unique niche where they directly interact with nearly all of the other cell types in the liver.

Along the luminal aspect of the sinusoid, LSEC interact quite intimately with Kupffer cells, the resident macrophages in the liver that account for about 20% of the nonparenchymal cells [17]. A more comprehensive account on this cell type is

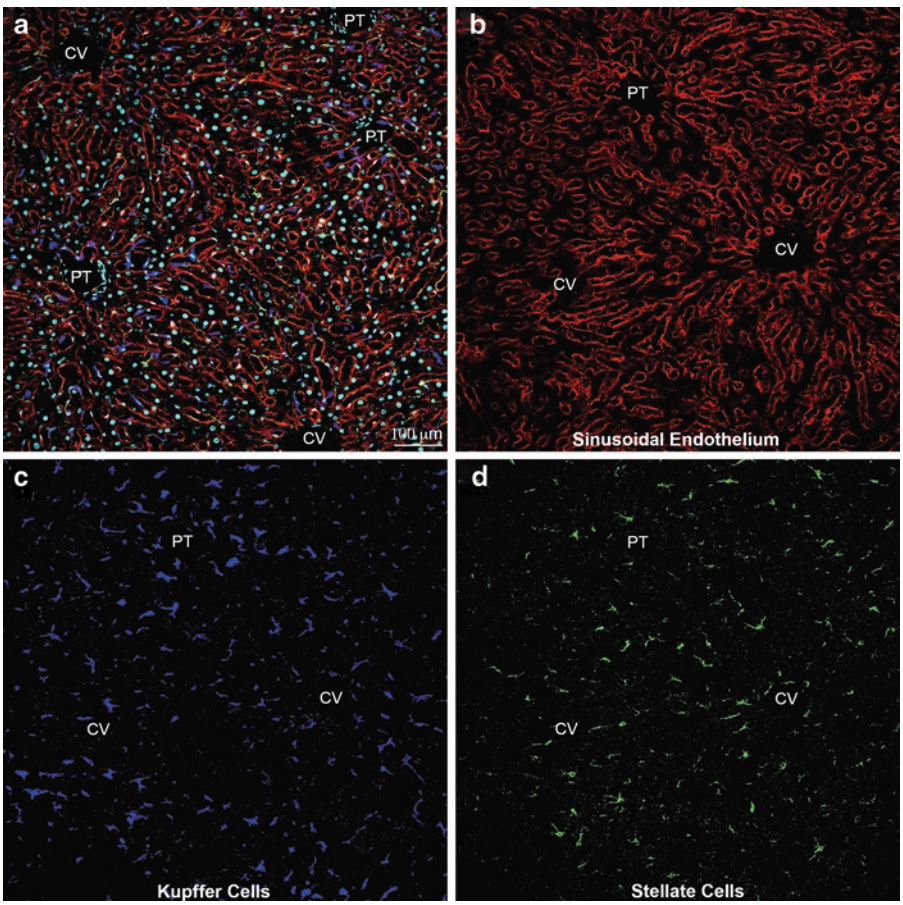

Fig. 7.4 Distribution of parenchymal and nonparenchymal cells in the liver. (**a**) Merged image from separate field with a Hoechst nuclear counterstain pseudocolored in cyan. Larger, round nuclei represent the hepatocytes. *CV* central venule; *PT* portal triad. (**b–d**) Low magnification confocal stack reconstruction image of a paraformaldhyde fixed, frozen rat liver section immunostained with antibodies specific for sinusoidal endothelial cells (goat anti-CD32b, *red* (**b**)), Kupffer cells (Mouse anti-ED2, *blue* (**c**)), and stellate cells (rabbit anti-GFAP, *green*(**d**))

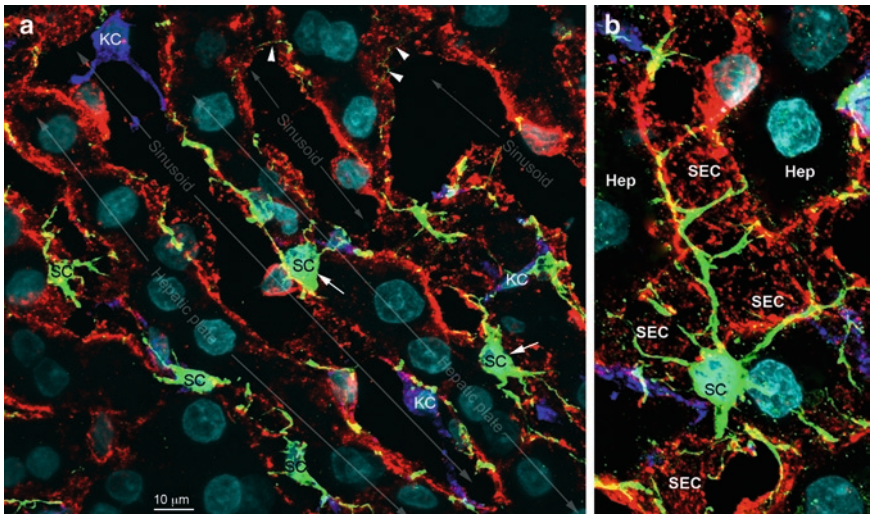

Fig. 7.5 High magnification reconstructed confocal stack from the same tissues as shown in Fig. 7.4. Sinusoidal endothelial cells are stained red, Kupffer cells are stained *blue*, and stellate cells are stained *green*. Nuclei are pseudocolored cyan. (**a**) Hepatic plates (with large, round nuclei) are bounded by the sinusoids lined by sinusoidal endothelium. Kupffer cells (KC) are found attached to the luminal side of the SEC. Stellate cells (SC) occupy spaces under the SEC and often extend processes through the hepatic plate to span between two sinusoids (*arrows*). Stellate cell processes, even very tiny projections, can be seen throughout the space of Disse (*arrowheads*). (**b**) Area of the liver lobule showing a stellate cell enveloping a large area surrounding a sinusoid

available in Chap. 6. Kupffer cells are an important first line of defense to eliminate and respond to particulate bacterial components that arrive from the gastrointestinal tract via the portal blood supply. Located throughout the lobule, they are more concentrated in portal areas, where they can act as sentinels and remove gut-derived products (Fig. 7.4). Kupffer cells migrate along the LSEC surface and often impede blood flow, facilitating interactions between the nonparenchymal cells and circulating leukocytes [18].

Pit cells, liver-associated natural killer cells, and part of the diverse hepatic leukocyte population, also occupy the sinusoidal lumen and interact with LSEC and Kupffer cells [19, 20]. Pit cells are about tenfold less abundant than Kupffer cells and possess the ability to kill tumor cells but are also thought to be involved in eliminating virally-infected cells [21–24]. Other leukocytes routinely found in the liver include dendritic cells, T cells, and B cells [14]. While dendritic cells are usually localized to spaces around the large vessels, B and T cells tend to circulate within the sinusoids, as well as collect in areas around the large vessels.

Abluminally, stellate cells (also called Ito cells, lipocytes, fat storing cells, or perisinusoidal cells) envelop the basolateral aspect of the LSEC between the vascular cells and the hepatocytes in the area called the space of Disse. Additional details on this cell type are provided in Chap. 5. Since they surround the LEC and are capable of contraction, they are often considered a sinusoidal pericyte [25, 26]. They comprise about 6–15% of the nonparenchymal cells and under quiescent conditions store retinoids, triglycerides, cholesterol as well as free fatty acids [16]. Stellate cells can also become activated under various inflammatory insults and transdifferentiate into a myofibroblastic phenotype [26]. Under activated conditions, the stellate cells lose their lipid stores, proliferate, and secrete a variety of cytokines and extracellular matrix proteins and as such are described as the main cell responsible for liver fibrosis and cirrhosis. Changes in extracellular matrix composition and increased matrix deposition within the space of Disse has profound effects on LSEC, causing loss of fenestrations (called capillarization or pseudocapillarization) and general LSEC dysfunction that likely contribute to disease progression [27, 28].

Also found in the space of Disse are splanchnic (sympathetic) and vagal (parasympathetic) nerves that emanate from the portal tracts and interact with LSEC, stellate cells, as well as the hepatocytes and regulate vascular tone [29]. However, there is a substantial variation of hepatic innervation with regard to species. Humans, cats, and guinea pigs show nerve endings throughout the lobule, while rats and mice show little intralobular innervation [30].

There is a complicated circuit of communication among the diverse nonparenchymal cell types that reside in the sinusoid [29]. Both Kupffer and pit cells, as well as any circulating leukocyte, like dendritic, B and T cells, can send pseudopodia through the LSEC fenestrations to interact with hepatocytes and stellate cells [19, 29, 31]. On the other hand, the basolateral microvilli of hepatocytes are often seen protruding through LSEC fenestrations [31]

(Figs. 7.1b and 7.2b) and can potentially interact with any passing cell in the lumen. The nature of these interactions are now being elucidated, and it is believed that they can affect the immune status of the liver if homeostasis is upset, in cases of sepsis, or other injuries. LSEC can directly modulate microvascular tone in the sinusoid by secreting factors such as endothelin-1 and PGF_{2a} that cause constriction of stellate cells, or relaxation by secreting PGE_2, PGI_2, nitric oxide, or adrenomedullin [16, 27].

Fenestrations: Function and Regulation of the Liver Sieve

Fenestrations are the undisputed hallmark of LSEC and it is well accepted that ultrastructural examination using electron microscopy techniques is still the only reliable imaging modality that allows for their critical evaluation [5]. Fenestrations permit the passive transport of solutes, and more importantly, regulate particulate traffic, between the blood supply and the underlying hepatocytes. Cholesterol metabolism by hepatocytes is critically regulated by LSEC fenestration diameter [32]. The sieving properties of the fenestrated LSEC allow for chylomicrons and their remnants that are smaller than fenestrations, access to the hepatocytes via the space of Disse (Fig. 7.2b). On the other hand, larger triglyceride-laden lipoprotein particles absorbed through the intestine and transported to the liver by way of the portal circulation are excluded until they are sufficiently reduced in size by lipases on systemic endothelium as well as LSEC and hepatocytes [16, 32]. In fact, there is belief among investigators that certain species, like rabbits or chickens, are more susceptible to atherosclerosis precisely because they possess smaller diameter fenestrations and lower sinusoidal porosity. These conditions allow for increased circulation times of cholesterol-rich chylomicrons, and animals challenged with high cholesterol diets suffer systemic circulatory complications over time resulting from this burden since small fenestrations do not permit access to the hepatocytes to metabolize the particles [8].

Wisse et al. postulated that the leukocytes and erythrocytes coursing through the sinusoids could massage the sinusoidal surface and assist in the "forced sieving" of fluid and particulates from the plasma into the space of Disse, facilitating their interaction with the underlying hepatocytes [9, 33]. Interestingly, there is a gradient of porosity and fenestration diameter along the hepatic lobule. Generally, the porosity increases from the portal triad to the centrilobular regions of the lobule, coincident with the increase in sinusoidal diameter along that axis [9, 34]. With this arrangement, forced sieving would be more marked in the narrower portal triad sinusoids (~4–5.9 μm) compared to the wider centilobular sinusoids (~5.6–7.1 μm) [8, 9].

Fenestrae are not rigid openings, but dynamic structures that are regulated by the actin cytoskeletal network. The proteins shown to regulate fenestration size include actin, myosin, small GTPases like Rho and Rac and the calcium-binding protein calmodulin [35–37]. Most studies that evaluate fenestration dynamics are performed on isolated cells in vitro as experimental conditions are more amenable to manipulation of conditions. The initial study that elucidated the mechanism for fenestral contraction was performed by Gatmaitan et al. Serotonin (5-hydroxytryptamine) rapidly (within 30–60 s) elicited fenestration contraction via the 5HT2 receptor on LSEC and this event is upstream of calcium signaling [38]. Following calcium influx, myosin light chains are phosphorylated resulting in increased actin-activated myosin ATPase activity in areas surrounding fenestrae, resulting in contraction. These events were further confirmed by using agents that modify intracellular calcium concentrations, and these similarly affected fenestration diameter. Calcium ionophore reduced fenestral diameter and this contraction could be inhibited by calcium chelation, or preincubating with calmodulin antagonists [8, 36, 38].

The fact that serotonin modulates LSEC fenestrations is intriguing. Serotonin is likely transported to the liver via the hepatic portal supply by platelets, which concentrate it intracellularly and release it by serotonin uptake receptors. Since 95% of the serotonin is synthesized in the gastrointestinal tract, rapid uptake and delivery to the hepatic microvasculature is readily achievable. Changes in serotonin secretion in the gut and its uptake by circulating platelets likely have downstream affects on LSEC fenestrae and corroborate the findings of cross talk between these two organ systems [39]. One case where this has been shown is liver regeneration following partial hepatectomy. Regeneration of the liver, as measured by hepatocyte proliferation, is known to be directly affected by serotonin [40]. Following partial hepatectomy, we [34] and others [41] have shown that there are fenestration diameter and porosity changes in the LSEC at various times following liver resection. It is tempting to suggest that serotonin levels might be regulating these LSEC changes, but further study is needed.

Many other substances are known to affect fenestration diameter and LSEC porosity both in vivo and in vitro [8, 38]. In general, drugs, toxins, or other agents that induce irreversible defenestration also induce atherosclerosis and hyperlipidemia since capillarized sinusoids do not allow for removal of lipoproteins from the blood. Defenestration is also usually accompanied by deposition of collagen, and basement membrane components such as laminin, as well as significant thickening of the LSEC. Recent reports also point to toxins and situations that increase reactive oxygen species (ROS) as major effectors of defenestration. For example, low levels (50–250 ppb) of trivalent arsenic, a major environmental contaminant in drinking water, reduced LSEC porosity

within 2 weeks in mouse models [42]. This capillarization was found to be the result of NADPH oxidase (NOX) generated superoxide and animals deficient in NOX activity were protected from fenestration loss [37]. Oxidative stress also contributes to the defenestration of liver that is coincident with aging [43, 44] and is likely exasperated by concurrent reduction in the levels of natural antioxidants like glutathione as tissue age. On the other hand, fenestrations can be induced or their number increased by various microfilament-inhibiting drugs, such as cytochalasin B, latrunculin A, and misakinolide [8].

Concurrent with capillarization is the upregulation of PECAM (platelet endothelial cell adhesion molecule or CD31) expression on the LSEC surface, especially at areas of cell–cell adhesion [37, 45]. DeLeve et al. had reported previously that upregulation of PECAM on rat LSEC is inversely correlated with porosity [45] and therefore constitutes a reliable marker for dedifferentiation-related defenestration. It is interesting that PECAM expression on LSEC is much lower than in other vascular endothelium, and anti-CD31 immunomagnetic isolation of rat liver endothelium resulted in the enrichment of endothelium that lacked fenestrations [45, 46]. Vascular endothelial cell growth factor (VEGF) is also critically important in the maintenance of LSEC porosity. VEGF, which is produced constitutively by neighboring hepatocytes and stellate cells, stimulates autocrine production of nitric oxide by LSEC, which then maintains the fenestrations in these cells [45]. In pathologies like liver cirrhosis where either VEGF or NO is reduced, capillarization of the sinusoid is also observed.

Reports in the literature had suggested that fenestrations were structurally related to caveolae (Latin *caveolae*: little caves), the 50–100 nm flask-shaped membranous invaginations found on a variety of cells, especially endothelial and smooth muscle cells, and involved in endocytosis and transcytosis [47]. Recent evidence now suggests the contrary as caveolin knock-out mice exhibit the same LSEC fenestration diameter and porosity as wild-type mice [48].

Other Novel Functions of LSEC

Besides providing a permeable barrier between the blood and the parenchyma, LSEC have substantial endocytic and scavenger capabilities. Unlike Kupffer cells that typically phagocytose large particles and insoluble material, LSEC rapidly and selectively clear soluble components and colloidal particles that are less that 0.23 μm from the blood by receptor-mediated endocytosis [49, 50]. At least five distinct types of scavenger receptors have been identified on LSEC surfaces that remove a variety of physiologic waste products of all types of biomacromolecules including proteins, polysaccharides, lipids, and nucleic acids [51]. Large volumes of waste products can be removed within minutes of injection, indicating their remarkable ability to clear blood of soluble debris and colloidal material.

Due to their ability to interact with leukocytes, their position downstream from the hepatic portal blood supply and their incredible scavenger activity, LSEC are now categorized as an antigen presenting cell and a major contributor to the innate immunity in the liver. The liver is an organ that appears to prefer practicing immunotolerence over promoting immunity under most circumstances [52]. In this environment, keeping the immune response in check, the presence of circulating endotoxins and variable antigen flux is critical to maintaining hepatic homeostasis. Unlike vascular endothelium, LSEC express the costimulatory MHC class I molecule required for cross-presentation of antigen to T cells. LSEC can cross-present antigens that can tolerize CD8+ T cells to a variety of signals like endotoxin [53], tumor cells [54], oral antigens [55], and allogenic antigens [56]. MHC class II presentation of antigen by LSEC to CD4+ T cells has been shown to induce differentiation of those cells toward a tolerogenic regulatory T cell phenotype [52]. It has also been shown that LSEC can suppress the activity of neighboring antigen presenting cells such as dentritic cells that could potentially induce T cell immunity [57].

Repopulation and Derivation of LSEC and their Role in Diseases

Injury to liver sinusoidal endothelium occurs under a variety of conditions [58, 59]. For the most part, diseases that directly involve liver vasculature are uncommon. Those involving the hepatic microvasculature encompass a discreet set of circumstances that affect the LSEC mostly as the result of toxic insult or ischemic conditions. Capillarization, is the loss of fenestrations, which was discussed above, is a common phenomenon that results from a variety insults, including loss of VEGF, and/or NO signaling observed in cirrhosis [27, 28], oxygen radical induced damage associated with aging [43], toxicants such as arsenic [42], or autoimmune antibodies directed toward LSEC [60].

Sinusoidal obstruction syndrome (SOS, also called hepatic veno-occlusive disease) has two known causes: consumption of pyrrolizidine alkaloids and injury resulting from chemotherapy and irradiation protocols often used in preparation for bone marrow transplantation. This pathology is independently described in Chap. 48. In both cases, obstruction of the sinusoid is caused by LSEC detachment and death coupled with blood cell invasion between LSEC and hepatocytes. As a result, the hepatic microvasculatures are blocked and the areas experience loss of perfusion, local necrosis,

and tissue death. In vivo models of SOS are induced by gavage of monocrotaline, a pyrrolizidine alkaloid plant toxin. DeLeve and coworkers has shown that sinusoidal repopulation following monocrotaline-induced LSEC death in rats is at least partially regulated by bone marrow-derived endothelial cell progenitors [61]. Infusing male bone marrow into female recipients on day 5 after treatment, the time of peak denudation of LSEC, indicated that ~27% of the LSEC were bone marrow derived when examined using Y-chromosome fluorescence in situ hybridization on the isolated LSEC on day 12.

Cold ischemia/reperfusion injury associated with liver transplantation represents another injury model to study endothelial cell repopulation. It is well known that cold storage of livers prior to transplantation results in significant LSEC denudation and death prior to, and is potentiated upon reperfusion [62–64]. Repopulation events were evaluated using syngeneic wild-type to GFP-transgenic rat liver transplantation. Under these conditions, ~5% of the LSEC that lined the sinusoids were double-positive for LSEC markers and GFP, indicating vasculogenic events occurred following orthotopic liver transplantation [65].

Others have shown in mouse models that partial hepatectomy of engrafted GFP bone marrow radiation chimeras results in the repopulation of ~70% of the LSEC by bone marrow derived progenitors [66].

Study In Vitro: Isolation and Culture of Liver LSEC

Isolation of liver LSEC is an important technique to study functional aspects of these cells. Since the LSEC comprise only ~3% of the liver volume, detecting changes in these cells apart from the hepatocytes that comprise 80% of the liver volume is challenging unless in situ techniques like immunohistochemistry or immunofluorescence are performed on intact tissue. Various techniques have been implemented that enrich LSEC for cell culture, biochemical analysis, and other functional studies. The protocols described below are general and can be applied to a variety of species with minimal modification. However, when isolating LSEC from human sources, only liver resections and not livers that have been previously stored for transplantation can be used if substantial numbers of LSEC are required. This is because cold-stored livers are known to have compromised LSEC, resulting in extremely low yields [62–64].

In all cases for cell culture techniques, the liver is digested to single-cell suspension with various proteolytic enzymes, such as collagenase, pronase, or commercial mixtures of collagenases and neutral proteases such as Blendzyme (Roche) based on the two-step protocol described by Seglen [67]. Once the tissue is dissociated, hepatocytes are easily removed from the suspension by low speed centrifugation, typically at $50 \times g$. The cells in the supernatant will contain a mixture of nonparenchymal cells (LSEC, Kupffer cells, leukocytes, and stellate cells) that require additional separation. At this point, various protocols can be applied to the suspensions.

The method of Braet et al. [68] involves differential centrifugation of the nonparenchymal component through Percoll step gradients, followed by differential plating technique, also called panning. Panning removes contaminating Kupffer cells in the preparation since they rapidly adhere to plastic in the absence of serum. This technique does not require any special equipment other than a preparative tissue culture centrifuge and is accessible for most laboratories and is popular for this reason (Fig. 7.6a).

Another popular technique involves centrifugal elutriation that takes the nonparenchymal component and separates the various cell populations by relative density using continuous flow centrifugation [69, 70]. Typically, this will give a high yield of relatively pure LSEC with minimal Kupffer cell contamination. The disadvantage is that elutriators are usually not readily available and are expensive to purchase. Set up of elutriation rotors on preparative centrifuges often dedicates them to this one particular purpose.

Recently, immunomagnetic isolation of LSEC has streamlined the isolation process. There are currently two major formats of immunomagnetic isolation and the main difference is the size of the beads to which antibodies are covalently attached. Larger beads, like those supplied by Dynal are approximately 2–3 μm in diameter (Fig. 7.6b). Smaller beads, like those from Miltenyi are only about 20–50 nm in diameter, allowing for the separation of cells by flowing them through a magnetized column (Fig. 7.6c). Only a few surface markers are known to be specific for positive selection of liver LSEC. For isolation of rat LSEC, the antibody SE-1, prepared and described by the Enomoto laboratory, was shown to specifically isolate LSEC [71, 72]. SE-1 antigen was recently found to be identical to the CD32b protein [73]. As mentioned above, CD31 (PECAM) is not a desirable marker to isolate fenestrated LSEC in the rat, since CD31 isolated only nonfenestrated endothelium that are likely derived from the large vessels [46]. Regardless of how viable LSEC are isolated, the cells maintain their fenestrations while in suspension and in early culture (Fig. 7.6d). Other surface markers used for LSEC immuno-isolation include CD105 (endoglin) [74], stabilin-2 [75], and a lymphatic hyaluronen receptor – LYVE-1 [76].

Other techniques have been successfully used to isolate LSEC, magnetically, without the use of antibodies. One technique is a modification of the cationic colloidal-silica protocol that takes advantage of the availability of the LSEC surface via vascular perfusion, and the negative charge normally found on cell membranes [77]. The cationic colloidal silica membrane isolation technique has been used by our

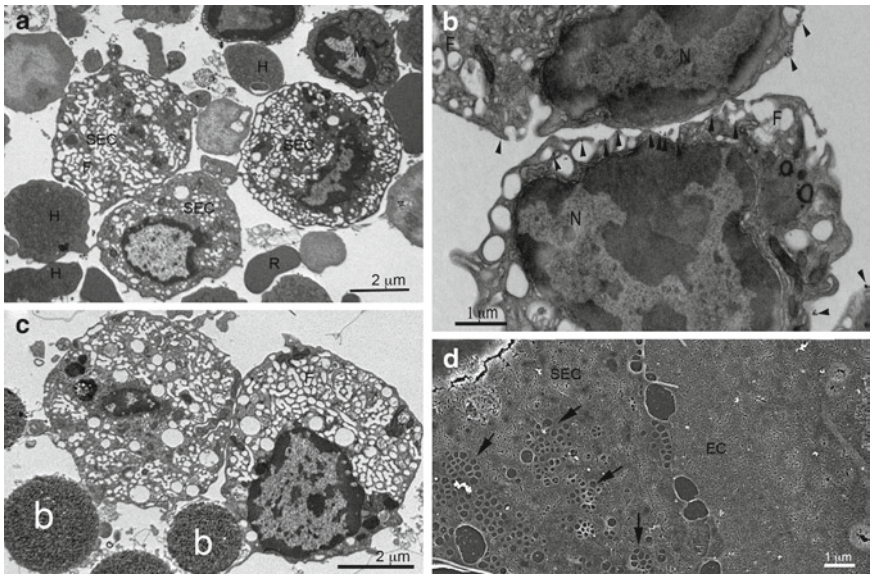

Fig. 7.6 Morphology of isolated rat sinusoidal endothelial cells. (**a**) SEC isolated using the Percoll differential centrifugation technique, showing SEC with fenestrations that are retained even though isolated and in suspension. Also observed are macrophages (M) as well as red blood cells (R) and hepatocyte remnants (H) in the suspension. This image shows the material collected from the SEC-enriched area of the 25–50% Percoll interface. (**b**) Isolation of SEC using Dynal magnetic beads (b) covalently linked to SE-1. Preparation is clear of debris and shows little contamination from other cell types. Fenestrations are observed in these suspended cells (F). (**c**) SEC isolated using SE-1 linked magnetic microbeads (Miltenyi), showing the difference in size (*arrowheads*) between the two immuno-magnetic isolation techniques. (N) SEC nucleus, (F) SEC fenestrations. (**d**) Cells isolated using the Percoll differential centrifugation technique and processed for SEM 24 h after plating onto collagen coated glass coverslips. A fenestrated SEC is observed next to an unfenestrated (large vessel) endothelium. Fenestrations are observed arranged in sieve plates (*arrows*)

laboratory to highly enrich sinusoidal cell-associated membrane proteins relative to nonsinusoidal tissue proteins [42, 78]. Similarly, cationic colloidal magnetite, which utilizes positively-charged beads about 20–50 nm in diameter, binds to cells with access to the vasculature and coats the surface with positively charged magnetite beads. Tissue is then dissociated using collagenase, hepatocytes are removed, and LSEC are then isolated from the nonparenchymal component using Miltenyi column. Using this technique, we successfully isolated LSEC from both rat [65] and mouse (unpublished results). Because other cells types are present in this isolate, such as Kupffer cells and other leukocytes that populate the sinusoid, selective panning to remove them is necessary if relatively pure cultures are needed. These isolates are amenable to evaluate the population of cells residing in the sinusoid using flow cytometry.

Once cells are isolated, a variety of media bases, serum concentrations, growth factors, and matrices have been used to culture LSEC. Regardless, LSEC start to lose their fenestrations and scavenger characteristics in culture almost immediately, [49] so caution should be exercised when employing cultured LSEC with regard to the specific question being addressed. Culture condition manipulation has resulted in increased maintenance of specific characteristics [45, 49], with VEGF and NO being one of the most important components required to maintain porosity.

Acknowledgments The author acknowledges the amazing technical assistance of the entire staff of the Center for Biologic Imaging, at the University of Pittsburgh Medical School, but especially Mark Ross for his dedication to all aspects of liver processing and imaging. Supported by NIH grant R01 CA76541 to DBS.

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Chapter 8
Hepatic Carbohydrate Metabolism

Dirk Raddatz and Giuliano Ramadori

Introduction

The liver plays a unique role in controlling carbohydrate metabolism by maintaining glucose concentrations in a normal range. This is achieved by a tightly regulated system of enzymes and kinases regulating either glucose breakdown, storage as glycogen, or synthesis in hepatocytes. This process is under the control of glucoregulatory mediators among which insulin plays a key role. The fact that insulin is secreted into the portal system, takes the same route as absorbed glucose, and that the liver eliminates a large portion of the portal insulin at the first pass highlights the role of the liver not only as glucose supply, but as a site of glucose uptake and storage.

When the hepatic function is impaired by either diminished hepatocellular function (e.g., fatty liver) by reduced number of hepatocytes (liver cirrhosis), alterations in hepatic glucose metabolism – mainly consisting of diminished glucose and insulin uptake following carbohydrate ingestion – occur, leading to peripheral "insulin resistance" (elevated glucose and insulin levels).

Knowledge of the processes involved in maintaining glucose homeostasis as well as insulin mechanisms of action in the liver alone and in concert with other hormones (e.g., glucagon, cortisol, catecholamines, IGF-1, GLP-1) is a prerequisite to develop new therapeutic approaches in diabetes type 2.

Hepatic Glucose Uptake, Glycogen and Lipid Synthesis

The liver displays the capacity to remove 30–40% of the glucose presented to it following glucose ingestion and therefore must be considered a significant site of postprandial glucose removal [1].

D. Raddatz (✉)
Department of Gastroenterology and Endocrinology,
University of Göttingen, Göttingen, Germany
e-mail: draddat@gwdg.de

It appears that the contribution to hepatic-glycogen synthesis following a meal will depend on the preexisting metabolic state [2, 3].

The liver as a sensory organ detects a glucose concentration gradient between the hepatic artery and the portal vein [4] by intrahepatic sensory-effector nerves, generating a cholinergic signal for an insulin-dependent net hepatic glucose uptake [5]. Interestingly, portal insulin may also stimulate intestinal glucose absorption via cholinergic hepatoenteral nerves [6]. The fact that insulin is released into the portal blood and reaches the liver together with glucose, and the observation that 70% of the insulin from the portal blood is taken up in the liver after the first passage [7] imply that in the postprandial state the most important function of insulin is to influence the utilization of glucose into the hepatocytes and to stimulate glycogen and lipid synthesis.

Due to the high capacity of the glucose transporter (Glut-2) in the liver intra- and extracellular glucose levels are in a tight equilibrium in hepatocytes. It has been shown that glucokinase (GK) is the rate limiting enzyme for further glucose utilization [8]. GK expression is enhanced by insulin [9]. In transgenic mice overexpressing hepatic GK, glycolysis and glycogen synthesis are increased and these animals have lower glycemia than controls after a glucose tolerance test, indicating that GK overexpression in liver increases glucose uptake, confirming the rate limiting role of GK in vivo [10]. Similar results were obtained with transgenic mice carrying additional or more extra copies of the entire GK gene. Moreover, it was shown that overexpression of GK may reverse diabetes in a mouse model of streptozotocin-treated mice [11], suggesting that influencing hepatic GK activity may be a valuable therapeutic strategy in treating diabetes mellitus. In fact, recent approaches with allosteric activators of GK are on the way [12].

With 10% of its weight (≈150 g), the liver has the highest specific glycogen content of any tissue (muscle approx. 1%, ≈300 g). Hepatic glycogen metabolism is controlled by coordinate action of the enzymes glycogen synthase (GS) and glycogen phosphorylase (GP), both regulated by phosphorylation. Insulin regulates glycogen metabolism by promoting the dephosphorylation and activation of GS [13]. A major function of glycolysis in the liver is to provide acetyl-CoA from

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_8, © Springer Science+Business Media, LLC 2011

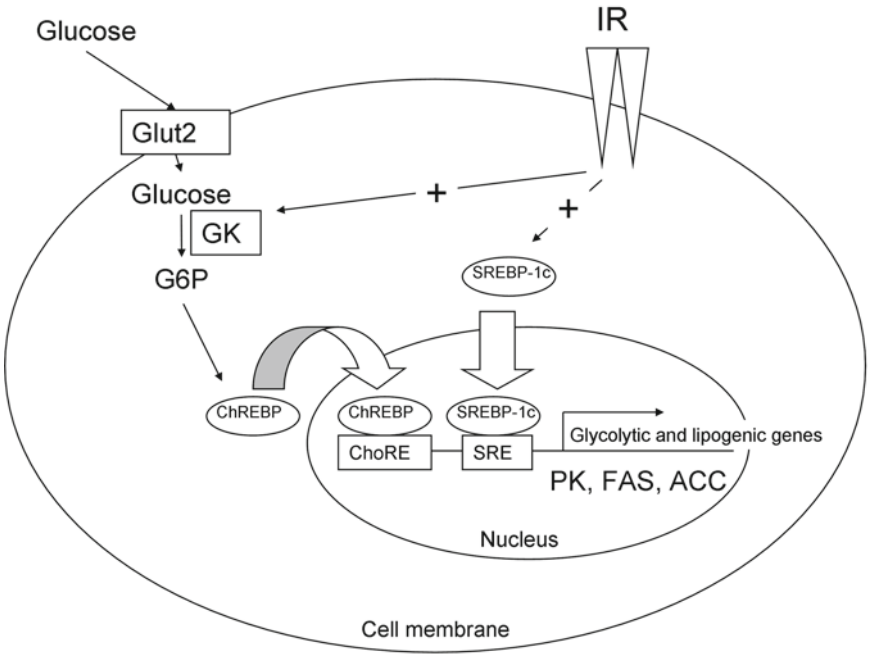

Fig. 8.1 Synergy of glucose and insulin in regulating glycolytic and lipogenic gene expression. On the intracellular level, this synergism is mediated by coordinate action of the transcription factors SREBP-1c (sterol responsive element binding protein) and ChREPB (carbohydrate responsive element binding protein). Except for GK (glukokinase) which is only induced by insulin, genes of the glycolytic and lipogenic pathways are also regulated by glucose (*FAS* Fatty Acid Synthase; *ACC* acetyl-CoA carboxylase; *PK* pyruvate kinase)

glucose for de novo lipid synthesis. The synthesis of fatty acids depends on two signals, glucose and insulin, to promote expression of lipogenic genes like acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The transcription SCREP-1c has emerged as a major mediator of insulin action on hepatic GK and lipogenic gene expression. Remarkably, SCREP-1c controls not only the rate of triglyceride synthesis in the liver but also the amount of storage in the liver [14].

With the exception of GK which is exclusively regulated by insulin, other genes of the lipogenic pathway are also regulated by glucose. Glucose- or carbohydrate-response elements (ChoRE) have been identified in the promoters of these genes and recently a glucose-responsive transcription factor named ChREBP (carbohydrate responsive element binding protein) has been identified. ChREBP is an important link of hepatic carbohydrate and lipid metabolism. Silencing ChREBP gene expression with a siRNA approach in hepatocytes and experiments with knockout mice revealed that ChREBP mediates the glucose effect on both glycolytic and lipogenic gene expression and that this transcription factor is a key determinant of lipid synthesis in liver [14, 15] (Fig. 8.1).

Regulation of Hepatic Glucose Production

The liver produces glucose by breaking down glycogen (glycogenolysis) and by de novo synthesis of glucose (gluconeogenesis) from non-carbohydrate precursors such as lactate, amino acids and glycerol. Both pathways are tightly regulated. The relative contribution in maintaining glucose homeostasis varies with time. While glycogenolysis occurs within 2–6 h after a meal, gluconeogenesis has a greater importance with prolonged fasting.

The rate of gluconeogenesis is controlled mainly by the activities of the unidirectional enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose-1, 6-bisphosphatase (FP2ase), and glucose-6-phosphatase (G6Pase). The gene transcription of these gluconeogenic enzymes is controlled by hormones, mainly insulin, glucagon, and glucocorticoids. While insulin inhibits gluconeogenesis by suppressing the expression of PEPCK and G6Pase, glucagon and glucocorticoids stimulate hepatic glucose production by inducing those genes. In diabetes, both type 1 and 2, an increased hepatic glucose production is a main contributor to fasting hyperglycemia.

Direct and Indirect Effects of Insulin on the Liver

The contribution of hepatic insulin receptor signaling to normal glucose homeostasis and pathogenesis of diabetes type 2 is a matter of ongoing debate. Systemic administration of insulin lowers blood glucose levels and inhibits HGP within minutes. Insulin exerts its effects on hepatic glucose fluxes via direct and indirect mechanisms [16, 17]. The direct effects can be further divided into *acute* insulin actions leading to rapid decrease in HGP [18] and *chronic* insulin actions inhibiting the gene expression of key enzymes of gluconeogenesis [19–21].

The indirect effects of insulin on hepatic glucose output include the suppression of lipolysis, the inhibition of glucagon secretion [22], and the activation of hypothalamic descending pathways [23]. There is a controversial discussion over which mode of insulin action is most important under physiological conditions Fig. 8.2.

In overnight fasted dogs, changes in the portal insulin concentration in the absence of changes in plasma glucagon, free fatty acids (FFA), or precursors of gluconeogenesis effectively inhibit HGP [24, 25]. Confirming these results, it has been recently shown that in addition, a rise in head insulin levels does not enhance insulin-suppressive effect on HGP [26], suggesting that insulin exerts its effect on HGP mainly directly. The importance of hepatic insulin receptors for a direct insulin action was also supported by the observation that in liver specific insulin receptor knockout (LIRKO) mice insulin is unable to suppress HGP sufficiently [27]. However, restoration of insulin receptor in LIRKO mice did not improve insulin's ability to suppress HGP [28], questioning the dominance of direct insulin effect in that certain mouse model. In fact, LIRKO mice display a certain hepatic phenotype with a liver approximately half the size of wildtype mice and changes in the expression of insulin regulated genes [29], which might have been responsible for the severe hepatic resistance in this model. To avoid changes in the liver phenotype, Buettner et al. [30] used an antisense oligonucleotide technique to acutely silence hepatic insulin receptors in mice. Despite a severe deterioration of hepatic insulin signaling, insulin was able to suppress HGP indicating that at least in mice insulin's indirect effects play the main role in inhibiting HGP.

Interestingly, ablation of insulin receptor in arcuate and paraventricular nuclei of the hypothalamus led to the inability of insulin to inhibit HGP in mice with intact liver insulin signaling [28], suggesting that inhibition of HGP by insulin involves central regulation. In summary, aforementioned studies from dogs clearly point to the dominance of direct insulin effects, while data from studies of mice suggest that insulin's indirect effects dominate. How to reconcile these apparent contradictory findings?

Apart from a number of methodological considerations which might at least explain in part these divergent findings as discussed by Cherrington [31], different physiology of dogs and mice have to be taken under consideration as recently discussed by Girard [32]. For instance, basal HGP per kilogram bodyweight in mice is 10–15 times greater than in dogs, plasma glucagon being similar. Removal of hepatic insulin receptor in mice could have led to an increased neural control of HGP as a protective response. Moreover, in fasted dogs gluconeogenesis contributes to less than 50% of HGP, while in rodents it contributes to 80–90%. Since hepatic gluconeogenesis is less sensitive to insulin than glycogenolysis, it is possible that inhibition of gluconeogenesis requires central nervous inputs. In fact, it has been shown previously that

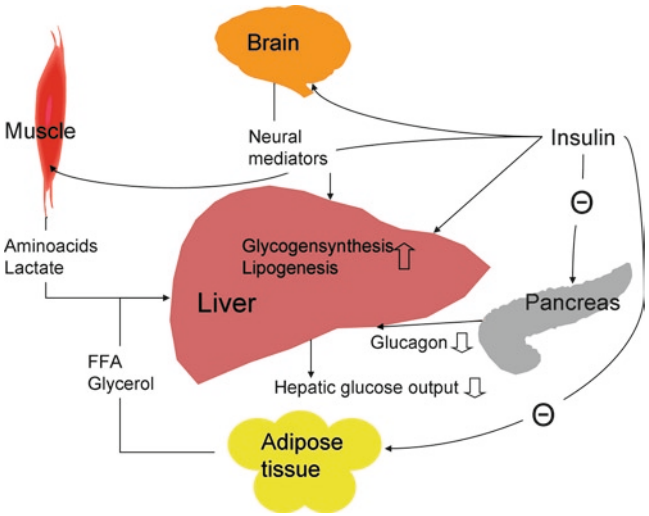

Fig. 8.2 Insulin actions on the liver. Insulin may influence hepatic glucose metabolism either directly in the liver or indirectly by influencing several humoral or nerval factors which might in turn act on the liver

an autonomic nervous input can modulate liver carbohydrate metabolism [33, 34].

As interesting as the above-mentioned data from mice and dogs may be, the crucial question is about the situation in men and especially in human disease. In men under physiological conditions, both insulin's direct and indirect effects play a role [24]. A summary of mechanisms by which insulin can act directly or indirectly is provided in Fig. 8.2. However, the relative contribution of insulin's direct and indirect effects however, may be crucial in the treatment of diabetes. The enhanced HGP in patients with type 2 diabetes is mostly attributable to an increase of hepatic gluconeogenesis [35]. Therefore, future therapeutic strategies should aim to suppress gluconeogenesis. Since gluconeogenesis is less sensitive to insulin than glycogenolysis, the use of insulin in this setting is questionable. In this context it is noteworthy that glucagon, which is increased in diabetes mellitus type 2 [36], stimulates hepatic gluconeogenic enzymes [37], so that inhibition of glucagon would be a desirable goal. GLP-1 analogs, recently established in the treatment of type 2 diabetes are known to inhibit glucagon secretion, so that those agents might provide a causal therapy for treating type 2 diabetes and possibly other conditions with an assumed "hepatic" insulin resistance.

Transcriptional Regulation of Gluconeogenic Enzymes

Transcriptional regulation of PEPCK and G6Pase, two key enzymes of gluconeogenesis, involves a "cross-talk" between a network of transcription factors. The PEPCK promoter is

known to be induced by various stimuli such as glucagon and adrenaline via cAMP, glucocorticoids, thyroid hormone [38], and transcription factors like CREB, HNF-3, HNF-4α and PPARα. The transcription factors, hepatocyte nuclear factor-4α (HNF-4α), and the peroxisome proliferative activated receptor (PPAR)-γ co-activator-1α (PGC-1α) are of particular interest in the transcriptional regulation of PEPCK and G6Pase genes. PGC-1α affects gluconeogenic gene regulation by directly binding to HNF-4α and to other transcription factors such as Foxo1 (Fig. 8.3) and is induced by cAMP and glucocorticoids in isolated hepatocytes and in liver in vivo [39]. In vitro studies have shown that the transcription factor Foxo1, which directly interacts with PGC-1α, regulates both PEPCK and G6Pase genes through an interaction with their consensus insulin response elements (IRE) [20]. Insulin signaling through the serine/threonine protein kinase Akt can attenuate the effects of increased levels of PGC-1α in fasting and other conditions by promoting the dissociation of PGC-1α from Foxo1 [40] (Fig. 8.3).

The transcription factor STAT-3, an important signal transducer used by the IL-6 family, also contributes to the transcriptional regulation of hepatic gluconeogenic genes. IL-6 reduces PEPCK- [41] and G6Pase- [42] gene-expression in isolated hepatocytes and in mice respectively. In a recent mouse model of liver-specific inactivation of STAT-3, genetically manipulated (L-ST3KO) mice show insulin resistance associated with increased PGC-1α expression, associated with a parallel increase in gluconeogenic gene expression [43].

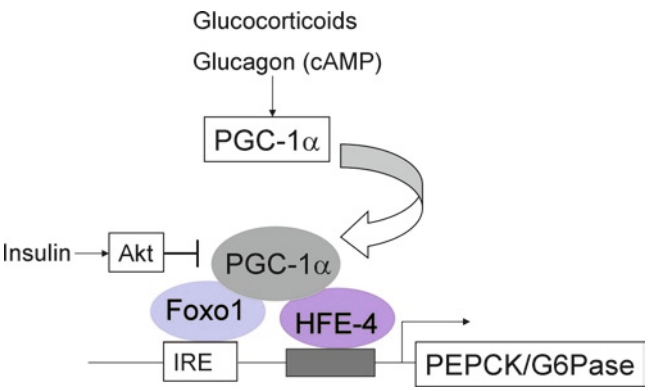

Fig. 8.3 Transcriptional control of gluconeogenic genes. PGC-1α and Foxo-1 play important roles in the suppression of hepatic gluconeogenesis by insulin. Foxo1 regulates PEPCK and G6Pase through insulin responsive elements (IRE). PGC-1α affects gluconeogenic gene regulation by binding to transcription factors like Foxo1 or HNF-4α. PGC-1α is induced by cAMP and glucocorticoids and by fasting and insulin deficient states in liver. Insulin signaling through Akt can attenuate the effects of increased levels of PGC-1α in fasting conditions by promoting the dissociation of PGC-1α from Foxo1

Overexpressing either G6Pase or PEPCK in vivo using transgenic mouse models results in impaired glucose tolerance, pointing at the significance of the deregulation of hepatic gluconeogenic genes in diabetes pathophysiology [44, 45]. In hepatocytes, G6P concentrations are determined by the balance between G6Pase and glucokinase (GK) activities, leading to the suggestion that the ratio of GK to G6Pase is crucial for the metabolic fate of glucose in liver [46]. In fact, G6Pase is increased in animal models of diabetes [47].

Overexpression of G6Pase in hepatocytes results in a reduction of glycogen synthesis and an increase in glucose production. Overexpression of G6Pase in rat liver in vivo results in glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content, and triglyceride accumulation in skeletal muscle, all typical features in diabetes type 2 [45]. However, overexpression of G6Pase does not increase fasting blood glucose consistent with the notion that the main function of G6Pase is to buffer G6P concentrations in the post-absorptive state.

Transgenic mice overexpressing PEPCK in liver show increased basal hepatic glucose production, but normal whole-body glucose disposal during a hyperinsulinemic-euglycemic clamp study compared to wild type controls [44], suggesting a severe hepatic insulin resistance. Accordingly, insulin signaling in the liver of these mice was diminished. In another transgenic mouse model with even higher overexpression of PEPCK in the liver, animals also displayed fasting hyperglycemia and peripheral insulin resistance [48]. Altogether, these models demonstrated that increased flux through gluconeogenesis, either via PEPCK or G6Pase overexpression is suitable to generate metabolic disturbances observed in diabetes mellitus type 2. However, recently Samuel et al. could show that PEPCK and G6P mRNA was not increased the liver of patients with DM undergoing bariatric surgery and in two mouse models of diabetes challenging this dogma [49].

The Role of Growth Hormone and Insulin-Like Growth Factor in Carbohydrate Metabolism

Although under physiological conditions insulin is the principal regulator of glucose metabolism, there is evidence that the growth hormone (GH)-insulin-like growth factor (IGF)-axis contributes to the carbohydrate homeostasis. GH has well known diabetogenic potency. In healthy subjects and subjects with diabetes type 1, GH increases hepatic glucose output (HGP) by increasing gluconeogenesis and glycogenolysis. Furthermore, GH decreases peripheral glucose utilization in the muscle. Stimulation of lipolysis with release of FFA

provides an additional mechanism for the diabetogenic properties of GH.

IGF-1, which is synthesized in hepatocytes upon GH stimulation is a 5,807 kDa single chain polypeptide, which has structural homology with pro-insulin. Ninety percent of IGF-1 is bound in a ternary complex comprising of IFG-1, IGF binding protein (IGFBP)-3 and an acid labile subunit (ALS). A schematic diagram of GH and IGF-1 action is provided in Fig. 8.4.

By acting through the type 1 IGF-receptor, IGF-1 can directly activate the insulin receptor substrate (IRS)-1 and phosphatidylinositol-3-kinase cascade, resulting in GLUT4 translocation to the plasma membrane and thus stimulating glucose uptake in the muscle. Historically, the role of the GH/IGF-1 axis has been analyzed in human and rodent hormone deficiency models. One problem in interpreting most studies on the role of IGF-1 has been that in addition to enhancing insulin effects, it also suppresses GH secretion. Therefore, it has been difficult to determine if the observed effects were due to a direct effect or have been mediated by a suppression of GH.

However, the development of tissue selective knockout mice has brought new insights in understanding the relative roles of those hormones in carbohydrate homeostasis.

A liver-specific gene-deletion knockout of the IGF-I gene resulted in a mouse model with reduced circulating IGF-I levels, that led to insulin resistance due to the secondary elevation of circulating GH levels [50]. Using the transgenic approach, IGF-I and insulin receptor function was inhibited by the overexpression of a dominant-negative IGF-I receptor in skeletal muscle. The result was a severe insulin resistance in muscle, followed by insulin resistance in fat and liver, and eventually beta-cell dysfunction and development of type 2 diabetes [51]. The interpretation of the authors was that IGF-1 might be necessary to sensitize the muscle to insulin.

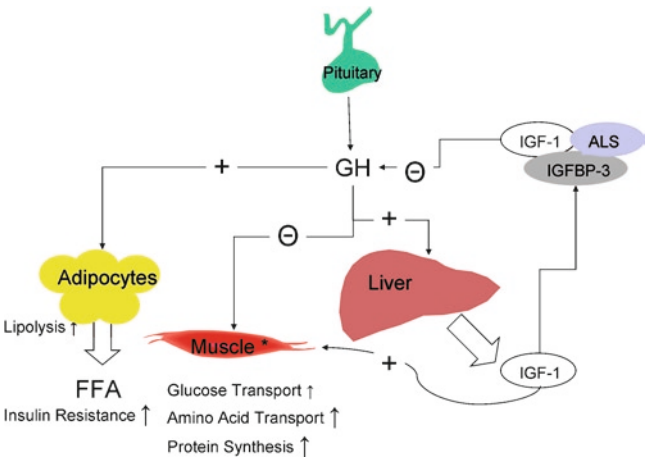

Fig. 8.4 Scheme of the GH/Igf-1 axis. IGF-1 is produced in the liver under the control of GH. In the serum it is bound in a ternary complex consisting of IGF-1, the acid labile subunit (ALS) and IGF binding protein (IGFBP)-3, suppressing pituitary production of GH in a negative feedback loop. GH opposes insulin action in adipose tissue by promoting lipolysis and generation of free fatty acids (FFA) which in turn induce insulin resistance. Although GH alone inhibits glucose utilization in muscle, the net effect of GH and IGF-1 working together in physiological conditions is synergistically with insulin

Crossing liver specific IGF-1 (LID) knockout mice with animals that do not express the acid-labile subunit (ALS), a protein which stabilizes the half-life of IGF-1, showed enhanced insulin sensitivity in muscle and fat tissue despite high GH levels, but no change in the liver [52]. These data suggest a dominating effect of IGF-1 in muscle and fat, while the major site at which GH blocks insulin action is the liver. To clarify this point, a fourth study was performed in which GH action was blocked in LID mice by crossing them with GH antagonist (GHa) transgenic mice [53]. The ability of insulin to inhibit hepatic glucose production was impaired in the LID mice. When GH action was blocked in LID + GHa mice, hepatic glucose production was suppressed to the levels observed in control mice. These results show that despite low levels of circulating IGF-1, hepatic insulin sensitivity in LID mice could be improved by inactivating GH action, suggesting that chronic elevation of GH levels plays a major role in insulin resistance mainly in the liver. In a recent study by O'Connell and Clemmons [54], a GH receptor antagonist was given to acromegalic patients for 6 months to block GH action. This study clearly demonstrated that GH receptor antagonist treatment significantly improved insulin sensitivity. Addition of IGF-1 in combination with the GH-receptor antagonist resulted in substantially greater improvement in the insulin sensitivity, suggesting that IGF-1 exerts additional effects on insulin sensitivity that are not mediated through suppression of GH action.

In summary, aforementioned data suggest that influencing the GH/IGF-1 axis may offer an alternative strategy in treating diabetes. However, future work will have to confirm the efficacy and safety of IGF-1 containing compounds.

Glucocorticoids and Hepatic Carbohydrate Metabolism

Glucocorticoid hormones play essential roles in the regulation of carbohydrate metabolism. Their effects primarily depend on their binding to intracellular receptors leading to altered target gene transcription, as well as on cell-type specific biotransformation between cortisol and its inactive 11-oxo-metabolite cortisone. The latter effect is accomplished by two different 11-alpha-hydroxysteroid dehydrogenase

isoenzymes (HSD). Whereas the type-1-enzyme transforms, in most instances, in vivo cortisone to active cortisol, the type-2-enzyme is a dehydrogenase of glucocorticoids, thus "protecting" the mineralocorticoid receptor against occupation by cortisol.

Alterations in HSD-activity are linked to metabolic disturbances in the cause of the metabolic syndrome [55, 56]. Knockout of 11β-HSD-1 in mice improves hepatic insulin action and protects against obesity and hyperglycemia [57, 58]. Conversely, selective overexpression of 11β-HSD-1 in adipose tissue in mice results in development of visceral obesity, hyperglycemia, hyperlipidemia, and hypertension [59].

In obese, nondiabetic humans, the liver accounts for all splanchnic cortisol production [60].

Interestingly, hepatic HSD-1-activity is under the control of growth hormone [61], a mechanism which might explain the positive effect of GH on body composition in GH-deficiency.

Pharmacological inhibition of HSD in animal models leads to lowered hepatic glucose production and increased insulin sensitivity [62] and may be a promising target in the treatment of the metabolic syndrome [63].

Glucose Homeostasis in Liver Disease

Insulin Resistance, the Common Link in Metabolic Syndrome, Nonalcoholic Fatty Liver Disease, and Hepatogenous Diabetes

Role of IRS1/2

Insulin mediates its action via binding its receptor on the surface of insulin-responsive cells. The stimulated receptor phosphorylates tyrosine sites of itself and several substrates including members of the IRS class, thereby promoting downstream signaling events [64, 65]. A reduction in expression and tyrosine phosphorylation of IRS1 in adipocytes has been reported in diabetic patients. Moreover, several polymorphisms of IRS1 have been linked to insulin resistance in men [66]. In contrast, polymorphisms of IRS2 were seldom found associated with IR, arguing against a major role of IRS2 polymorphisms in the pathogenesis of type 2 diabetes [67].

While IRS1 is the major substrate leading to glucose uptake in adipose tissue and muscle, earlier IRS2 knockout models suggested a dominant role of IRS2 in hepatic metabolism [68]. However, later studies on liver specific IRS2 knockout mice challenged this view of being the most important IRS in the liver, since affected animals displayed only a mild insulin resistance and a slight reduction of hepatic insulin signaling [69], or even no insulin resistance [70], but no diabetes, suggesting that IRS1 alone is sufficient to allow almost normal hepatic insulin signaling.

IRS are unique docking molecules whose actions are tightly regulated by phosphorylation at several sites. Control over insulin signaling can be achieved by autoregulation, where downstream events inhibit upstream components via feedback control.

Alternatively, unrelated pathways such as inflammatory signals may inhibit this pathway downstream of the IR, thus inducing insulin resistance. TNFα and high levels of FFA stimulate inhibitory phosphorylation of serine instead of tyrosine residues of IRS-1, thereby inhibiting downstream signaling and insulin action [71–73].

In recent times, it became apparent that inflammatory pathways may become activated by metabolic stress from inside the cell as well as from extracellular mediators (Fig. 8.5).

Intracellular Activators of Inflammatory Pathways

Reactive oxygen species (ROS), produced by mitochondria in case of hyperglycemia are important intracellular activators of inflammatory pathways [74, 75]. In addition, intracellular stress may arise from the endoplasmic reticulum (ER), faced with the requirements of adipocytes to cope with increased substrate fluxes and altered tissue architecture in case of obesity [76, 77]. As recently shown, experimental lowering of ROS levels ameliorates IR in different models of IR, suggesting ROS to be a common mechanism in different settings of IR [78]. Interestingly, during viral infection, intracellular stress pathways are activated by an excess of viral proteins in the ER, which might be of importance in the development of IR in chronic viral diseases as chronic hepatitis C.

Inflammatory Pathways Involved in Insulin Resistance

JNK-1, -2, and -3, three serine/threonine kinases belonging to the MAPK family, play a major role in the development of IR in obesity [79]. In response to ER-stress, cytokines and FFA JNK is activated, and phosphorylates IRS-1 on Ser307, thereby impairing insulin action [80].

JNK activity is increased in obesity. Modulation of JNK-1 activity in the liver affects systemic glucose metabolism, underscoring the importance of this particular pathway in the liver [79]. The two kinases PKC and IKK play a role in counteracting insulin action especially in response to lipid metabolites. Lipid infusions lead to a rise in intracellular metabolites like diacylglycerol and fatty acyl CoA. This rise is accompanied by an activation of PKC and an increase in Ser307 phosphorylation of IRS-1 [81].

IKKb directly serine phosphorylates IRS-1 and secondly phosphorylates inhibitor of NFκB (IκB), thereby activating

the NFκB pathway, which in turn stimulates a battery of proinflammatory mediators including TNFα [82] (Fig. 8.5). Support for the relevance of this pathway comes from mice heterozygous for IKKb, being partially protected against IR triggered by lipid infusions.

Of note, the IKK pathway seems to play a particular role in the liver. Cai et al. showed that mice with a hepatic overexpression of IKK develop a diabetes-type-2-phenotype, characterized by hyperglycemia, profound hepatic insulin resistance, moderate systemic insulin resistance, and increased hepatic production of proinflammatory cytokines, providing evidence that lipid accumulation in the liver promotes hepatic and to a lesser extend systemic IR [83].

The mTOR complex 1 and its downstream kinase S6K1, integrators of nutrient and insulin signaling are also critically involved in mediating nutrient effects on IR (Fig. 8.5). Recently, Ser1101 was identified as another S6K1 site in IRS-1. Phosphorylation of Ser1101 was increased in liver of obese (db/db) or WT, but not of S6K1−/− mice, implicating S6K1 as the kinase involved [84]. In accordance with these findings, rapamycin, an mTOR inhibitor, improved insulin actions in a short-term in vivo study [85]. In contrast, long-term treatment with rapamycin increased rather than decreasing the insulin resistance [86], which might be explained by an increased activity of stress-response kinases in muscle and islets.

Adipokines

Recently, it became clear that adipose tissue communicates with the rest of the body by synthesizing and releasing a number of secreted cytokine-like molecules, collectively designated as "adipokines," which along with FFA, significantly effect total body glucose metabolism and insulin sensitivity. Steatosis and steatohepatitis could be attributable to the combined effects of insulin resistance, and a relative failure of adipokine mediators to combat the effect of hyperinsulinemia and fasting hyperglycemia on hepatic lipid turnover.

Adiponectin

Adiponectin, also known as ACRP30, is a 30 kDa protein abundantly and selectively expressed in white adipose tissue. Its role in insulin resistance and atherosclerosis has been well established. Two adiponectin receptors have been cloned in mouse and humans, and both are expressed in liver [87]. In the liver, adiponectin increases insulin sensitivity and regulates FFA metabolism by suppressing lipogenesis and activation of FFA oxidation [74].

Adiponectin/ACRP30-knockout (KO) mice showed delayed clearance of FFA in plasma, high levels of tumor necrosis factor-alpha (TNFα) mRNA in adipose tissue, and high

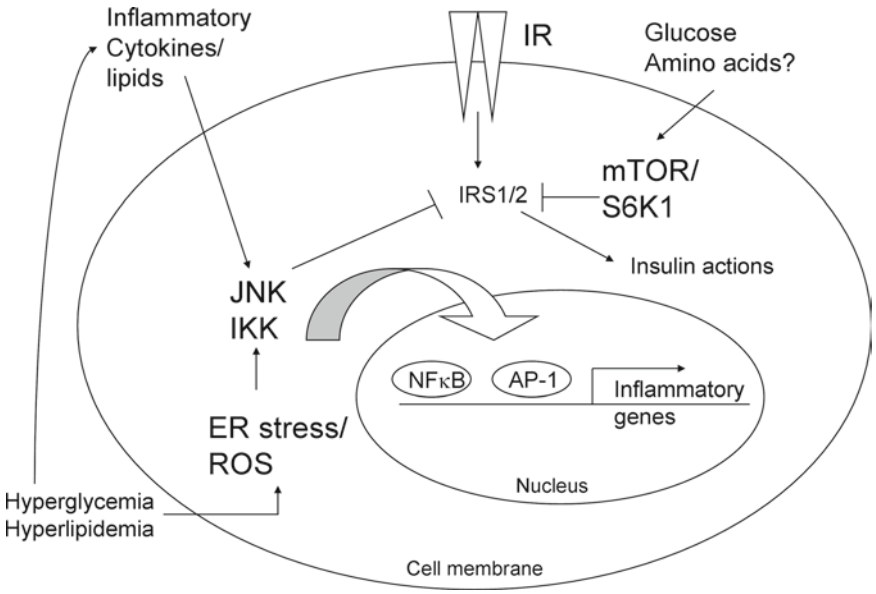

Fig. 8.5 Molecular mechanism of insulin resistance. Inflammatory pathways can be initiated by extracellular mediators such as cytokines, hyperglycemia, and lipids or by intracellular stresses such as ER stress or excess ROS production by mitochondria. Signals from all of these mediators converge on inflammatory signaling pathways, including the kinases JNK and IKK. These pathways lead to the production of additional inflammatory mediators through transcriptional regulation via the transcription factors AP-1 and NFκB as well as to the direct inhibition of insulin signaling. In addition the kinase mTOR and its downstream kinase S6K1 are candidates for negative regulation of IRS, mediating signals of nutrient overload (glucose and amino acids). *Arrows* represent activation, *bars* represent inhibition

plasma TNFα concentrations. Moreover, KO mice exhibited severe diet-induced insulin resistance with reduced IRS-1-associated PI3-kinase activity in muscle [88].

Adiponectin levels correlate negatively with liver fat and hepatic IR in NAFLD [89], suggesting that hypoadiponectinemia is part of a metabolic disturbance characterized by central fat accumulation. Moreover, low adiponectin levels are associated with a more extensive inflammation in NAFLD patients [90], suggesting an anti-inflammatory role of adiponectin in controlling hepatic inflammation. This is highlighted by the fact that adiponectin administered in ob/ob mice alleviates liver steatosis and attenuates inflammation in NAFLD mouse models [91]. However, adiponectin levels in the liver and plasma are not always correlated. In NASH adiponectin-receptor, (AdipoRII)-mRNA is negatively correlated with the histological grade of fibrosis, but not with serum or hepatic adiponectin. No correlation was observed between adiponectin-levels and liver adiponectin-expression [92]. In advanced liver fibrosis adiponectin-levels are even higher than normal [93], a phenomenon which might be explained by a reduced hepatic extraction.

Leptin

Leptin plays a pivotal role in regulating food intake, energy expenditure, and neuroendocrine function. Leptin stimulates the oxidation of fatty acids and the uptake of glucose, and prevents the accumulation of lipids in nonadipose tissues, which can lead to functional impairments known as lipotoxicity. Moreover, it exerts a proinflammatory influence regulating T-cell response [94]. Long term leptin-treatment improves hepatic and peripheral glucose metabolism in insulin resistant lipodystrophic patients and improves liver histology [95]. Leptin-receptors are expressed on hepatic stellate cells and leptin is believed to be involved in liver fibrosis [96].

Leptin-deficient mice develop obesity; however, they fail to develop liver fibrosis during steatohepatitis or in response to chronic toxic liver injury. Restoration of physiological levels of circulating leptin, but not correction of the obese phenotype by dietary manipulation, restored fibrosis indicating an essential role in developing liver fibrosis [97]. Elevated serum-leptin levels have been detected in patients with liver cirrhosis, a disease frequently associated with hypermetabolism and altered body weight [98]. However, implantation of a transjugular intrahepatic portosystemic shunt (TIPS) improved nutritional status in patients with cirrhosis while leptin levels increased (most probably via decreasing hepatic degradation), suggesting that increased leptin levels are not a major reason for poorer body composition in liver cirrhosis [99].

The significance of leptin in human NAFLD has been questioned recently [100]. A more comprehensive overview about the role of leptin in liver disease is published elsewhere [101].

Resistin

Resistin is a 10 kDa adipose tissue-specific hormone that was identified when screened for genes selectively upregulated during adipogenesis, but decreased by PPARγ agonist treatment. Injection of resistin into wild-type mice resulted in reduced glucose tolerance and insulin action, whereas injection of neutralizing antibodies into diabetic obese mice improved insulin action [102]. A significant role for resistin in regulating insulin sensitivity was further confirmed in resistin knockout mice which display low glucose levels after fasting, suggesting an impact on hepatic glucose production [103].

Transgenic mice that have high circulating levels of resistin in the setting of normal weight display higher fasted blood glucose and an impaired glucose tolerance than their non-transgenic littermates. Metabolic studies revealed that chronically hyperresistinemic mice have elevated glucose production and this increase in glucose production may be partly explained by an increased expression of hepatic PEPCK [104].

Insulin resistance caused by resistin infusion was attributed to an increase in the rate of glucose production and not to an increase in glucose uptake, indicating that resistin has effect on hepatic, but not peripheral insulin sensitivity in rat [105] and mouse [106] models of insulin resistance. However, other groups have shown resistin-mRNA in most mouse models of insulin resistance to be down-regulated [107]. In addition, resistin expression in human adipocytes was very low [108]. In fact, human resistin expression was higher in monocytes [109]. Furthermore, some studies have failed to show a link between resistin levels and BMI or insulin sensitivity [108, 110], whereas others argue for such a connection [111, 112].

In a cirrhotic rat model using bile duct ligation (BDL), the BDL-induced cirrhotic rats showed significantly higher resistin mRNA and protein levels were evident as compared to sham animals [113]. Resistinlevels are also increased in human liver cirrhosis. However, as in case of obesity, the link between resistin and insulin sensitivity is not clear.

While in one study serum resistin correlated with insulin secretion and inversely with insulin-sensitivity [114], another study failed to show such a correlation. Moreover, after liver transplantation resistin levels remained unchanged while insulin sensitivity improved [115], challenging the concept of resistin directly influencing insulin sensitivity in humans. Another study in NAFLD-patients failed to show a correlation between resistin and BMI, HOMA-IR, insulin and glucose, but could show a positive correlation between resistin and histological inflammation, supporting a link between histological severities of the disease but not between resistin and insulin resistance in NAFLD [116].

Taken together, most of these studies shed doubt on the role of resistin in insulin resistance associated with obesity and/or liver disease. However, there is probably a role for resistin in regulating body composition both in obesity and in liver disease. Further studies in humans and the identification of a receptor will be necessary to determine the clinical relevance of resistin in humans.

Tumor Necrosis Factor-α

TNFα plays a central role in IR. TNFα impairs insulin signaling by inhibiting the function of IRS-1 through serine phosphorylation [117]. Both in vivo and in vitro experiments have demonstrated that adiponectin and TNFα suppress each other's production and also antagonize each other's action [88]. TNFα knockout mice demonstrate less IR with high-fed feeding [118]. Overproduction of TNFα in liver tissue has been proposed to play a key role in the pathogenesis of fatty liver. Fatty liver disease in ob/ob mice is significantly improved by inhibition of hepatic TNFα production or infusion of anti-TNFα neutralizing antibodies [119].

IL-6

IL-6 is expressed at high levels in omental adipose tissue and is released following a meal [120]. IL-6 is elevated in subjects with hyperinsulinemia and obesity [121, 122]. On the other hand, muscular IL-6 release in an autocrine fashion has a beneficial effect on glucose disposal [123] and insulin signaling in muscle during exercise [124] designating IL-6 as a "myokine" [125]. Although fat-fed IL-6 knockout mice demonstrate a higher plasma glucose following glucose tolerance testing, these mice were characterized by a lack in overt diabetes and obesity when compared to wild-type mice [126]. Somehow confusing, a previous study with another IL-6 knockout strain showed that IL-6 KO mice developed maturity onset diabetes and insulin resistance [127]. The reason for this discrepancy is not clear. A possible explanation might be the use of a different background strain or the introduction of subtle genetic differences into the IL-6 knockout lines.

Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis

A detailed account on NAFLD and NASH is provided in Chap. 34. A summarized account is mandated in this section on carbohydrate metabolism. IR is a key feature of NAFLD [128], a condition which ranges from fatty liver to nonalcoholic steatohepatitis (NASH). It has been shown that glucose disposal is reduced to approximately 50% in nondiabetic NAFLD patients compared to normal subjects, and a similar extent in patients with diabetes mellitus type 2 [129]. If IR causes NALFD or vice versa, or if both conditions occur in parallel are still not completely understood. Also, the matter of whether hepatic or peripheral IR has the primacy in NALFD is still a matter of debate.

The recurrence of NALFD after liver transplantation is one argument against a primary hepatic defect. Recent data indicate that most probably the primary site of IR in NAFLD is the periphery, where lipolysis in adipose tissue is suppressed insufficiently, followed by a reactive hyperinsulinemia. As a consequence, there is an increase of FFA into the liver, followed by hepatic TG deposition and finally a hepatic-insulin resistance as summarized by Bugianesi et al. [130] in a recent review.

However, in a dog fat-fed model, subcutaneous and visceral adiposity was accompanied by hyperinsulinemia, modest peripheral IR, but a complete inability of insulin to suppress hepatic glucose production, suggesting that IR of the liver may be the primary defect in the development of insulin resistance associated with obesity [131].

The etiopathogenic sequence of hepatic IR being a consequence of liver steatosis has been shown in rats fed with a high-fat diet or [132] in lipoprotein lipase transgenic mice [133]. Moreover, a correction of steatosis reverses hepatic IR in leptin deficient (ob/ob) mice [134].

In humans, mitochondrial defects on a genetic basis eventually worsened by environmental factors have been suggested to be a cause of impaired β-oxidation in the liver. In fact, mitochondrial abnormalities have been described in NASH livers [135], favoring the liver as primary site of insulin resistance. Hepatic TG-deposition is a process, which is normally linked to increased insulin sensitivity. It has been suggested that hepatic lipid accumulation results from a selective intracellular sensitivity, such that while signaling to glycogen storage and HGP is impaired, signaling to lipid synthesis is preserved. This apparent contradiction might be dissolved by a recent animal model in which the hepatic transcription factor Foxo1 was shown to have dual properties, since in a state of impaired insulin signaling, Foxo1 activity increases, leading to excessive glucose production while at the same time TG synthesis and fatty acid oxidation decreases [136]. However, further studies will be required to establish the role of Foxo1 in human disease.

Insulin-resistance is a target for specific treatment of NAFLD, and insulin-sensitizing agents (metformin or thiazolidinediones) as well as lifestyle changes to reduce visceral adiposity are the most promising therapeutic options. Future trials need to be performed in order to test the long-term effectiveness of these treatments on the basis of clinically relevant histological outcomes.

Hepatitis C and Diabetes

A detailed account of hepatic phenotype in hepatitis C infection is provided in Chap. 38. Here, the evidence of the role of hepatitis C in contributing to metabolic disease is provided. Since the discovery of the hepatitis C virus (HCV) in 1989, it has been noticed that HCV does not solely affect the liver. In fact, a number of diseases like cryoglobulinemia and glomerulonephritis occur in patients with HCV infection. A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has also been known, implying hepatitis C as a metabolic disease. Furthermore, epidemiological studies have suggested a linkage between type 2 diabetes and chronic HCV infection [137, 138]. For hepatitis B, the situation is less clear. However, the majority of epidemiologic studies found a higher prevalence of diabetes in patients with HCV as compared to HBV infection [138, 139].

Earlier studies have been criticized because HCV might have been transmitted due to frequent measurement of blood sugar, and additional factors influencing glucose tolerance such as advanced cirrhosis or obesity and aging were difficult to rule out in epidemiologic studies. However, in a recent epidemiologic study, HCV was shown to be independently associated with an impaired glucose tolerance [140]. IR can be found in patients with HCV infection even in the absence of hepatic fibrosis [141]. In addition, the presence of diabetes in patients with HCV infection, but without cirrhosis was shown to be independently linked to the risk of developing hepatocellular carcinoma [142].

Moreover, basic studies performed in experimental systems were able to prove the association of HCV infection and diabetes. Transgenic mice expressing HCV core protein have elevated serum levels of insulin, although they do not develop diabetes. In insulin tolerance test, glucose levels were significantly higher in transgenic than in normal mice, signalizing insulin resistance. In addition, hyperinsulinemic-euglycemic clamp experiments and measurements of muscle glucose uptake revealed that this insulin resistance was a central, hepatic one [143].

A closer analysis of the insulin-signal pathway in these animals revealed a disturbed tyrosine phosphorylation in transgenic animals, which was sensitive to an inhibition of TNFα by applying anti-TNFα antibodies [144]. Although a direct impact on insulin signaling cannot be excluded, these data suggest a crucial role for TNFα in HCV-associated insulin resistance. Disturbed insulin signaling with impaired tyrosine phosphorylation of IRS-1 was also detected in ex vivo insulin stimulated liver biopsies explanted from patients with HCV infection [145].

IRS-1 and 2 have been found to be down-regulated in patients with HCV, and HCV core downregulated the expression of IRS-1 and -2 in human hepatoma lines and in transgenic mice. HCV has been shown to induce suppressor of cytokine signaling 3 (SOCS3), which is capable of ubiquitinating and thereby inactivating IRS [146], thus providing an additional mechanism of IR in HCV.

IR in HCV infection may have significant clinical implications, since IR and hyperinsulinemia have been shown to be independent predictors of progression of liver fibrosis [141], suggesting a causal role of insulin in liver fibrosis.

Strategies which improve insulin sensitivity thereby preventing hyperinsulinemia should therefore be of use in those patients in whom an antiviral therapy was not successful. Therefore, weight control should be an advice to patients with persistent HCV infection. If the use of drugs like thiazolidinediones or GLP-1 analogs will be beneficial in this situation is an intriguing question and clinical studies are urgently required.

Hepatogenous Diabetes

The changes in carbohydrate metabolism occurring in liver cirrhosis, a condition of loss of hepatic function and capacity, highlight the notion that the liver is the main regulator of glucose metabolism and important site of insulin resistance. The term “hepatogenous diabetes” was coined by Naunyn in 1898, who discovered the coincidence of diabetes and liver cirrhosis [147]. In the 1970s, observation was confirmed by larger epidemiologic studies. The prevalence of diabetes and impaired glucose tolerance among patients with liver cirrhosis are by far higher than in subjects without liver diseases. Sixty to eighty percent of cirrhotic patients are reported to be glucose intolerant and up to 20% develop overt diabetes [148–151]. Moreover, the development of diabetes is an established risk factor for long-term survival in cirrhosis [152].

Glucose clamp studies have shown that insulin-induced glucose metabolism is reduced in cirrhosis [153]. Insulin resistance occurs early in the natural history of cirrhosis and is not associated with degree or etiology of liver damage [154]. Insulin resistance is associated with reduced skeletal muscle glucose disposal [155], whereas whole body glycolysis and carbohydrate-induced lipogenesis remain unaffected [156]. Although chronic hyperinsulinemia has been undoubtedly linked to insulin resistance in cirrhosis, the underlying mechanisms and the resulting sequence of endocrine and molecular alterations are only partially resolved.

Several factors, including hyperinsulinemia, elevated contrainsulinary hormones like growth hormone, glucagon, and catecholamines, an increase in FFA, or decreased insulin-like growth factor have been proposed to contribute to cirrhosis-associated abnormalities in carbohydrate metabolism as discussed in detail elsewhere [157]. Moreover, disease

related putative liver factors, like HCV (discussed above) may be involved in developing insulin resistance and/or diabetes in liver disease.

Diabetes type 2 is characterized by an impaired augmentation of postprandial insulin secretion by gastrointestinal hormones like GLP-1 ("incretin" effect). If this is also the case in hepatogenous diabetes remains to be elucidated. However, if this should be the case, it is not due to diminished GLP-1 secretion, since GLP-1 response in the portal blood is not different between cirrhotic patients with and without diabetes [158].

Experimental data from animal models revealed that hyperinsulinemia in cirrhosis is not due to an increase of insulin-secretion from islets, but could be explained by a decreased hepatic clearance of insulin [159]. It is known that about 70% of the portovenous insulin is extracted during the first pass through the liver [7]. In fact, in human liver cirrhosis, hepatic insulin clearance is diminished [156, 160]. Both, portosystemic shunting and an impaired liver function are suggested to play a role in hyperinsulinism observed in liver disease. Furthermore, portosystemic shunting is involved in hyperglucagonemia, a phenomenon frequently observed in liver cirrhosis [161].

Data from liver transplanted patients support the view that a disturbed hepatic insulin clearance is of major importance in developing hyperinsulinism, since insulin suppression of hepatic glucose production could be restored after transplantation. However, in about one third of the patients, diabetes did not disappear because of persistence of a reduced beta-cell function [162].

Summary

Although an important role of the liver in carbohydrate metabolism has been known for decades, the recent years taught us important details about the liver's communication with other organs, the lipid metabolism and about the intracellular mechanisms regulating glucose homeostasis. Moreover, it became apparent that there is a close interrelationship between liver disease and alterations of the carbohydrate metabolism, which is characterized by insulin resistance, culminating in the suggestion to include NAFLD in the catalog of the metabolic syndrome, a disease of pandemic dimension. Transgenic and gene knockout mice proved to be valuable tools to identify factors and pathways involved in regulating glucose homeostasis. Dissection of insulin signaling taught us that the direct effect of insulin on the liver is probably more important in maintaining glucose homeostasis than previously believed. Moreover, a deepened insight into the regulation of intracellular glucose fluxes may give us an idea about how therapy of insulin resistance and associated diseases could look like in the future.

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Chapter 9
Hepatic Protein Metabolism

Wouter H. Lamers, Theodorus B. M. Hakvoort, and Eleonore S. Köhler

Introduction

The body cannot store protein to any extent under steady state conditions. The equivalent of all amino groups of the protein component of the food has, therefore, to be deaminated. The resulting ammonia has to be detoxified and excreted (largely as urea), whereas the carbohydrate component has to be burned, or stored as glycogen, or fat. Hyperammonemia is frequently associated with, or is even ascribed to failing liver function, but few studies have summarized the available data on substrate load and functional capacity of the liver to metabolize ammonia. This chapter discusses the quantitative aspects of protein metabolism in the body with significant emphasis on the role of liver in this phenomenon and highlights the role of periportal vs. pericentral hepatocytes.

Protein Ingestion and Metabolism to Urea

A 25g mouse consumes ~3.5 grams of chow a day [1]. The minimum maintenance content of protein in mouse food is 12% [2], but regular mouse chow typically contains ~20% protein, while a high-protein diet contains >40% protein. As a result, the mice cited have to produce ~2, ~3, and ~6 mmol urea per day, or ~75, ~115, and ~250 mmol urea.kg^{-1}.day^{-1}. For a "typical" 70 kg human consuming 0.3, 1.0, or 2.5 g protein/kg^{-1}.day^{-1} [3], these numbers are ~100, ~300, and ~800 mmol urea per day, or ~1.4, ~4.5, and ~11 mmol urea.kg^{-1}.day^{-1}. These numbers correspond well with the urea synthesis rates as determined with isotopes in mice [4, 5] and in humans [3, 6–9], and with the observed daily urea excretion rates (for a review, see [10]). The species difference arises from the fact that metabolic rates scale to approximately three fourth power of the animal's body mass [11, 12].

Urinary Excretion of Urea Accounts for 70–80% of its Production

Studies on urea synthesis rates in humans in vivo have consistently shown that urea excretion via the urine accounts for only 70–80% of urea synthesized over a wide range of protein intakes [3, 6, 8, 13–15], with some studies reporting a value as low as ~60% [9] (Fig. 9.1). This apparent "leak" is generally ascribed to urea excretion to the gut [9, 16]. Microbial urease can make this urea available for the resynthesis of amino acids. However, measured as the contribution of labeled lysine, fecal microbial amino-acid synthesis from $^{15}NH_4^+$ or $[^{15}N]_2$-urea contributes only 5–10% to the appearance rate of essential amino acids in plasma in man [17–19]. In agreement, fecal nitrogen loss, and reabsorption of ammonia and reentry into the urea cycle are quantitatively more important fates of intestinal urea than the rescue of nitrogen for amino-acid synthesis [3, 14, 20, 21]. Urea hydrolysis in the intestine is most prominent in the fasting period [21]. The finding that oral $^{15}NH_4^+$ is a ~65% better precursor for intestinal amino-acid synthesis than $[^{15}N]_2$-urea [22] indicates that microbial urease expression is a determining factor, which varies with the feeding cycle [23].

Although as much as 50% of urea that is excreted to the intestine during fasting may be hydrolyzed [21], Young and co-workers [3, 21] argue that the resulting ammonia is recycled to urea in the liver, whereas Jackson c.s [9, 24]. claim that as much as 80% is reincorporated into amino acids (the disagreement centers about the degree to which the ^{15}N tracer is exchanged). The numbers from both sets of studies [9, 21] nevertheless, indicate that, in the postprandial period in man, ~30–90 μmol intestinal ammonia.kg^{-1}.h^{-1} from endogenously synthesized urea returns again to the liver. This corresponds to ~10% of the protein intake.

W.H. Lamers (✉)
AMC Liver Center, Academic Medical Center, University of Amsterdam, Meibergdreef 69-71, 1105 BK, Amsterdam, The Netherlands; NUTRIM School for Nutrition, Toxicology and Metabolism of Maastricht University Medical Center, Universiteitssingel 50, 6229 ER, Maastricht, The Netherlands
e-mail: w.h.lamers@amc.uva.nl

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_9, © Springer Science+Business Media, LLC 2011

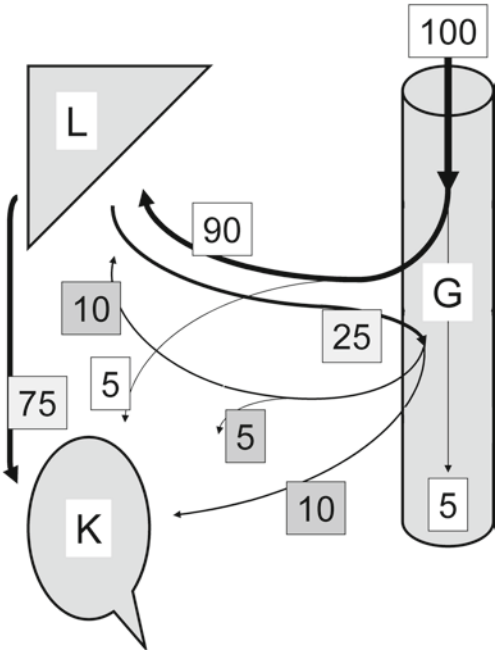

Fig. 9.1 Fate of the amino groups of dietary proteins. The scheme demonstrates the extensive recycling of urea. The numbers refer to rodents and humans fed an average protein-containing diet, and are expressed as a fraction of the dietary intake. Numbers on a white background: of all ingested dietary proteins, ~5% remains undigested and will eventually be catabolized by the colonic flora. If no acidosis or alkalosis exists, the amino groups of another ~5% is excreted as ammonia by the kidney. The amino groups of the remaining ~90% will eventually be metabolized to urea by the liver. Numbers on a light gray background: of all urea synthesized by the liver, ~75% is excreted by the kidney, while the remaining ~25% is excreted to the gut. Numbers on a dark gray background: ~60% of the urea excreted to the intestine (equivalent to ~15% of the ingested amino groups) will be hydrolyzed by the intestinal microbial flora and used for amino-acid synthesis (~5%) or returned to the blood (~10%). The remaining urea (~10% of the ingested amino groups) is reabsorbed into the blood. For more details, see main text. *L* liver; *G* gut; *K* kidney

Intestinal Microbial Urea Recycling in Ruminants

The recycling of urea for microbial amino-acid resynthesis in the human intestine is a quantitatively weak reflection of a similar process in ruminants. In ruminants, urea excretion to the gut occurs almost exclusively in the stomach and small intestine [25]. In cows, the fraction of endogenous urea production that is excreted to the gut decreases from ~100% in animals on a low-protein diet to ~30% in animals on a high-protein diet, and appears to reflect the fact that the gut entry rate of urea depends on the plasma urea concentration rather than protein intake [26]. A nearly fixed fraction (30–35%) of the urea that enters the gut returns to the ornithine cycle, whereas the remainder is used for amino-acid synthesis in enteral bacteria [26]. As a result, the recycling of urea through the digestive tract functions as a buffered source of amino acids when protein in the food becomes limiting, but does not enhance amino-acid utilization at higher, growth-promoting protein supplies in the food.

In contrast to the findings in ruminants, studies in pigs [25, 27] and the only comparable study available in humans [3] indicate that urea excretion to the gut increases with increasing protein intake and endogenous urea production. Apparently, the circulating urea concentration does not directly determine the rate of urea excretion into the gut in pigs and humans, as it does in ruminants (see previous paragraph).

Ammonia Excretion

Most of the ammonium excreted by the kidney is produced in the kidney itself from amino acids, chiefly glutamine. In normal control humans, only ~5% of the daily production of ammonia is excreted into the urine, but if acidosis develops, this number can rise to almost 30% [28]. Under alkalotic conditions, ammonia excretion into the urine virtually ceases (~1%).

Digestibility of Dietary Proteins

The true oro-ileal digestibility of proteins in simple-stomached animals is 90–95% [29–31]. Most of the remaining protein is metabolized by bacteria in the colon. In agreement, luminal colonic ammonia levels were found to increase with the protein content of the diet. In rats on a low-protein diet (8 en%), the ammonia concentration was 20–25 mM in the proximal colon and 30–40 mM in the distal colon, irrespective of whether the diets contained 12 or 48 en% fat [32]. In rats on a high-protein diet (32 en%), the ammonia concentration in the proximal colon was ~50% higher than in rats on a low-protein diet (30–40 mM) and ~2-fold higher in the distal colon [32]. In another rat study, a 60 en%-protein diet resulted in ~2-fold higher ammonia levels in the proximal colon than a 20 en%-protein diet (~20 vs. ~10 mM, respectively), without differences in the distal colon (10–15 mM). The differences in colonic ammonia content were reflected in the ammonia concentration of the colonic vein (~100 vs. ~175 μM) [33]. In agreement, experimental introduction of ammonia into the pig colon showed a direct relation between colonic ammonia content and the appearance of ammonia in the portal vein [34].

Intestinal Ammonia Production from Glutamine

The now classical studies of Windmueller and Spaeth [35–37] revealed that glutamine rather than glucose is the major metabolic fuel of the enterocytes of the small intestine, that the amount

of glutamine utilized is similar in germ-free and conventional animals, and in the fed and the fasted state, and finally, that both luminal and vascular glutamine can be utilized by the enterocytes to meet requirements in both the fed and the fasted state. First-pass catabolism of glutamine, glutamate, and aspartate is near complete, while that of most other amino acids amounts to 30–50%. Only alanine, arginine, and tyrosine are produced [38, 39]. The limited role of intestinal bacteria in ammonia production can be deduced from the findings that ammonia levels in germ-free and conventional anhepatic rats rose equally fast [40], and that circulating ammonia levels decline only moderately upon antibiotic treatment (for a review, see [41]).

In rats in vivo, intestinal glutamine utilization was reported to be 200–900 μmol.kg^{-1}.h^{-1} [38, 42, 43], while glutamine utilization of the intestine was ~500 μmol.kg^{-1}.h^{-1} in postabsorptive mice [44] and 20–30 μmol.kg^{-1}.h^{-1} in fasting humans [45]. Glutaminolysis in enterocytes accounts for ~65% of total small-intestinal ammonia production in the fed condition and for ~95% in the fasted condition [38]. These numbers are based on arterial-portal-vein concentration differences and, therefore, do not reveal the glutamine contributed by the food. When glutamine was provided both enterally and vascularly, glutaminolysis was ~1.5-fold higher than when glutamine was provided from either side alone [46]. Irrespective of these considerations, the numbers indicate that intestinal glutaminolysis accounts for ~10% of the daily ammonia supply for urea synthesis in both rodents and humans.

Role of Intestine in Ammonia Production

We have discussed three sources of intestinal ammonia production. Glutamine deamination contributes ~10% of the total daily ammonia production, urea recycling ~10%, and colonic bacterial catabolism due to incomplete small-intestinal protein digestion ~5% (Fig. 9.1). These numbers are similar in the fed and postabsorptive periods [38], and imply that ~75% of ammonia that is converted into urea is carried to the liver as amino acid (Fig. 9.2).

Hepatic Ammonia Detoxification

The maximal activity of urea synthesis in human liver amounts to 1–3 mmol urea.kg^{-1}BW.h^{-1} [47–49]. The capacity to synthesize urea in cirrhotics is ~80% of that in controls [48]. In rat liver, this number amounts to 3.5–5.5 mmol urea.kg^{-1}BW.h^{-1} [50–53]. We recently determined the capacity to synthesize urea from ammonia in perfused mouse liver in situ and found it to be 4–5 mmol urea.kg^{-1}BW.h^{-1} in animals fed with 18% protein diet (He et al. unpublished). Assuming a protein consumption of 70–140 g (human), 2.7 g (rat), and 0.6 g (mouse;

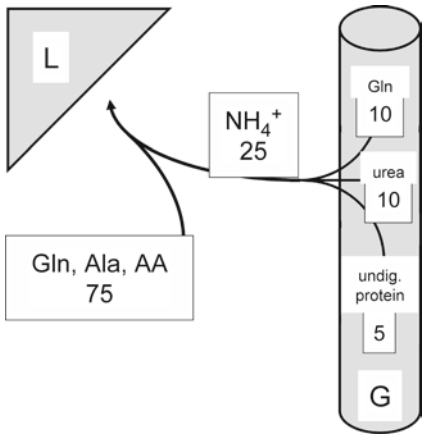

Fig. 9.2 Sources of substrate for urea synthesis. The portal vein carries intestinal ammonia derived from glutaminolysis (equivalent to ~10% of the ingested amino groups), microbial intestinal urea hydrolysis (~10%), and colonic microbial catabolism of undigested protein (~5%) to the liver. The remaining amino groups for urea synthesis (equivalent to ~75% of the ingested amino groups) are carried by glutamine, alanine, and other amino acids. For more details, see main text

the latter two numbers are based on a daily consumption of 15 and 3.5 g pellets, containing 20% protein, per "typical" 300 g rat or 25 g mouse, respectively [1]), ~1,300, ~25, and ~6 mmol ammonia per day needs to be detoxified (see Sect. "Protein Ingestion"). Since ammonia detoxification proceeds relatively independent of feeding (~85 and ~60% in the postabsorptive state relative to the fed state in humans and rats, respectively [21, 38]), these numbers amount to 0.2–0.4, ~1.7, and ~5 mmol.kg^{-1}BW.h^{-1} urea synthesis for humans, rats, and mice, respectively. This comparison reveals that human liver has a ~5-fold overcapacity to detoxify ammonia, whereas this number is only ~2-fold in rats and barely exceeds one in mice. The ~4-fold increase in ammonia detoxifying activity per gram of liver after an 80% partial hepatectomy in humans due to substrate accumulation [49] is in agreement with this conclusion. In further agreement, the liver remnant of rats that have undergone a 65% partial hepatectomy 24 h earlier can indeed double its urea output in response to substrate accumulation [54, 55]. For mice, no data are available. The limited reserve capacity of the liver to detoxify ammonia explains why the capacity to synthesize urea corresponds directly with the protein content of the food in both man [56] and in rats [51, 57], and suggests that mammals limit their capacity to synthesize urea to avoid loss of amino acids at low protein intake.

Sources of Hepatic Urea Synthesis

The main sources of the liver for urea synthesis are ammonia and amino acids, especially alanine and glutamine (Fig. 9.3). The liver can, however, also become a producer of glutamine,

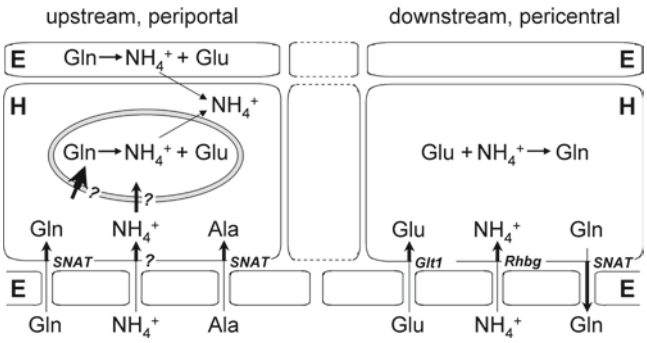

Fig. 9.3 Amino-acid and ammonia transport into the liver. An upstream, periportal and a downstream, pericentral hepatocyte (H) and sinusoidal endothelial cell (E) are shown. Glutamine and alanine are transported mainly by SNAT transporters. Ammonia is transported into pericentral hepatocytes by the Rhbg transporter and glutamate by the Glt1 transporter. The carrier for concentrative transport of glutamine across the mitochondrial membrane (?) is not yet identified. Furthermore, it is unknown whether ammonia accumulates inside periportal hepatocytes by a concentrative transporter (?) or results from spill over of mitochondrial glutaminase-2 or endothelial glutaminase-1. For more details, see main text

particularly in the fasted state [38]. Ammonia reaching the liver has a threefold higher chance of being metabolized to citrulline than to aspartate, whereas the reverse is true for alanine [58, 59]. Glutamine has a twofold smaller chance of ending up in citrulline than ammonia [60]. The more contentious issues pertain to the relative contributions of ammonia and glutamine to hepatic urea and glutamine synthesis. Due to the extensive glutamine utilization by the enterocytes, postabsorptive portal glutamine levels amount to 50–70% of arterial glutamine levels. Under these conditions, portal ammonia levels were ~4-fold higher and portal alanine levels almost twofold higher than the corresponding arterial levels in mice (He et al. unpublished data).

Zonation of Ammonia-Metabolizing Enzymes and Transporters

The expression of the enzymes involved in hepatic ammonia and glutamine metabolism is highly heterogeneous. Urea-cycle enzymes are found in the upstream, periportal hepatocytes [61–63], whereas glutamine synthesis is confined to the downstream, pericentral hepatocytes [64]. Periportal hepatocytes also express the liver-type glutaminase (glutaminase-2) [65], which may play a crucial role in citrulline synthesis by producing high amounts of ammonia intramitochondrially. Mitochondrial carbonic anhydrase (type V), also a crucial enzyme in mitochondrial citrulline synthesis, is nearly hepatocyte-specific in its organ distribution [66, 67], but its zonal distribution has not been established definitively [67]. In a typical mouse liver, the porto-central axis is ten hepatocytes long [68]. Of these, the upstream 7–8 cells express the urea cycle enzymes and the most upstream 3–4 cells glutaminase-2. The presence of glutaminase-2 in the upstream hepatocytes and of glutamine synthetase in the downstream hepatocytes implies that the liver can both consume and produce glutamine. Glutamate dehydrogenase is present in the mitochondria of periportal and pericentral hepatocytes [69, 70]. The putative role of the aquaporin AQP9 in urea export from the hepatocytes [71] also remains questionable, since this transporter is predominantly expressed in the sinusoidal membrane of the pericentral rather than the periportal hepatocytes [72, 73], and its systemic deficiency does not cause a perturbation in urea production or transport [73].

The hepatocytes in the downstream comprise 2–3 cell layers, and are well equipped to take up ammonia and glutamate from the blood by strongly and exclusively expressing the plasma membrane glutamate transporter Glt1 (SLC1A2) [74, 75] and the ammonia transporter Rhbg (SLC42A2) [76]. Glutamate accumulates in pericentral hepatocytes [70] via import from the circulation [74] and the selective expression of the glutamate-supplying enzyme ornithine aminotransferase [77]. The role of pericentral glutamine synthesis in ammonia detoxification appears, nevertheless, limited. Circulating ammonia levels were not increased in Rhbg-deficient mice [78], while only a twofold increase in systemic ammonia levels was found in hepatocyte-specific β(beta)-catenin-deficient mice that were fed a high-protein diet [79]. These mice lack expression of all pericentral ammonia-transporting and metabolizing proteins in their pericentral hepatocytes.

The major transporters of glutamine and alanine in liver appear to be the sodium-coupled neutral amino-acid transporters (SNATs) 3–5 (SLC38A3, -4, -5) [80–83] (for review, see [84]). SNAT1 is expressed in fetal liver, but disappears neonatally [85], whereas SNAT2 is not expressed in normal adult liver, but is strongly upregulated with a periportal distribution in diabetic liver [83]. SNAT3 mRNA [83, 86, 87] and protein [81] are present in a shallow centro-portal gradient. SNAT4 mRNA, in contrast, has a pronounced pericentral localization [83]. SNAT5 mRNA has, like SNAT3, no clear zonal distribution [87]. The SNAT3 [88] and SNAT5 transporters [82] appear to prefer alanine over glutamine, whereas SNAT4 prefers glutamine over alanine [80]. However, it should be kept in mind that alanine can also be transported via several other transporters that are expressed in the liver [89].

Amino-Acid Transport into the Liver

The upstream, periportal hepatocytes are committed to urea synthesis, mainly from ammonia, glutamine, and alanine (Fig. 9.3). The conversion of precursors to urea nitrogen is

very fast [90, 91]. Although this finding points to an important role for transporters, none of them is, as far as known, specifically expressed in the upstream region of the liver (see previous paragraph). The ammonia concentration in rat liver is 0.2–0.5 mmol/kg [92], whereas that of glutamine is 3–4 mmol/kg [93–95] and that of alanine is ~2 mmol/kg [95]. In mice, we found the hepatic concentration of glutamine to be 1–1.5 mmol/kg and that of alanine ~3 mmol/kg (He et al. unpublished data). These findings show a two to fourfold increase in concentration of ammonia, alanine, and glutamine in the hepatic tissue compared to the surrounding blood.

The electrogenic properties of the SNATs imply that these transporters both facilitate a Na^+-dependent uptake of glutamine or alanine and increase the cytosolic pH of the hepatocytes by the simultaneous export of H^+ ions, whereas export of glutamine or alanine has the reverse effect. Since all enzymes involved in urea synthesis have their activity optimum at pH ~8 (for a review, see [92]), the SNAT-dependent import of glutamine and alanine facilitates urea synthesis. The concentration of glutamine is increased a further three to fivefold in a pH-dependent manner across the mitochondrial inner membrane by a yet unidentified transporter [93, 94]. This stepwise increase in glutamine concentration brings about a mitochondrial glutamine concentration that is in the range of the K_m of glutaminase-2 for glutamine (~25 mM).

Ammonia Transport into the Liver

The (human) liver effectively detoxifies intestinal ammonia up to at least 6 $mmol.h^{-1}$ [96]. Although portal ammonia levels rise to ~0.5 mM in these cases, the high K_m value of carbamoylphosphate synthetase for ammonia (1–2 mM) [97] implies that this rate-limiting enzyme of the urea cycle functions at <25% of maximum activity under these conditions unless there is a concentrating mechanism for ammonia in the upstream hepatocytes. However, no measurable levels of transporters of the Rh glycoprotein family of ammonia transporters were found in periportal hepatocytes [76]. Alternatively, the increase in intracellular ammonia levels may depend on glutaminase activity. Ammonia may escape as product of mitochondrial glutaminase-2 before it is captured by carbamoylphosphate synthetase (Fig. 9.3) [98]. This mechanism assumes that metabolite channeling between the mitochondrial enzymes glutaminase-2, carbamoylphosphate synthetase, and ornithine transcarbamoylase to form citrulline from glutamine is not very efficient, if at all existing [60, 90, 99]. Alternatively, ammonia may originate from the recently described glutaminase-1 activity in (porcine) sinusoidal endothelium (Fig. 9.3) [100]. The latter two options would imply that glutamine rather than ammonia is the real substrate for citrulline (and urea) synthesis.

pH and Ammonia Detoxification

A normal liver is able to effectively correct to systemic levels the substantial changes in portal ammonia and amino-acid concentrations that result from feeding and metabolism [95, 96]. As an example, circulating ammonia levels are ~10-fold lower than portal ammonia levels up to at least 2 mM portal ammonia in guinea pigs [101]. Similarly, increasing glutamine concentrations (0–2.3 mM) in the perfusate induced an almost linear response in liver glutamine metabolism with glutamine production decreasing between 0 and 0.5–0.6 mM glutamine, and switching to increasing glutamine consumption at higher glutamine concentrations [87]. In this latter study, the relation between the afferent glutamine concentration and hepatic glutamine metabolism could be entirely ascribed to glutamine transport via the SNAT transporters. In agreement with the electrogenic properties of these receptors, ammonia detoxification is very sensitive to changes in the acid-base balance. Most importantly, acidosis decreases liver ammonia detoxification [102], because the reduced amino-acid transport into the liver [103] and the reduced glutamine transport into the mitochondria [94] decrease substrate availability. The inhibitory effects of acidosis on substrate transport are magnified by the alkaline pH, optimum of the activity of most enzymes involved in urea synthesis [92]. At the same time, ammonia excretion, a vehicle of H^+, into the urine increases up to ~6-fold in acidosis [28]. This ammonia is almost exclusively derived from glutamine. The net effect of a developing acidosis is, therefore, a shift in glutamine catabolism from the liver to the kidney. The capacity of the kidney to eliminate ammonia is, however, limited to ~30% of the daily production of ammonia [28]. Perhaps equally important is that the pH dependence of ammonia detoxification reduces the already small margin (two to fourfold excess capacity) between the hepatic capacity to metabolize ammonia and the supply of this toxic substrate if acidosis develops.

Ammonia Detoxification in the Compromised Liver

In addition to inborn errors of metabolism, liver cirrhosis is arguably the best studied cause of hyperammonemia. Hyperammonemia due to liver failure increases ammonia detoxification as glutamine in peripheral organs, such as muscle, and increases ammonia elimination in the kidney via

glutaminolysis in rodents and man [104–106]. In anhepatic animals, plasma glutamine levels can increase to ~4 mM [104]. In addition, the intestine switches from glutaminolysis to glycolysis as source of energy [107, 108], even though there are no signs of hypoxia [107]. Instead, this change in metabolism is attributed to the hypermetabolic state [109], a condition that frequently develops after significant injury to the body. These findings demonstrate that ammonia detoxification is a distributed property, with many organs that express the enzyme glutamine synthetase [110] able to contribute. The decreased glutamine consumption by the splanchnic region [107, 108] also supports ammonia detoxification by reducing its production. The capacity of the kidney to eliminate ammonia produced from glutamine appears to be limited [104], even if acidosis is invoked [28] (see Sect. "Ammonia Excretion").

Conclusions

Thus, it is relevant to emphasize that there is extensive recycling of urea through the gut, even in simple-stomached mammals. It is also important to emphasize that about 25% of the amino groups that have to be detoxified by the liver are supplied as ammonia, even though the periportal hepatocytes apparently do not have ammonia-concentrating transporters. Finally, the ammonia detoxification by the liver is highly pH-sensitive, so that a developing acidosis can quickly eliminate the small surplus ammonia-detoxifying capacity of the liver.

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Chapter 10
Hepatic Lipid Metabolism

Jiansheng Huang, Jayme Borensztajn, and Janardan K. Reddy

Introduction

The liver is a major regulator of lipid metabolism in the body. It plays a central role in the synthesis and degradation (oxidation) of fatty acids. Fatty acids serve as an important source of energy as well as energy storage for many organisms and are also pivotal for a variety of biological processes, including the synthesis of cellular membrane lipids and generation of lipid-containing messengers involved in signal transduction [1]. Fatty acids can generally be stored efficiently as non-toxic triglycerides (triacylglycerols/fat), which generate more than twice as much energy, for the same mass, as do carbohydrates or proteins. Accordingly, liver is a key player in energy homeostasis: first, as it converts excess dietary glucose into fatty acids that are then exported to other tissues for storage as triglycerides as lipid droplets [2]; second, under conditions of increase in synthesis and decreased oxidation of fatty acids the liver contributes to the progressive accumulation of excess unspent energy in the form of energy-dense triglycerides in adipocytes of adipose tissue, which provide virtually limitless capacity to store energy and finally, under chronic energy over-load situations the liver may serve as a surrogate reservoir for storing considerable quantities of excess fat, leading to the development of hepatic steatosis and steatohepatitis [3]. For molecular pathogenesis of fatty liver, please see Chap. 29. Independent chapters are also included on non-alcoholic fatty liver disease (NAFLD) (Chap. 34) and alcoholic liver disease (Chap. 35). This ability of the liver to store lipids is viewed as a protective mechanism, neutralizing the potential toxicity of long-chain fatty acids [4].

In addition to synthesis, oxidation, and secretion of fatty acids for transport and storage in extrahepatic tissues, the liver also functions in the maintenance of plasma lipid levels, a) through its ability to assemble and secrete lipoproteins into the circulation and b) through its central role in the removal of lipoproteins from circulation [5]. Accordingly, an understanding of fat metabolism in liver is important for delineating the pathophysiological implications of altered energy balance and in developing pharmacological strategies for preventive and therapeutic approaches. The emphasis of this chapter is on the sources and synthesis of fatty acids, very low density lipoprotein assembly and secretion, and fatty acid oxidation.

J.K. Reddy (✉)
Department of Pathology, Northwestern University,
Feinberg School of Medicine, Chicago, IL, USA
e-mail: jkreddy@northwestern.edu

Sources and Synthesis of Fatty Acids

Fatty acids utilized by the liver for energy generation, for storage as triglycerides, and incorporation into lipoproteins, are generally derived from: (a) hepatic uptake of plasma non-esterified fatty acids (NEFAs) transported in the circulation after release by adipose tissue, as well as after hydrolysis of circulating triglyceride-rich lipoproteins; (b) breakdown of triglycerides of chylomicron remnants taken up by hepatocytes; (c) hepatic cytoplasmic triglyceride stores that manifest as lipid droplets, big or small, in all cell types; and (d) synthesis in situ (lipogenesis).

Hepatic Uptake of Plasma NEFAs Released by Adipose Tissue and Hydrolysis of Circulating Triglyceride-Rich Lipoproteins

Fatty acids deposited as triglycerides in white adipose tissue represent the primary energy store in animals. Under conditions of caloric deficit (e.g., starvation) or increased energy demand, triglycerides are hydrolyzed and free fatty acids are released into the circulation. Thus, in these situations, most of the circulating fatty acids taken up by the liver are mobilized from adipose tissue triglycerides. The hydrolysis of triglyceride is catalyzed by adipose tissue lipases in sequential steps leading to the formation of NEFAs and glycerol. Adipose triglyceride lipase (ATGL) and hormone-sensitive

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_10, © Springer Science+Business Media, LLC 2011

lipase (HSL) are the major triglyceride lipases involved in this process (Fig. 10.1). ATGL specifically performs the first step in lipolysis generating diacylglycerol and fatty acid [6], and subsequent action of HSL most efficiently hydrolyses diacylglycerol, further releasing fatty acids. Together, ATGL and HSL are responsible for more than 95% of the triglyceride hydrolase activity present in white adipose tissue [7]. Also involved in the mobilization of fatty acids from stored sites are members of perilipin family [8]. The initial hydrolysis of triglyceride to diacylglycerol is known to be the rate-limiting step, since hydrolysis of diacylglycerol is tenfold faster. The released free fatty acids become bound to circulating albumin and are carried to the liver, as well as other tissues, for uptake and utilization. Fatty acid mobilization from fat is tightly controlled by hormones such as insulin, glucagon, epinephrine, and adrenocorticotropic hormone. Mice deficient in HSL secrete less insulin and are glucose intolerant and reveal adipocyte size increase and decrease plasma free fatty acids, mostly due to the attenuation of lipolysis [9, 10]. Of interest is that the surface of the lipid storage droplet coated with a variety of proteins has emerged as a central site of regulation of lipolysis (see below). The mechanism by which HSL attacks the lipid droplets is not yet firmly established, but may be involved in the activation of a lipid binding domain on HSL and translocation from the cytosol by a carrier protein [11].

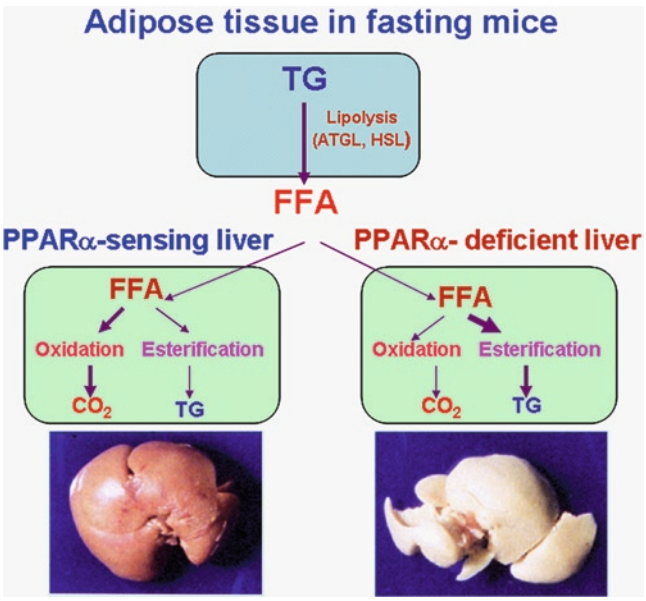

Fig. 10.1 Adipose tissue lipolysis during fasting, fatty acid influx into liver and PPARα sensing. Under conditions of caloric deficit such as fasting, or increased energy demand, trigylcerides (TG) in adipose tissue stores are hydrolyzed and free fatty acids (FFAs) are released into the circulation. The hydrolysis of TG is catalyzed by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). Nuclear receptor PPARα in liver, senses the influx of FFAs and upregulates all three fatty acid oxidation systems, reduces fatty acid esterification to TGs and minimizes hepatic steatosis (*see* wild type mouse liver on the *left*). If PPARα sensing is deficient as in PPARα/ACOX1 double knockout mouse liver (*right*), fatty acid oxidation systems are not upregulated to burn the influxed FFAs. This results in increased TG synthesis contributing to the development of hepatic steatosis. Modified from Yu et al. [91] with permission

Although circulating fatty acids derived from adipose tissue stores are important during starvation as well as during increased energy demand states, plasma fatty acids taken up by the liver in the postprandial state are derived mainly from the catalytic action of lipoprotein lipase (LPL). This enzyme is present on the endothelial surface of capillaries of skeletal and cardiac muscles and in adipose tissue and is responsible for the hydrolysis of circulating triglyceride-rich lipoproteins synthesized and secreted by the intestine (chylomicrons) or liver (very low density lipoproteins/VLDL) [12]. The action of LPL-mediated lipolysis of chylomicrons requires apoC-II as a cofactor acquired from plasma high density lipoproteins (HDLs) [12]. Most of the fatty acids generated from the LPL-mediated hydrolysis of chylomicrons and VLDL triglycerides are taken up by the tissues where the enzyme is located to be either oxidized (muscles) or stored (adipose tissue) [13]. However, a considerable fraction of the generated unesterified fatty acids become bound to plasma albumin and, in this form, are transported to the liver where they are taken up [13, 14]. It has been estimated that >50% of long chain fatty acids bound to albumin can dissociate and bind to liver cells in single pass through the liver [15]. The six-member family of fatty acid transport proteins (FATP-1 through FATP-6) present as integral transmembrane proteins facilitates the uptake of long chain and very long chain fatty acids into cells [14, 16]. These proteins exhibit fatty acid synthetase activity implying that fatty acids are rapidly converted to acyl-CoAs in the cell after translocation across the plasma membrane. The mechanism whereby the fatty acids enter the hepatocytes has not yet been fully elucidated, but it appears that FATP5 plays a major role in hepatocellular uptake of fatty acids [17]. Furthermore, hepatocytes contain cytoplasmic fatty acid binding protein (FABP1) and fatty acid translocase (FAT/CD36) are also known to be involved in the cellular uptake of fatty acids [18]. Whether fatty acids enter into hepatocytes by passive diffusion through the plasma membrane or the entry is facilitated exclusively by fatty acid membrane protein transporters remains unclear [19].

Chylomicron Remnants

As indicated above, a major source of fatty acids for the liver in the postprandial state are triglycerides associated with chylomicrons. These lipoproteins are synthesized in the intestine and are responsible for the transport of most of the

absorbed dietary fatty acids and cholesterol. However, the chylomicrons themselves do not contribute to the hepatic fatty acid pool because they are not cleared from circulation by the liver. Instead, chylomicrons, as well the triglyceride-rich VLDL, are acted upon by LPL in the peripheral vascular bed, which hydrolyzes the triglycerides in the core of the particles generating unesterified fatty acids, some of which bind to plasma albumin for transport to the liver [12]. As a result of LPL action, chylomicrons are converted into remnant particles, which are smaller than their parent chylomicrons but retain most of their original cholesterol content as well as a considerable amount of triglycerides. Unlike the chylomicrons, remnants are readily cleared from circulation by the liver where they are disassembled, thus providing fatty acids to hepatocytes. The efficient uptake of chylomicron remnants – but not intact chylomicrons – by the liver is the result of a complex and as yet not a fully elucidated process that involves the interaction of apoprotein E on the surface of the particle first with glycosaminoglycans in the space of Disse, followed by its binding to the low density lipoprotein receptor (LDLR) on the surface of hepatocytes and finally endocytosis of the particle [20].

Hepatic Cytoplasmic Lipid Droplet Stores

All eukaryotes, from yeast to humans, synthesize triglycerides and store this excess energy in the form of cytoplasmic lipid droplets for use when needed [21, 22]. Lipid droplets, once considered inert or static, are emerging as metabolically dynamic structures. During times of energy scarcity, this stored energy from lipid droplets is retrieved by the action of lipases [23]. Although the lipid droplet-rich adipocytes of adipose tissue are the principal sites of energy storage and retrieval, all other cell types in animals, including hepatocytes, can accommodate under normal physiological conditions, limited quantities of energy in the form of smaller, less conspicuous, lipid droplets. These smaller lipid droplets provide immediate energy source for hepatocytes and other non-adipocyte cells, and serve as fatty acid source for utilization in intracellular signaling cascades [24]. However, in steatotic states, hepatocytes can accumulate massive amounts of energy-rich macro- or micro-vesicular lipid droplets and lead to the development of fatty liver disease [25].

The entry and sequestration of lipid in lipid droplets as well as lipolysis to release the packaged fatty acids are physiological processes regulated by evolutionarily conserved families of lipid-droplet surface proteins, including the members of the perilipin (perilipin amino-terminal/PAT proteins) and Cide (cell-death-inducing DFFA-like effector) families [26]. The perilipin family includes five members: Perilpin-1 (perilipin), perilpin-2 (adipocyte differentiation-related protein/ADRP/adipophilin), perilipin-3 (tail-interacting protein of 47 kDa/TIP47), perilpin-4 (S3–12), and perilipin-5 (oxidative tissue-enriched/OXPAT) [27]. These five members share sequence similarity and are differentially expressed in different cell types [28]. These proteins are amphiphilic proteins that are associated with the phospholipid monolayer surrounding lipid droplets and participate in lipid droplet maturation and metabolism [29]. Perilipin-2 and -3 are expressed in most cell types. Although perilipin-4 (S3–12) is expressed mostly in adipocytes and to a lesser extent in heart and skeletal muscles, it can be induced in liver cells in response to PPARγ overexpression reflecting its function in hepatic adiposis [30]. Perilipin-5 is expressed in tissues with high fatty acid oxidation capability including the liver [31]. Fasting and PPARα activation are known to induce perilipin-5 expression in liver, consistent with its role in fatty acid oxidation [32–35] (Fig. 10.1). It appears that perilipin-4 and perilipin-5 are reciprocal in function, in that the former is associated with lipid storage and the latter with fatty acid oxidation [31, 36]. Perilipins decorate lipid droplets either constitutively (perilipins 1 and 2), or in response to lipolytic challenge (perilipins 3–5) [37]. Because of this differential presence, lipid droplets may be heterogeneous in a given cell with reference to perilipin composition [38]. Evidence suggests that perilipin-1 and perilpin-2 serve as physical barriers to lipolytic enzymes under basal conditions, but in response to lipolytic stimulation, these two proteins can also facilitate interactions with lipases [39]. Perilipin-1 is more effective at attenuating lipid droplet lipolysis than perilipin-2 [40]. Therefore, absence of perilipin-1 or perilipin-2 reduces the amount of triglyceride in adipose tissue and liver, possibly due to increased lipolysis resulting from the removal of barrier to lipase action [41]. The relative contributions of various members of perilipin family in lipid droplet composition, assembly, and hydrolysis of triglycerides in the progression of fatty liver disease remain to be clarified [42].

In recent years, the importance of the three members of the Cide family of lipid droplet proteins CideA, CideB, and CideC (also known as Fsp27), in lipid droplet metabolism is being increasingly recognized [43]. Similar to perilipins, Cide proteins are also associated physically with lipid droplets and appear to modulate droplet size and lipid metabolism in liver during lipid overload conditions [44]. The expression of perilipin-1, CideA, and CideC is markedly elevated in liver with severe steatosis [31, 45]. CideC expression is elevated in the liver following PPARγ overexpression [46]. Mice deficient in perilipin-2, CideB, or CideC do not develop fatty liver disease implying that these proteins normally function to facilitate lipid storage in the cell [47]. Although the lipid droplet biology is gaining enhanced attention, the state of knowledge is rudimentary since very little

is known about the ~100 lipid droplet associated proteins [48]. It is noteworthy that the storage of lipid in the liver may also protect the cells against lipotoxicity [49–51]. The packaged lipids in droplets may also be important in transportation of lipid to specific cellular destinations or for specific functions. Moreover, the lipid droplets may provide a shelter for some special proteins when they are at a higher level in the cells [22, 52].

De Novo Lipogenesis

Lipogenesis encompasses fatty acid synthesis and their utilization for phospholipid and triglyceride generation. The human body is able to synthesize all fatty acids with the possible exception of two polyunsaturated fatty acids, namely linoleic acid (C18:2) and α-linolenic acid (C18:3). Liver and adipose tissue are the major sites of fatty acid synthesis and mammary glands also generate fatty acids during lactation. Lipogenesis requires acetyl-CoA precursors that are generated during certain metabolic processes as these precursors provide all the carbon atoms necessary for fatty acid synthesis [53]. Liver is the principal organ responsible for the conversion of excess carbohydrate (glucose), beyond organism's energy needs, to fatty acids via a series of metabolic steps that are regulated by several factors, including nutritional, hormonal, and genetic elements [54]. Glucose is first converted to pyruvate, which enters the Krebs cycle in the mitochondria to yield citrate [55]. Citrate is then transported into the cytosol and broken down by ATP citrate lyase to yield acetyl-CoA and oxaloacetate. Acetyl-CoA is converted to malonyl-CoA, the rate-limiting step in the lipogenesis pathway catalyzed mainly by acetyl-CoA carboxylases (ACC1 and ACC2) [56]. Successive molecules of malonyl-CoA, which serves as a two carbon donor, are added to the acetyl-CoA primer by a multifunctional enzyme complex, the fatty acid synthase (FAS) [57]. Palmitic acid (C16:0) is the predominant fatty acid generated by FAS [58]. FAS is expressed in the liver and adipose tissue, but in the humans, the liver appears to be the major site for de novo lipogenesis [59]. Palmitic acid is desaturated by stearoyl-CoA desaturase-1 (SCD-1) to palmitoleic acid or elongated to yield stearic acid (C18:0). SCD-1 catalyzes the conversion of stearoyl-CoA to oleoyl-CoA, which is a major substrate for triglyceride synthesis [60]. Oleic acid (C18:1) is formed as a result of desaturation of stearic acid and is regarded as the end product of de novo fatty acid synthesis. However, the saturated C16 fatty acid, first synthesized during *de novo* lipogenesis, is the essential precursor for almost all the newly synthesized fatty acids including the formation of very long chain fatty acids [61].

Very long chain ($>C_{22}$) saturated, monounsaturated, and PUFAs are synthesized by elongation and desaturation reactions performed by enzymes located in the endoplasmic reticulum [62]. Palmitoyl-CoA is elongated by type III fatty acid synthetases, now known as elongases (elongation of very long chain fatty acids; ELOVLs) [63, 64]. Seven ELOVL enzymes (ELOVL1–7) are known in mammals, and they reveal different fatty acid substrate preferences for catalyzing the elongation reaction [64]. ELOVL1 and ELOVL3 exhibit high activity toward all of the saturated C20- to C26-CoAs, while ELOVL2 elongates C20 and C22 PUFAs [65]. Fatty acids with chain lengths longer than C26 are elongated by ELOVL4 [65]. ELOVL5 has been shown to be responsible for the elongation of C18 substrates and ELOVL6 participates in the elongation of C_{12}–C_{16} fatty acids [65]. ELOVL7 exhibits significant activities to C18-CoAs and less to C16:0-CoA. ELOVL1 and ELOVL7 are expressed in many tissues, suggesting that ELOVL7 elongates C18:0-CoA to C20:0-CoA, which is then transferred to ELOVL1 [63, 64].

Very Low Density Lipoprotein Assembly and Secretion

Fatty acids taken up by the liver and which do not undergo oxidation, are mainly esterified and collected in a common cytosolic triglyceride pool [66]. Conditions in which the delivery of fatty acids to the liver is increased may be associated with increased hepatic triglyceride content. However, under normal conditions, triglycerides do not accumulate in the cytoplasm of hepatocytes but are mobilized and, together with cholesterol and phospholipids, are assembled into VLDL particles for secretion into the circulation. The secretion of VLDL into the blood is crucial in preventing the accumulation of triglycerides by liver cells that may lead to steatosis and its resulting pathologic consequences.

The synthesis of VLDL requires the availability of apolipoprotein B (apoB), a complex protein that serves as the scaffolding upon which the VLDL is assembled. ApoB occurs in two forms, apoB-100 and apoB-48 [67]. ApoB-100 is essential for the assembly of VLDL in the liver, and apoB-48 is essential for the assembly of chylomicrons in the intestine. Both apoBs are the products of a single gene. ApoB-100 is synthesized as a 4536-amino acid polypeptide and apoB-48 – a truncated form containing 48% of the protein from the N-terminus – is synthesized as a result of apoB mRNA editing, a process that requires a multi-component enzyme complex containing an RNA-specific cytidine deaminase, apobec-1, and an RNA-binding subunit, apobec-1 complementation factor (ACF) [68]. In humans, the RNA editing occurs in the intestine but not in the liver. In rodents, apoB-48 is also synthesized in the liver and secreted into the circulation

associated with VLDL. Thus, human VLDLs carry only apoB-100 whereas rodent VLDL particles carry either apo B-100 or apoB-48 [67].

The synthesis of apoB in the liver for the assembly of VLDL apparently occurs at a constant rate. However, only a fraction of newly-synthesized apoB protein serves as the scaffold for VLDL assembly [69]. The assembly of VLDL has been proposed to occur in two sequential steps [70]. In the first step, apoB, during its co-translational translocation through a protein channel in the membrane of the rough endoplasmic reticulum, acquires a small quantity of triglycerides, phospholipids and cholesterol ester, forming a small, dense, VLDL precursor. At this stage, the apoB, if not properly folded or if it acquires an insufficient amount of lipids, is rapidly degraded and this process appears to be the principal determinant of the amount of apoB that is secreted into the circulation associated with the VLDL [71]. The acquisition of triglycerides by apoB is mediated by microsomal triglyceride transfer protein (MTP), which functions in the endoplasmic reticulum lumen as a chaperone shuttling triglycerides and phospholipids to the newly synthesized apoB [72]. In a second step, the small, dense VLDL precursor undergoes maturation by the further acquisition of triglycerides, a process that is still poorly understood, but which is thought to occur primarily by fusion between the newly lipidated particle with triglyceride droplets in the smooth endoplasmic reticulum [73]. This process does not appear to require the mediation of MTP [74, 75]. Under physiological conditions, the production of VLDL depends, primarily, on the availability of fatty acids that are taken up by the liver. However, overproduction of VLDL can occur resulting in hypertriglyceridemia. The mechanisms underlying VLDL overproduction are poorly understood although insulin appears to play an important role. High levels of this hormone increase levels of MTP expression; increase apoB availability, and induce the transcriptional regulation of hepatic lipogenic enzymes, all of which can lead to increased VLDL production and secretion [76].

Fatty Acid Oxidation

Breakdown of the major energy fuels namely, carbohydrates, amino acids, and fats, generates ATP, which is the universal cellular energy source. For ATP to be synthesized from these complex fuels, they first need to be broken down into their basic components. In general, carbohydrates are hydrolyzed into simple sugars, such as glucose and fructose, proteins to amino acids and fats (triglycerides) to fatty acids. Mitochondria use these energy-generating fuels and play a dominant role in ATP generation. The extent to which these fuels contribute to ATP production within an organism varies, but fatty acids are considered the major source of energy for many cell types except the brain, which uses glucose and also ketone bodies for ATP generation [4, 77].

Liver plays a central role in the fatty acid oxidation for energy generation and for the production of substrates for the synthesis of ketone bodies for use by extrahepatic tissues under fasting conditions for energy. In liver cells, fatty acids are oxidized in three cellular organelles, with β-oxidation confined to mitochondria and peroxisomes, and the CYP4A catalyzed ω-oxidation taking place in the endoplasmic reticulum [4, 78]. The major pathway for the catabolism of fatty acids is mitochondrial fatty acid β-oxidation [4, 79]. The following is a brief overview of the fatty acid oxidation processes in the liver.

Mitochondrial β-Oxidation

Mitochondrial β-oxidation is responsible for the degradation of the major portion of the short- ($<C_8$), medium- (C_8–C_{12}), and long- (C_{14}–C_{20}) chain fatty acids [74]. Although long-chain fatty acids (LCFAs) are β-oxidized predominantly in mitochondria, the peroxisomal β-oxidation system does indeed contribute to the oxidation of LCFAs as an overflow contingency. On the other hand, very long-chain fatty acids (VLCFAs) ($>C_{20}$) are almost exclusively, but incompletely β-oxidized (chain-shortened) in peroxisomes and the resulting chain-shortened acyl-CoAs are shuttled to mitochondria for the completion of the oxidation (see below). Since LCFAs constitute the bulk of dietary fat, their mere abundance makes them the predominant source of energy production by ATP generating mitochondrial oxidative phosphorylation. Importantly, mitochondrial β-oxidation conserves double the energy compared with peroxisomal β-oxidation, because the energy generated during the first step of peroxisomal β-oxidation dissipates as heat [79, 80].

Mitochondrial fatty acid β-oxidation is a complex process which is regulated at several levels, but mainly by carnitine palmitoyltransferase 1 (CPT1), the carnitine concentration, and malonyl-CoA, which inhibits CPT1. It should be noted that, after entry into the cell, the fatty acids are activated to acyl-CoA esters by acyl-CoA synthetases and targeted into the mitochondrial β-oxidation spiral [4]. Because the mitochondrial inner membrane is impermeable to long-chain acyl-CoAs, they are transported across to the mitochondrial matrix by the so-called carnitine shuttle. This rate-controlling shuttle utilizes three proteins: CPT1, carnitine acylcarnitine translocase (CACT), and CPT2 [81]. CPT1 exchanges the CoA group of long-chain acyl-CoA for carnitine to form long-chain acylcarnitines, which are transported across the mitochondrial inner membrane by carnitine acylcarnitine translocase [82]. CPT2 located at the mitochondrial inner

membrane releases the carnitine group from acylcarnitines in exchange for a CoA group and delivers the CoA esters to mitochondrial matrix for oxidation. The released carnitine shuttles back to the cytosol for reuse [81, 82].

In the mitochondria, fatty acyl-CoAs ($<C_{22}$) are completely β-oxidized to acetyl-CoA subunits and this process occurs in a sequence of four reactions and each reaction may be catalyzed by more than one enzyme [4, 79, 83]. The β-oxidation is a repetitive process in which acyl-CoAs are progressively shortened by releasing two carboxy-terminal carbon atoms as acetyl-CoA subunits, which either condense into ketone bodies (acetoacetate, acetone, and β-hydroxybutyrate) in liver to serve as oxidizable fuels for extrahepatic tissues, or enter into the tricarboxylic acid cycle for further oxidation into water and carbon dioxide [84].

The mitochondrial β-oxidation has two distinct components. The first, which is mitochondrial inner membrane bound, is active with long-chain fatty acyl-CoAs (C_{12}–C_{20}) and after 2–3 cycles, generates medium-chain acyl-CoAs for further oxidation in the mitochondrial matrix. The inner membrane-bound mitochondrial β-oxidation system involves four enzymatic reactions performed by two membrane bound proteins. The first protein, a very long-chain acyl-CoA-dehydrogenase (VLCAD), catalyzes the α-β-dehydrogenation of the acyl-CoA ester, the initial, rate-limiting first step of mitochondrial fatty acid β-oxidation, to generate trans-2,3-enoyl-CoA [77]. The second membrane bound protein involved in long-chain acyl-CoA oxidation is a mitochondrial trifunctional protein (MFP, L-PBE), with long-chain enoyl-CoA hydratase/long-chain 3-hydroxy acyl-CoA dehydrogenase/long-chain 3-ketoacyl-CoA thiolase activities responsible for performing steps 2–4 of β-oxidation. In the second step, enoyl-CoAs are hydrated by 2-enoyl-CoA hydratases to generate L-3-hydroxyacyl-CoAs, which in the third step are catalyzed by short chain L-3-hydroxyacyl-CoA dehydrogenase to produce 3-ketoacyl-CoAs. The final step is catalyzed by 3-ketoacyl-CoA thiolases [77, 85]. After 2–3 cycles using these membrane bound enzymes, the resulting medium acyl-CoAs ($<C_{14}$) are metabolized by mitochondrial matrix based second β-oxidation system. This matrix based chain-length specific system consists of LCAD (C_8 to C_{20}), MCAD (C_4–C_{12}), and SCAD (C_4–C_6) that catalyze the first step to yield enoyl-CoA [77, 86]. The second, third and fourth steps of this matrix based system are carried out by the 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, all which are present in the mitochondrial matrix as individual enzymes (not as trifunctional protein) encoded by separate genes [77, 87]. Each mitochondrial β-oxidation cycle results in the production acyl-CoA shortened by two carbon atoms and an acetyl-CoA. The resulting shortened acyl-CoA reenters the fatty acid oxidation cycle and the acetyl-CoA is used for ketone body generation for export to organs such as heart, or it can enter the citric acid cycle for ATP generation.

Peroxisomal β-Oxidation

In the liver, both mitochondria and peroxisomes participate in the β-oxidization of fatty acids. While short- and medium-chain fatty acids are β-oxidized rather exclusively in mitochondria, LCFAs can be oxidized in both mitochondria and peroxisomes. However, VLCFAs ($>C_{20}$), such as C24:0 and C:26:0 are β-oxidized exclusively by the peroxisomal β-oxidation system as mitochondria lack very long-chain fatty acyl-CoA synthetase to convert these VLCFAs into acyl-CoA esters for entry into the mitochondria. Some pertinent differences between the mitochondrial and peroxisomal β-oxidation systems are listed below.

- Mitochondria lack very long-chain fatty acyl-CoA synthetase, hence VLCFAs ($>C_{20}$) cannot enter these organelles. Peroxisomal membrane on the other hand has at least two acyl-CoA synthetases: a long chain acyl-CoA synthetase and a very long-chain acyl-CoA synthetase capable of activating LCFAs and VLCFAs, respectively [88]. While LCFAs can be oxidized both in the mitochondria and peroxisomes, the presence of very long-chain fatty acyl-CoA synthetase on peroxisomal membrane accounts for the exclusively streamlined β-oxidation of VLCFA within the peroxisomes.
- The first oxidation step in the peroxisomal β-oxidation of fatty acids is catalyzed by fatty acyl-CoA oxidase 1 (ACOX1) in the classic inducible pathway, but unlike in mitochondria, the β-oxidation in peroxisomes is not coupled to ATP synthesis. Instead, the high-potential electrons are transferred to O_2 to yield H_2O_2, which is further converted into H_2O and O_2 by peroxisomal catalase. The energy released during peroxisomal fatty acid oxidation is dissipated as heat.
- Unlike the mitochondrial system, peroxisomal β-oxidation does not go to completion, as the appropriately chain-shortened acyl-CoAs are exported to the mitochondria for the completion of β-oxidation.
- Peroxisomal β-oxidation generated chain-shortened acyl-CoAs are shuttled to mitochondria, either as carnitine esters and/or as free fatty acid for the completion oxidation. Peroxisomes contain carnitine acetyltransferase and carnitine octanoyltransferase for conjugation and transport of short- and medium-chain acyl-CoAs respectively. Mitochondria on the other hand use carnitine shuttle with carnitine palmitoyl transferase-1 (CPT1) and CPT2 as major players.

It is noteworthy that the peroxisomal β-oxidation is uniquely geared toward the metabolism of less abundant and relatively more toxic and biologically active VLCFAs ($>C_{20}$), 2-methyl-branched fatty acids, dicarboxylic acids, prostanoids, and C_{27} bile acid intermediates, among others [89]. VLCFAs are not completely β-oxidized in peroxisomes, but

this system serves to shorten the chain length for further completion of oxidation in mitochondria [77, 90]. Although this system normally functions in the shortening of VLCFA-CoA, it also breaks down LCFA-CoA when the mitochondrial β-oxidation is decreased or overwhelmed. Long-chain dicarboxylic acids produced by the microsomal ω-oxidation of LCFAs and VLCFAs (see below) are also metabolized by the peroxisomal β-oxidation system [91]. Dicarboxylic acids are generally considered more toxic than VLCFAs and are known to inhibit mitochondrial fatty acid oxidation system and thus may contribute to the development of hepatic steatosis. Peroxisomal β-oxidation also acts in the synthesis and metabolism of docosahexanoic acid (DHA) and retroconversion of DHA to eicosapentaenoic acid.

Similar to the mitochondrial β-oxidation system, the peroxisomal β-oxidation spiral consists of four sequential steps with each metabolic conversion carried out by at least two different enzymes [90, 92]. These enzymes are separated into two pathways, inducible and non-inducible, with each pathway consisting of three separate enzymes. The first step of the peroxisomal β-oxidation in each pathway is catalyzed by a different ACOX, with an inducible classic ACOX1 exhibiting specificity for straight-chain VLCFA-CoA esters, dicarboxylic acids, and eicosanoids the non-inducible ACOX2 acting on CoA esters for 2-methyl branched-chain fatty acids [77, 93]. This first step converts acyl-CoA into enoyl-CoA. The second and third reactions, hydration and dehydrogenation of enoyl-CoA esters to 3-ketoacyl-CoA are catalyzed by one of two bi/multi-functional enzymes (PBE/MFP) with enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (L-PBE/MFP1) in the inducible pathway, or D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase (D-PBE/MFP2) in the non-inducible pathway. D-PBE/MFP2 of this pathway can substitute for the L-PBE/MFP1 function in the inducible [4, 90, 94]. The fourth step in the peroxisomal β-oxidation converts 3-ketoacyl-CoAs to acyl-CoA that is two carbon atoms shorter than the original molecule and acetyl-CoA. This function in the inducible pathway is performed by straight-chain 3-oxoacyl-CoA thiolase and in the noninducible pathway by the sterol carrier protein x (SCPx), which possesses thiolase activity [94, 95]. The functional significance of peroxisomal β-oxidation system is to metabolize potentially toxic substrates such as VLCFAs and shuttle the chain-shortened metabolites to mitochondrial β-oxidation system for further degradation and to prevent hepatic steatosis.

Microsomal ω-Oxidation

Liver also utilizes the microsomal ω-oxidation system to metabolize fatty acids (C_{10}–C_{26}) as an alternative pathway to β-oxidation, especially when β-oxidation is defective. In the ω-oxidation pathway, the first step involves the conversion within the endoplasmic reticulum of the ω-methyl group of the fatty acid into a ω-hydroxyl group by P450 enzymes belonging to the CYP4A/F subfamilies. In the human CYP4A11 of CYP4A family and CYP4F11 of the CYP4F family appear to be the predominant catalysts for fatty acid ω-oxidation [96, 97]. The resulting ω-hydroxy fatty acid is then dehydrogenated to a dicarboxylic acid in the cytosol. The dicarboxylic fatty acids generated by ω-oxidation require β-oxidation in mitochondria and or peroxisomes to shorter chain dicarboxylic acids for excretion into the urine [98]. Prior to entering β-oxidation spiral, dicarboxylic acids are converted into dicarboxylyl-CoAs by acyl-CoA synthase present in endoplasmic reticulum. Medium-chain dicarboxylyl-CoAs are oxidized in mitochondria, whereas long- and very long-chain dicarboxylyl-CoAs are metabolized exclusively by the classic inducible peroxisomal β-oxidation system in humans. Although ω-oxidation is a minor catabolic pathway accounting for <10% of total fatty acid oxidation in the liver, this system constitutes a critical route in the elimination of potentially toxic levels of free fatty acids. First, ω-oxidation is upregulated when β-oxidation is defective, and this poses a problem as the dicarboxylic acids so generated cannot be adequately metabolized to avert accumulation of toxic levels of dicarboxylic acids. A defect in β-oxidation leads to dicarboxylic acid toxicity [77, 99]. Significant quantities of dicarboxylic acids are also generated under conditions of fatty acid overload in the liver, for example, in obesity and diabetes, and in conditions where mitochondrial oxidation is inadequate to oxidize fatty acids.

Fatty Acid α-Oxidation

Plants synthesize fatty acids such as phytanic acid with odd numbers of carbon and these 3-methyl branched fatty acids cannot be β-oxidized, but use an alternate α-oxidation pathway present in peroxisomes [100]. Oxidation of phytanic acid proceeds as follows: phytanic acid is activated to phytanoyl-CoA by long-chain acyl-CoA synthetase, which is then hydroxylated to 2-hydroxyphytanoyl-CoA by phytanoyl-CoA 2-hydroxylase. Subsequently, 2-hydroxyphytanoyl-CoA is cleaved to yield formyl-CoA and pristanal. Pristanal is oxidized to pristanic acid and upon activation to pristanoyl-CoA, it enters peroxisomal β-oxidation spiral, eventually generating propionyl-CoA and acetyl-CoA. In essence, 3-methyl branched fatty acids in mammalian liver are subjected to α-oxidation in the initial steps and to β-oxidation process subsequently to generate acetyl-CoA and 2-methyl propionyl-CoA [100].

Ketogenesis

Under conditions contributing to increased fatty acid uptake and oxidation in the liver, large amounts of acetyl-CoA are generated. When acetyl-CoA concentrations exceed the capacity of tricarboxylic acid cycle to metabolize, acetyl-CoAs are utilized to generate ketone bodies called acetoacetate, β-hydroxybutyrate, and acetone. These products of ketogenesis, can be used as energy source by tissues such as brain, heart, skeletal muscle, liver, and kidneys [84, 101]. Accordingly, ketogenesis depends on the capacity of liver to fully oxidize fatty acids. It should be noted that the release of glucose by the liver is essential to meet the energy needs of many cell types. During fasting conditions, after the hepatic glycogen reserves are exhausted, the organism relies on ketone bodies which become the main alternative fuel source. Ketone bodies released from liver provide up to 80% of the total energy needed. This glucose sparing effect of ketone bodies is extremely important in cerebral tissue in infants due to the high proportional glucose utilization [101].

Regulation of Hepatic Lipid Metabolism: Clues from Knockout Mouse Models

Over the years, the importance of lipid metabolism in liver in the development of alcoholic steatohepatitis and chronic fatty liver disease is well recognized. Currently, lipid metabolic dysfunctions in liver, in particular steatosis and steatohepatitis, related to obesity and metabolic syndrome are receiving considerable attention. Accordingly, an understanding of the regulatory factors influencing overall energy balance and attempts at pharmacological intervention are of paramount importance.

Several genetic disorders affecting the mitochondrial and peroxisomal fatty acid oxidation system genes at different levels of the catabolic spiral have been discovered in humans [4, 80, 91]. These defects have provided fascinating insights into the role of fatty acid catabolism in maintaining energy homeostasis and causing dysfunction. In addition, during the past 25 years, several gene knockout mouse models have been generated to investigate the functional roles of many genes involved in various aspects of lipid metabolism.

The following provides a brief overview of the regulatory molecules that influence lipogenesis, and fatty acid oxidation [102]. Abnormalities in lipogenesis, fatty acid oxidation, and in fatty acid intake and export, contribute to the development of hepatic steatosis and these functions in liver are influenced by many factors [103]. In this regard, attention is focused on two key transcription factors, the sterol regulatory element-binding protein 1-c (SREBP1-c) that controls the expression of genes involved in lipogenesis, and peroxisome proliferator-activated receptor-α (PPARα), which controls the expression of genes of the fatty acid oxidation systems in liver [104–106]. Two other members of PPAR subfamily, namely, PPARβ/δ and PPARγ, also play key roles in overall lipid metabolism of the organism [106]. Fatty acids and their metabolites control the expression of these factors. Retinoid X receptor α (RXRα), a heterodimerization partner of PPARs and several other nuclear receptors [105, 107], are also involved in lipid homeostasis. Evidence is also emerging about the role of microRNAs in the regulation of genes that participate in liver lipid metabolism [108].

Regulation of Hepatic Lipogenesis

As de novo synthesis of fatty acids in liver is regulated by insulin and glucose, it is essential to understand their role in activating the membrane-bound lipogenic transcription factor SREBP-1c, an important member of SREBP family of transcription factors [109]. Insulin is a key regulator of SREBP-1c, one of the three SREBP isoforms in humans and rodents [110]. SREBP-1c and SREBP-2 are the predominant forms in the liver. SREBP-1a and SREBP-1c activate hepatic fatty acid synthesis through regulation of lipogenic genes, such as ACC2, FAS, SCD-1 and glycerol phosphate acyltransferase [111]. ACC2-generated malonyl-CoA inhibits fatty acid entry and oxidation in mitochondria, because malonyl-CoA inhibits CPT1 activity. As noted above, mutation of ACC2 results in increased fatty acid oxidation due to reduced malonyl-CoA production [112]. SREBP-1c induces lipogenic genes by binding to sterol response element (SRE) sites in their promoters, which is impaired in SREBP-1c–/– mice. SREBP-1c gene contains two response elements for lipogenic transcription factor liver X receptor (LXR), and the activation of LXR by oxysterol ligands induces the transcription of SREBP-1c [102]. Disruption of SREBP-1c gene expression in ob/ob mice improves hepatic steatosis, while overexpression of SREBP-1c in the liver leads to increases in hepatic glycogen and triglyceride contents [113]. Glucose regulates lipogenesis as glucose is converted to fatty acids. Glucose-mediated stimulation of lipogenesis is controlled by transcription factor carbohydrate response element-binding protein (ChREBP) [114]. ChREBP activates the expression of genes involved in the synthesis and uptake of fatty acids, including those encoding ACC, FAS, SCD-1, and ATP citrate lyase [102, 115]. Glucose exerts the ChREBP activation by regulating the entry of ChREBP from the cytosol into the nucleus thus influencing the DNA binding and transcriptional activity of this transcription factor [116].

The transcriptional activities of ACC1, ACC2, and FAS in liver are regulated by both glucose and insulin [102, 117].

Deficiency of ACC1 is embryonically lethal in mice, whereas ACC2 null mice are viable but exhibit enhanced metabolic rate, manifesting as continuously elevated fatty acid oxidation and reduced adiposity [118, 119]. In ACC2 null mice, CPT1 activity increases resulting in an increased rate of fatty acid oxidation [120]. It would appear that malonyl-CoA, generated by ACC1 and ACC2, work independently and that there is minimal, if any, overlap of their functions. FAS, which catalyzes the first committed step in fatty acid biosynthesis to generate mostly the saturated fatty acid palmitate, also plays an important role in fatty acid oxidation (see below). When FAS gene is conditionally inactivated in mouse liver, it causes decreased fatty acid oxidation suggesting that this enzyme is involved in generating ligands that activate the lipid sensing transcription factor, PPARα, a critical regulator of fatty acid oxidation [90, 121]. SCD-1 is also modulated by a number of dietary, physiological and hormonal factors including insulin and glucose, and is critical in maintaining intracellular lipid flux. SCD-1 has also been suggested in representing a key step in the partitioning of lipids between storage and oxidation [122]. Mice with global deletion of SCD-1 are resistant to high-fat diet induced obesity and SCD-1 deficiency also prevents the development of hepatic steatosis [123]. Significant increase of SCD-1 expression and reduced energy expenditure in ob/ob mice result in marked hepatic steatosis, hyperlipidemia and increase of lipid secretion.

Regulation of Fatty Acid Oxidation

Oxidation of fatty acids in liver involves the participation of several enzymes that are affected by a multiplicity of factors including more prominently the nuclear receptor PPARα [90, 91]. Enzymes such as CPT1 and CPT2, and substrates (fatty acid metabolism intermediates), such as malonyl-CoA are known to influence mitochondrial fatty acid β-oxidation [82]. Likewise, several structurally diverse compounds called peroxisome proliferators [4, 90, 91], and certain metabolic pathways that generate or degrade biological (endogenous) PPARα ligands, also affect fatty acid catabolism, especially by activating PPARα, which is referred to as lipid and peroxisome proliferator-sensor in liver [77]. PPARα transcriptionally regulates all key enzymes of peroxisomal and mitochondrial β-oxidation pathways, as well as microsomal ω-oxidation system [4, 90, 91, 124–127]. Several genetic disorders in humans involving the enzymes of the mitochondrial and peroxisomal fatty acid β-oxidation pathways have been identified that have provided considerable insights into the regulatory aspects of energy homeostasis and in particular implications of disturbances in fatty acid metabolism. Genetic defects affecting mitochondrial β-oxidation in humans include: deficiencies of CPT1, CPT2, CACT, VLCAD, MTP, MCAD, M/SCHAD, and among others [4, 77, 91]. Mouse models of gene disruption affecting mitochondrial β-oxidation include: CPT1a, CPT1b, LCHAD/MTP, VLCAD, LCAD, MCAD, and SCAD among others [90, 91]. Most of these gene knockout mouse models exhibit severe or lethal phenotype and, in most part, mimic human disease with some exceptions [90, 128, 129].

In both humans, and mouse models, peroxisomal β-oxidation pathway disruptions have been studied in considerable detail [4, 50, 89, 91, 125, 127–129]. Three genetic disorders affecting peroxisomal β-oxidation spiral, namely ACOX1 deficiency, DPBE/MFP1 deficiency and SCPx deficiency have been identified and studied in detail in humans [4, 91, 127]. Gene knockouts in the mouse include disruptions of genes encoding ACOX1, L-PBE/MFP1, D-PBE/MFP2, and SCPx [4, 89, 91, 128, 129].

The following focuses, first, on the role of the transcription factor PPARα in regulating the fatty acid oxidation system genes, and second, as to how disruption of these genes contributes to high levels of unmetabolized substrates that function as PPARα ligands [128]. It is also worth noting that some of these substrates/ligands may be generated by a proximal enzyme in the oxidation pathway. It is becoming increasingly clear that some of the enzymes in fatty acid oxidation systems contribute to the formation or degradation of biological ligands of PPARα [4, 90, 92, 128].

First, all three members of the PPAR subfamily are known to function as sensors for fatty acids and fatty acid derivatives and thus control important metabolic pathways involved in lipid and energy metabolism [90, 91, 126]. Of these, PPARα is a key regulator of mitochondrial, peroxisomal, and microsomal fatty acid oxidation enzyme systems in liver [4, 90, 130–133]. Exposure to peroxisome proliferators, which are synthetic PPARα ligands, results in massive induction of these fatty acid oxidation systems in liver culminating in increased energy combustion [4, 77]. This receptor is also activated by both saturated and poly-unsaturated fatty acids and their derivatives. PPARα also plays an important role in lipoprotein synthesis and inflammatory responses and in the development of liver cancer [90]. PPARα knockout mice have demonstrated unequivocally that this receptor is indeed the bona fide receptor for transcriptionally activating the fatty acid oxidation genes and inducing the peroxisome proliferator-mediated pleiotropic responses including liver tumors [130, 134]. PPARα null mice display normal basal levels of the inducible peroxisomal β-oxidation enzymes in liver but possess generally lower levels of mitochondrial β-oxidation enzyme activities [131]. Under fasting conditions, PPARα senses the fatty acid influx into the liver and up-regulates all three fatty acid oxidation systems to combust the energy and minimize hepatic steatosis [33–35]. Mice deficient in PPARα fail to upregulate fatty acid burning

enzymes in liver and become grossly steatotic when fasted [33–35] (Fig. 10.1). When maintained on a diet deficient in methionine and choline, PPARα null mice develop severe steatohepatitis [135].

Second, increasing evidence suggests that PPARα senses certain endogenous lipid metabolic intermediates as ligands and participates in their metabolic breakdown by inducing downstream lipid metabolism genes [90, 128]. Studies with ACOX1 null mice revealed that disruption of this gene encoding the first and rate-limiting enzyme of the fatty acid β-oxidation system results in profound activation of PPARα in liver [94, 128] (Fig. 10.2). Animals with ACOX1 deficiency have high levels of VLCFAs and since VLCFA-CoAs are incapable of entering the fatty acid oxidation pathway due to ACOX1 deficiency, these unmetabolized substrates act as ligands to hyper activate PPARα in liver [4, 90, 128]. Recent studies with gene knockout mouse models suggest that enzymes such as ACOX1, L-PBE/MFP1, D-PBE/MFP2, and SCPx of peroxisomal β-oxidation spiral are necessary for the degradation of endogenously generated PPARα ligands [50, 78, 80, 89–91, 102, 104, 128, 129]. It is also becoming clear that other enzymes such as FAS, FACS, and certain lipoxygenases are required for PPARα ligand generation in vivo [90, 104, 121]. For example, FAS deficient mice reveal a phenotype resembling PPARα deficiency. FAS generated 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC), functions as a physiologically relevant PPARα ligand [104] (Fig. 10.2). Thus, absence of an enzyme such as FAS fails to generate a ligand, where as absence of an enzyme such as ACOX1 fails to degrade endogenous PPARα ligand(s). These observations establish that fatty acid metabolism in liver is intricately regulated to prevent to harmful accumulation of products that may lead to hepatic steatosis and steatohepatitis, or that fail to upregulate PPARα under conditions of stress such as that induced by excess fatty acid influx into liver during fasting or excess energy overload.

MicroRNAs in the Regulation of Lipid Metabolism

Recent studies suggest that microRNAs play a role in regulating lipid metabolism [136–139]. For example, ectopic expression of let-7 blocks 3T3 L1 cell growth and completely inhibits terminal differentiation [136]. miR-103(1), miR-103(2), and miR-107 within introns of the pantothenate

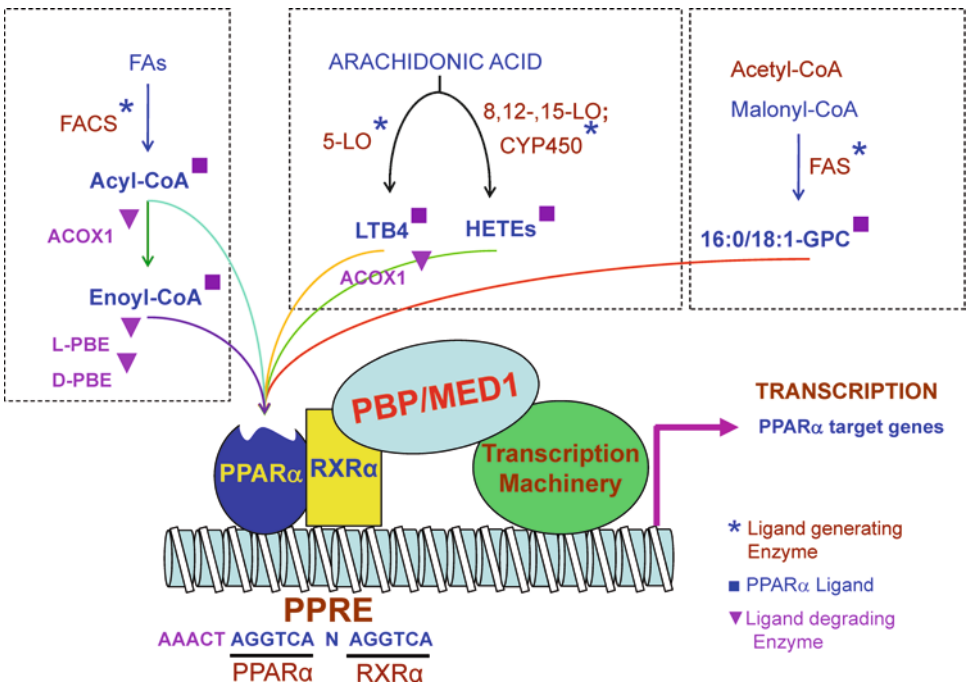

Fig. 10.2 Biological ligands of PPARα. Lipid metabolism generates intermediary metabolites and if some of these, for example acyl-CoAs generated by fatty acyl-CoA synthetase (FACS) remain unmetabolized as in the absence of ACOX1, they can act as biological (endogenous) PPARα ligands in liver. Gene knockout mouse models have provided valuable insights regarding PPARα ligand degrading and ligand generating enzymes in liver. The model also shows peroxisome proliferator response element (PPRE) hexanucleotide direct repeat separated by one nucleotide (DR1) and the putative PPAR and RXR heteridimer binding half-sites. Also shown are the basal transcription machinery and the transcription coactivator PBP/MED1 as they appear critical for PPAR regulated transcriptional activity. From Pyper et al. [90] with permission

kinase gene appear to be important in the regulation of cellular acetyl-CoA synthesis [137]. miR-122 is a liver specific microRNA, expressed in rodents and appears to regulate cholesterol and hepatic lipid metabolism [138]. Inhibition of miR-122 in mice results in a significant improvement in liver steatosis, which may result from a reduction of several lipogenic genes and stimulation of hepatic fatty-acid oxidation. Recent studies showed that PPARδ and PPARα coactivator Smarcd1/Baf60a are the target genes of miR-122 [138].

Several microRNAs have been proposed to be associated with obesity. The expression of miR-335 in liver is elevated in obese mouse models, including ob/ob, db/db, and KKAy mice and its expression accounts for increase of body, liver size, and white adipose tissue stores [139]. miR-335 levels are closely correlated to the expression of PPARγ, aP2, and FAS expression, the markers for adipocyte differentiation. Thus, the induction of miR-335 may contribute to the pathophysiology of obesity and to the development of hepatic steatosis [139]. Recent microarray data showed that miR-151, -192, -34a, -24, -10b, -132 are upregulated in hepatic steatosis [140]. The identification of target genes for these micro-RNAs and authentication of changes in their expression require further attention to appreciate the regulatory implications of these pivotal molecules in hepatic lipid metabolism. Manipulating microRNA levels may be an another layer of gene regulation, and may present a potential for therapeutic targets.

Summary

Lipids constitute an important source of energy. Liver plays a central role in lipid metabolism as it is critical for lipogenesis and fatty acid catabolism. Several enzymes participating in these energy balancing processes are affected by a variety of pathophysiological conditions and regulated by certain transcription factors. Evidence indicates that SREBPs and PPARα play prominent roles in controlling the hepatic expression of genes responsible for fatty acid synthesis and oxidation, respectively. Elucidation of the role of enzymes and of transcription factors that regulate the expression of these enzymes is critical for understanding the intricacies involved in the pathogenesis of hepatic steatosis and for the development of therapies. Several genetic disorders affecting the mitochondrial and peroxisomal fatty acid oxidation systems have been identified, and in recent years, gene knockout-mouse models have also been generated to investigate in detail the role of a given gene in modulating the metabolic pathways when their function is disrupted. These genetically altered mouse models are also providing considerable insights into the role of enzymes in generating and degrading endogenous ligands for transcription factors. The substrates and/or products generated by specific enzymatic processes appear to function as biological ligands for the transcription factors that control the expression of genes responsible for lipid metabolism in liver.

Acknowledgment This work was supported by NIH Grant DK083163 (J.K.R).

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Chapter 11
Detoxification Functions of the Liver

Udayan Apte and Partha Krishnamurthy

Introduction

The body is exposed to a variety of chemicals everyday in the form of pharmaceutical agents, household chemicals, dietary supplements, and environmental contaminants, many of which are extremely toxic. The primary defense mechanisms against xenobiotics in the body are the drug metabolizing enzymes (DMEs) involved in metabolism and excretion of xenobiotics [1]. Liver is the primary organ involved in the metabolism of xenobiotics including chemicals and pharmaceutical agents. Other organs including kidney and intestine have minor drug metabolism capabilities but liver is the site of metabolism for a vast majority of drugs and chemicals [2]. The chemical reactions involved in drug metabolism generally convert chemicals into more water-soluble metabolites rendering them easier for eventual excretion in the urine. Whereas many reactions catalyzed by the DMEs result in more water-soluble products, which are relatively less harmful, a number of the DME-mediated reactions result in the production of metabolic intermediates, which are highly reactive and induce tissue damage [3]. Therefore, the detoxification function of the liver is not homogenous and metabolism of each chemical in the liver should be investigated on a case-by-case basis. Nevertheless, there are some general principles that apply to the entire drug metabolism process.

The chemical reactions that are involved in drug metabolism process can be divided into four broad categories as hydrolysis, reduction, oxidation and conjugation reactions [3]. Traditionally, drug metabolism process is divided into Phase I and Phase II, where enzymes involved in hydrolysis, reduction and oxidation are considered as Phase I enzymes and the enzymes involved in conjugation reactions are considered as Phase II enzymes. The so-called Phase I enzymes include cytochrome P450 (CYP450), flavin containing monooxygenase (FMO) and aldehyde and alcohol dehydrogenase enzymes, which mainly conduct hydrolysis, oxidation, and reduction reactions [2]. The Phase II enzymes generally conduct conjugation reactions such as glucuronidation, glutathione conjugation, sulfation, methylation, acetylation, and amino acid conjugation. Generally, the chemical first undergoes Phase I biotransformation and the product of Phase I metabolism becomes a substrate for the Phase II conjugation reactions. This traditional classification of DME has been challenged in recent years mainly because of new evidence suggesting that many chemicals can be first conjugated and later become substrates for CYP450 enzymes. Similarly, many drugs are directly conjugated and do not undergo CYP450 mediated metabolism unless in case of an overdose. For example, acetaminophen, which is known to induce liver injury and acute liver failure, undergoes conjugation to glucuronic acid and sulfonic acid and only in the case of an overdose, it is metabolized by CYP2E1 [4]. Furthermore, the original idea behind considering conjugation reactions as Phase II was that the conjugated product was thought to be less toxic. However, substantial evidence in recent years has shown that many conjugation processes can result in generation of toxic intermediates. A classic example is trichloroethylene (TCE) a known hepatotoxicant and chemical carcinogen, which undergoes Phase I metabolism and Phase II glutathione conjugation to produce a highly nephrotoxic intermediate called *S*-(1,2-dichlorovinyl)-L-cysteine [5].

Majority of the DMEs in the liver are present in hepatocytes thus making hepatocytes, the center of the detoxification process not only in the liver, but also in the entire body [2]. There are a large number of enzymes known to be involved in drug metabolism in the liver and the list is growing. The most important enzymes in terms of drug metabolism and toxicity include the classic CYP450, FMO, aldehyde dehydrogenase, alcohol dehydrogenase, monoamine oxidases, epoxide hydrolases, glutathione transferases, glucuronosyltransferases, sulfotransferases, methyltransferases, and *N*-acetyl transferases. Whereas majority of DMEs are present in smooth endoplasmic reticulum, (also called microsomal, since upon isolation

U. Apte (✉)
Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas, USA
e-mail: uapte@kumc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_11, © Springer Science+Business Media, LLC 2011

using differential centrifugation, ER forms lipid micelles called as microsomes) many are present in the cytoplasm (cytosolic). Because of this spatial variation within the cell, DMEs are sometimes also referred to by their location along with the reaction they catalyze (For e.g., microsomal epoxide hydrolase). Certain enzymes such as glutathione transferase are also present in the mitochondria.

In recent years, a third dimension of drug metabolism process, which has received much attention, is the drug transport [6]. Both hepatocytes and biliary epithelial cells in the liver express a variety of influx and efflux transporter proteins, which are critical in determining the cellular levels of xenobitics. Studies have highlighted the importance of drug transporters in the bioavailability of various chemicals and drugs, and transporters are now considered as the Phase III of the drug metabolism process.

Major DMEs in the Liver

Whereas there are many enzymes involved in catalyzing specific reactions in drug metabolism and detoxification in the liver, few have a much higher impact on liver biology and human health in general than others. These include CYP450s and FMO in the so-called Phase I metabolic reactions and the glutathione transferases, glucuronosyltransferases, and sulfotransferases in the Phase II reactions [2]. The following section describes the general mechanism of action of these enzymes with details on some of the more important isoforms of these enzymes.

Cytochrome P450

CYP450 form the largest group of DMEs with 57 known human isoforms [2]. CYP450 are versatile enzymes and can catalyze oxidation of a wide range of the substrates including drugs, chemicals, and endogenous substrates [7]. CYP450 are heme-containing proteins and the heme (iron) is generally present in the ferric (Fe^{3+}) state. Upon reduction to ferrous state (Fe^{2+}), the CYP450 enzymes form a complex with substrates such as oxygen and carbon monoxide (CO). The CYP450-CO complex was found to absorb light at 450 nm, which gave these enzymes their name. However, depending on the isoforms, these enzymes can absorb light at various wavelengths between 447 and 452 nm following binding to CO. This peculiar absorbance at 450 nm is because of a formation of thiolate bond between a highly conserved cysteine residue next to the heme moiety and the ligand. Upon dissociation of this thiol bond, CYP450 becomes catalytically inactive and shows maximum absorbance at 420 nm.

The basic CYP450 reaction is a mono-oxygenation reaction in which the substrate (RH) is oxidized by the incorporation of one oxygen atom to generate the product (ROH) (Fig. 11.1a). The second atom of oxygen is used to produce water by using reducing equivalents donated by NADPH [7]. Most CYP450 enzymes function in collaboration with another protein called NADPH oxidoreductase, a flavin containing enzyme. NADPH oxidoreductase is critical for CYP450 activity because CYP450 enzymes bind to the substrates directly, but not to NADPH. The FMN and FAD, which are parts of NADPH oxidoreductase, aid in transfer of electrons from NADPH to CYP450. This is mainly true for CYP450 present in endoplasmic reticulum, whereas for CYP450 present in mitochondria, two other proteins called ferrodoxin and ferrodoxin reductase are involved in electron transfer from NADPH to CYP450. In addition to NADPH oxidoreductase, another protein called cytochrome b_5 is also involved in the electron donation in CYP450 enzymes. Cytochrome b_5 increases the rate of CYP450 reaction and also increases the substrate-binding affinity of CYP450 enzymes. Whereas several CYP450 isoforms have been identified, both NADPH oxidoreductase and cytochrome b_5 do not have any isoforms.

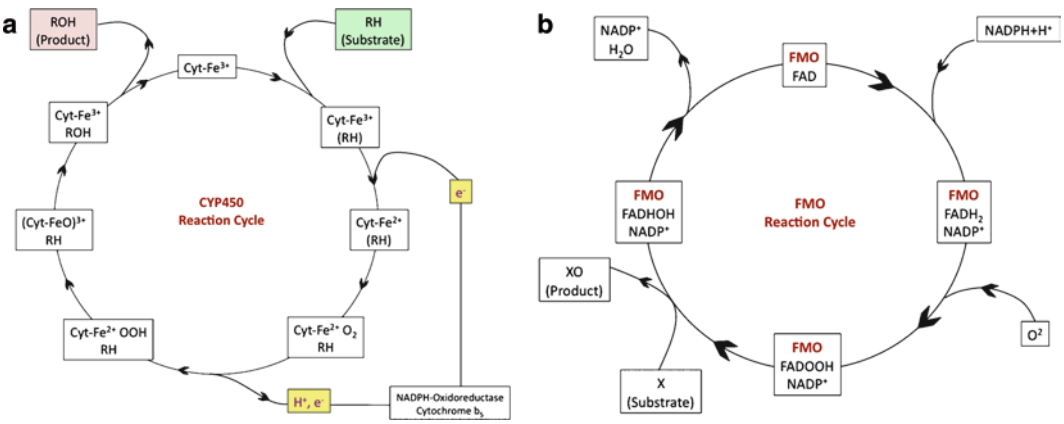

Fig. 11.1 Reaction cycles of (**a**) CYP450 and (**b**) FMO enzymes. See text for details

CYP450 Isoforms

There are 57 known isoforms of CYP450 in humans (Table 11.1). The classification of CYP450 isoforms is based on amino acid sequences of individual enzymes [8]. The enzymes are divided into families (CYP1, CYP2, CYP3, etc.), subfamilies (CYP1A, CYP2E, CYP3A, etc.), and individual genes (CYP1A1, CYP2E1, CYP3A4, etc.). The CYP450 enzymes grouped into each family and subfamily share common characteristics including substrates, type of reactions catalyzed, chemical inducers and inhibitors. Whereas each of the 57 CYP450 enzymes are unique and have specific functions, enzymes from some CYP450 enzymes have deeper impact on liver, and overall physiology and pathobiology than others [9]. These CYP450 enzymes important for liver biology are further discussed here.

CYP1A2

The most well-characterized and physiologically relevant enzymes in CYP1A family include CYP1A1 and CYP1A2 [10]. In humans, the main hepatic CYP1A family member is CYP1A2. However, the CYP1A1 is not expressed in the human liver,but it is present in many extrahepatic tissues. The expression of CYP1A family members is regulated mainly

Table 11.1 CYP450 isoforms expressed in humans

CYP450 family	Specific isoform	Function
CYP1A	CYP1A1, 1A2	Xenobiotic metabolism
CYP1B	CYP1B1	Xenobiotic metabolism
CYP2A	CYP2A6, 2A7, 2A13	Xenobiotic metabolism
CYP2B	CYP2B6,	Xenobiotic metabolism
CYP2C	CYP2C8, 2C9, 2C18,2C19	Xenobiotic metabolism, fatty acid and ecosinoid metabolism
CYP2D	CYP2D6	Xenobiotic metabolism
CYP2E	CYP2E1	Xenobiotic metabolism
CYP2F	CYP2F1	Xenobiotic metabolism,
CYP2J	CYP2J2	Xenobiotic metabolism, fatty acid and ecosinoid metabolism
CYP2R	CYP2R1	Vitamin D metabolism
CYPS, U, W	CYP2S1, 2U1, 2W1	Unknown
CYP3A	CYP3A4, 3A5, 3A7, 3A43	Xenobiotic metabolism, bile acid synthesis
CYP4A	CYP4A11, 4A22	Fatty acid and ecosinoid metabolism
CYP4B	CYP4B1	Fatty acid and ecosinoid metabolism
CYP4F	CYP4F2, 4F3, 4F8, 4F11, 4F12, 4F22	Xenobiotic metabolism, fatty acid and ecosinoid metabolism
CYP4V, 4X, 4Z	CYP4V2, 4X1, 4Z1	Unknown
CYP5A	CYP5A1	Fatty acid and ecosinoid metabolism, thromboxane A synthesis
CYP7A	CYP7A1	Bile acid synthesis
CYP7B	CYP7B1	Bile acid synthesis
CYP8A	CYP8A1	Fatty acid and ecosinoid metabolism, prostaglandin synthesis
CYP8B	CYP8B1	Bile acid metabolism
CYP11A	CYP11A1	Steroid synthesis
CYP11B	CYP11B1, 11B2	Steroid synthesis
CYP17A	CYP17A1	Steroid synthesis
CYP19A	CYP19A1	Steroid synthesis
CYP20A	CYP20A1	Unknown
CYP21A	CYP21A2	Steroid synthesis
CYP24A	CYP24A1	Vitamin D metabolism
CYP26	CYP26A1	Retinoic acid metabolism
CYP26B	CYP26B1	Retinoic acid metabolism
CYP26C	CYP26C1	Vitamin D metabolism, retinoid acid metabolism
CYP27A	CYP27A1	Bile acid synthesis
CYP27B	CYP27B1	Vitamin D metabolism
CYP27C	CYP27C1	Unknown
CYP39A	CYP39A1	Bile acid metabolism
CYP46A	CYP46A1	Bile acid metabolism
CYP51A	CYP51A1	Bile acid metabolism, cholesterol biosynthesis

via aryl hydrocarbon receptor (AhR)-mediated mechanism. The chemicals that are known to induce CYP1A enzymes include AhR agonists such as 3-methylcholanthrene, TCDD, and other polyaromatic hydrocarbons. CYP1A2 catalyzes O-dealkylation, epoxidation, and N-hydroxylation reactions that are commonly associated with metabolic activation of many chemical carcinogens. Numerous chemicals are known to inhibit CYP1A2 including drugs such as acyclovir, ciprofloxacin, and Verapamil [2].

CYP2B Family

The CYP2B family is represented by CYP2B6 in the human liver, an isoform that is regulated by nuclear receptors CAR and PXR [11]. The inducers of CYP2B family members are agonists of CAR and PXR including phenobarbital, antitubercular drugs such as rifampicin, and herbal preparations such as St. John's wort. CYP2B6 catalyzes N-demethylation of substrates, which include various pharmacological compounds such as bupropion, propofol, ketamine, and tamoxifen. The inhibitors of CYP2B6 include pharmaceutical agents such as clopidogrel.

CYP2C Family

There are four isoforms from the CYP2C family expressed in the humans including CYP2C8, 2C9, 2C18, and 2C19 [12]. Together, these enzymes play a critical role in metabolism of endobiotics as well as xenobiotics. The CYP2C enzymes are regulated by CAR/PXR and the agonists of these nuclear receptors induce CYP2C enzymes. The substrates of CYP2C enzymes are varied and include paclitaxel, rosiglitazone (CYP2C8), various NSAIDs including diclofenac, ibuprofen, naproxen (CYP2C9), omeprazol and lansoprazol (CYP2C19). CYP2C8 is of particular importance as it can metabolize glucoronide conjugates of various drugs leading to significant toxicity. Some of the substrates of CYP2C enzymes including pioglitazone and rosiglitazone are also CYP2C inhibitors.

CYP2D6

CYP2D6 is involved in metabolism of a large number of pharmaceutical agents of various chemical structures and chemical classes. Therefore, CYP2D6 has a deep impact on human health in general and drug metabolism in specific [13]. Some of the substrates of CYP2D6 include dexamethorphan, codeine, chlorpromazine, imipramine, propanolol and tamoxifen [2]. CYP2D6 exhibits extensive genetic polymorphism in the human population and humans can be divided into four distinct groups depending upon their CYP2D6 levels including, poor metabolizers, intermediate metabolizers, extensive metabolizers, and ultra-rapid metabolizers. CYP2D6 polymorphism is associated with toxicity of many drugs including debrisoquine, codeine, and perhexiline.

CYP2E1

No other CYP450 isozyme has caught more attention of liver biologists than CYP2E1, which is involved in metabolic activation of a number of hepatotoxic chemicals including alcohol, acetaminophen, carbon tetrachloride, halothane, benzene, chloroform, trichloroethylene, and diethylnitrosamine [14]. CYP2E1 is expressed primarily by the centrilobular hepatocytes and converts its substrates into highly reactive metabolites causing extensive hepatic necrosis. Metabolic activation of acetaminophen, the active ingredient of Tylenol® and many other antipyretic-analgesic formulations, to a reactive metabolite *N*-acetylbenzoquinoneimine (NAPQI) is the mandatory initial step involved in acetaminophen hepatotoxicity and subsequent acute liver failure [4]. The inducers of CYP2E1 include chemicals such as alcohol, isoniazid, and physiological conditions such as diabetes. The inhibitors of CYP2E1 include diallyl sulfide and disulfiram. CYP2E1 has been studied extensively for its role in alcoholic liver disease, acetaminophen hepatotoxicity, as well as non-alcoholic steatohepatitis and remains at the center of research focused at liver injury.

CYP3A4 Family

The isozymes that belong to CYP3A family including CYP3A4, 3A5, 3A7, and 3A43 are the most abundant CYP450 enzymes in human liver [15, 16]. The substrates of CYP3A family of enzymes include a wide variety of chemicals, drugs and, herbal preparations. CYP3A4 is estimated to metabolize approximately 70% of all drugs and thus is critical for human health. CYP3A enzymes are regulated by the nuclear orphan receptor CAR and CAR agonists are potent inducers of CYP3A. A wide variety of chemicals inhibit CYP3A4 activity including verapamil, clotrimazole, and ketoconazole.

CYP4A11

This member of CYP4A family, which includes 12 isozymes in humans, is of particular interest to liver biologists as it is regulated by PPAR-α, especially in rodents [17]. PPAR-α agonists such as clofibrate are potent inducers of CYP4A11 and levels of CYP4A11 have been used as markers of PPAR-α activation. CYP4A enzymes are involved in metabolism of endobiotics including fatty acids and eicosanoids, and are involved in prostaglandin metabolism.

Regulation of DME Expression

The DMEs, especially CYP450 enzymes are inducible and their expression can be induced by a large number of CYP450-family specific inducers. In most cases (with the exception of CYP2E1), CYP450 inducers are ligands for nuclear receptors and induce their expression via nuclear receptor based mechanism [18]. The four nuclear receptors that play a major role in regulation of CYP450 are constitutive androstane receptor (CAR), pregnane X receptor (PXR), aromatic hydrocarbon receptor (AhR), and peroxisome proliferator activated receptor-alpha (PPAR-α). Apart from these classic nuclear receptors, NF-E2-related factor 2 (Nrf-2) is also involved in regulation of CYP450 enzymes in response to oxidative stress. Some nuclear receptors such as HNF4α are master regulators of hepatic differentiation and regulate expression of CAR and PXR, and thus, also regulate CYP450 enzymes.

AhR regulates CYP1A1, 1A2, 1B1, and 2S1 expression. AhR functions in conjunction with aryl hydrocarbon receptor nuclear transporter (ARNT). CAR and PXR regulate a large number of CYP isoforms from CYP2A, 2B, 2C, and 3A families and PPAR-α regulates members of CYP4A family. CAR, PXR, and PPAR-α all require retinoid X receptor (RXR) as a binding partner for their function.

CYP2E1 is a unique enzyme not regulated by the commonly involved nuclear factors. However, substantial evidence indicates that CYP2E1 levels are negatively regulated by insulin. Similarly, recent data obtained from tissue specific knockout mice implicates β-catenin in stimulation of CYP2E1 expression.

Zone Specific Expression of CYP450

Metabolic zonation of hepatocytes is a hallmark of liver biology. It is known that the CYP450 expression is relatively higher in the centrilobular region (zone 3) of the liver lobule [19]. The zonal expression of DME is regulated by various factors including oxygen tension in the circulation, blood pressure and zonal expression of several transcription factors that regulate DME gene expression. Recent studies indicate that transcriptional coactivator β-catenin may be involved in zone specific expression of various CYP450 enzymes including CYP2E1 and CYP1A2 [20, 21]. Data from hepatocyte specific knockout mice indicates that β-catenin regulates the expression of CYP2E1 and 1A2 in the centrilobular hepatocytes. The zone specific expression of DME in the liver is the main reasons behind zone specific injury of many hepatotoxic chemicals such as acetaminophen (APAP). For example, in case of APAP, an overdose of APAP will result in specifically centrilobular (zone 3) necrosis because, CYP2E1, the main enzyme involved in metabolic conversion of APAP into NAPQI is expressed mainly by the centrilobular hepatocytes.

Flavin Monooxygenase

Apart from CYP450 enzyme, the other significant DMEs in the liver are the members of FMO family. FMO enzymes play important roles in detoxification function of the liver and are involved in the metabolism of a large variety of nitrogen, phosphorous, and sulfur containing chemicals [2, 22]. There are five FMO isoforms known designated as FMO1 to FMO5. FMO6 has also been identified, but it is a nonfunctional enzyme produced by mRNA from a pseudogene. The tertiary structure of FMO is not known and many aspects of FMO biology remain undiscovered partly due to their heat labile property (FMO can be destroyed at 50°C for 1 min) and lack of specific chemical inducers and inhibitors.

FMO uses FAD as an electron donor and is involved in metabolizing nucleophilic targets including tertiary and secondary amines, thiols, thioethers, thiocarbamates, and phosphines. It has been postualted that in vivo, the FMO enzymes bind a peroxyflavin moiety produced from binding of $FADH_2$ to oxygen (Fig. 11.1b). In the initial step, NADPH reduces FAD to produce $FADH_2$. The resultant free $NADP^+$ remains bound to the FMO enzyme. When a substrate (X) binds to FMO, it is oxidized (XO) followed by the removal of $NADP^+$ from the FMO enzyme complex to return FAD to its oxidized state.

The substrates of FMO are varied and include pharmaceutical agents such as cimetidine, and chemicals such as nicotine, 2-acetylaminofluorene, hydrazines, thiols and thioamides such as thioacetamide, and thiobenzamide, and phosphines such as diphenymethylphosphine [22].

DMES Involved in Conjugation Reactions

The conjugation reactions, which were previously grouped as Phase II drug metabolism reactions, form an important part of the detoxification function of the liver. The conjugation reactions involved in drug metabolism include glucuronidation, sulfation (or sulfonation), acetylation, methylation, amino acid conjugation, and glutathione conjugation (Table 11.2). With a few important exceptions, the conjugation reactions convert the substrates into a more water-soluble and less toxic product [2]. The conjugation enzymes catalyze reactions where various molecules such as glucose, glucuronic acid, amino acids, and glutathione are conjugated

Table 11.2 Overview of conjugation reaction involved in the liver

Type of conjugation reaction	Conjugating enzyme	Cofactor	Number of human isoforms
Glucuronidation	UDP glucoronosyl transferase (UGT)	UDP-glucuronic acid UDP-glucose UDP-xylose UDP-galactose	22 Isoforms classified into 8 families
Sulfation	Sulfotransferase (SULT)	3′-Phosphoadenosine-5′-phsphosulfate	13 Total, 6 expressed in the liver
Glutathione conjugation	Glutathione transferase (GST)	Glutathione	17 Isoforms divided into 8 different classes
Acetylation	*N*-acetyl transferase (NAT)	Acetyl Co-A	2 Distinct isoforms with several genetic polymorphisms
Methylation	Methyl transferase	*S*-adenosyl methionine	10 Distinct enzymes classified according to substrates used
Amino acid conjugation	ATP dependent acid:CoA ligase and Acyl:CoA amino acid *N*-acyltransferase	Glycine, glutamine, taurine, serine and proline	2 Known enzymes but more enzymes have been postulated

with the substrate to give rise to more hydrophilic products. The conjugation reactions are classified into two types of reactions. Glucuronidation, sulfation, acetylation, and methylation involve conjugation of substrates with an activated or "high energy" cofactor. Amino acid conjugation and glutathione conjugation involves conjugation of an activated and highly reactive xenobiotic intermediate to either amino acids, or glutathione.

Glucuronidation

Except animals from the cat family, glucuronidation is the prominent conjugation reaction observed in mammals. Glucuronidation is catalyzed by enzymes called UDP-glucuronosyltransferases (UGTs), which use several forms of modified sugar molecules as cofactors and conjugate a wide variety of pharmaceutical agents, chemicals, and endogenous substrates [23]. Whereas UDP-glucuronic acid is the most common cofactor used by UGTs, other cofactors including UDP-glucose, UDP-xylose, and UDP-galactose are also used. UGTs are expressed in a number of tissues but the levels of UGTs are highest in the liver smooth endoplasmic reticulum.

In humans, a total of 22 UGT isoforms are known and majority of them are expressed in the liver. The human UGTs are classified into four families including UGT1, UGT2, UGT3, and UGT8. Out of these, UGT1 and UGT2 are the best-characterized enzymes and pertinent to the detoxification function of the liver. UGT1A family of UGTs is transcribed from a single UGT1A gene locus. Due to alternative exon usage and splicing, 13 different transcripts are produced from UGT1A gene locus (UGT1A1 – UGT1A13) but only nine functional enzymes can be identified in vivo (UGT1A2, 1A11, 1A12, and 1A13 are pseudogenes). Ten different UGT2 enzymes, which belong to UGT2A and UGT2B subfamilies, are known. Studies have shown that out of all the UGTs expressed in the liver UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 are the most important for the drug metabolism function of the liver.

The substrates of glucuronidation reaction are varied and include a large number of drugs, chemical and endobiotic. These include commonly used antipyretics such as acetaminophen and ibuprofen, other drugs such as naproxane, diclofenac, propofol, oxazepam, zedovudin etc. The endogenous compounds conjugated by UGTs include mainly include bilirubin, thyroid hormones, and steroids including 17-β-estradiol and testosterone. Because UGTs are involved in conjugation of endobiotics such as bilirubin, they play a critical role in liver and physiological homeostasis and genetic polymorphism in some UGTs such as UGT1A1 are associated with Crigler–Najjar syndrome and Gilberts syndrome. A large number of genetic polymorphisms have been identified in various UGT isoforms including in UGT1A1 and several UGT2B family members including UGT2B4, 2B7, 2B15 and 2B28.

There is substantial evidence that UGT expression is regulated by nuclear receptors. In fact, in the rat, UGTs can be categorized into four groups depending upon their time of expression and type of inducers. Group 1 UGTs are expressed embryonically and are induced by AhR-agonists such as 3-methylcholantherene. Group 2 UGTs are expressed perinatally (day 5 after birth) and are induced by CAR agonists such as phenobarbital. Group 3 and 4 UGTs are expressed around puberty and are regulated by PXR agonists such as PCN, and PPAR-α agonists such as clofibrate, respectively.

Sulfation

Sulfation or sulfonation is a conjugation reaction catalyzed by enzymes called sulfotransferases (SULTs) [2]. Sulfation involves conjugation of the substrates with sulfonate moiety donated by a cofactor called 3′-phosphoadenoside-5′-phosphosulfate (PAPS) [24]. Many substrates that undergo glucuronidation also undergo sulfation. However, sulfation is a high-affinity low-capacity process mainly because of the relatively lower concentration of PAPS (4–80 μM as compared to 200–350 μM of UDP-glucuronic acid). Whereas many organs including kidneys, lungs and intestine express SULTs, these enzymes are primarily present in the liver.

There are two types of SULT enzymes known depending upon their cellular location and function. The membrane-bound SULTs are present in the Golgi apparatus, are involved in sulfation of endobiotics such as cholecystokinin and play minimal role in detoxification of xenobiotics. The soluble SULTs present in cytosol are critical for xenobiotic metabolism and conjugate a large variety of substrates to produce hydrophilic sulfuric acid esters excreted in the urine. In humans 11 distinct genes encode for 13 distinct SULT isoforms that have been classified into SULT1, SULT2, and SULT4 gene families. Out of the 13 known SULTs, six are expressed in the liver. Studies have shown that both rodent and human SULTs can be regulated by nuclear receptors such as CAR and PXR. In humans, compelling evidence suggests that agonists of PXR including rifampin are potent inducers of SULTs and such drug-mediated induction of SULT is of critical clinical importance as it can change the bioavailability and elimination of many pharmaceutical agents.

The SULTs can conjugate a variety of endobiotics and xenobiotics including dopamine, 17-β-estradiol, tamoxifen, 2-acetylaminofluorene, and acetaminophen.

Glutathione Conjugation

One of the most important conjugation reactions pertaining to detoxification function of the liver is the glutathione conjugation because it is directly involved in conjugating a number of hepatotoxic chemicals and serves as a defense mechanism against oxidative stress [25]. Glutathione (GSH) is a tripeptide made of three amino acids including glycine, cysteine and glutamic acid. GSH conjugation is catalyzed by an enzyme called glutathione transferase (GST) expressed in all tissues, but is in especially higher quantities in the liver. GSTs can be found in the cytosol, mitochondria, endoplasmic reticulum, peroxisomes, and nucleus and are also involved in cellular functions distinct from drug metabolism.

Whereas the substrates of GSH conjugation are structurally varied, they all have three common chemical properties. These include presence of an electrophilic atom, ability to react with GSH in nonenzymatic fashion up to certain extent, and relatively high hydrophobicity. Depending upon the chemistry, GSH conjugation reactions are classified into "displacement" reaction where GSH displaces the electron-withdrawing group of the substrate, and "addition" reaction where GSH is added to an activated double bond or ring structure in the substrate. GSH conjugation subsequently results in mercapturic acid synthesis via a multistep process. The first step is catalyzed by GST, and results in the formation of GSH conjugate followed by formation of cysteinyl-glycine conjugate catalyzed by γ-glutamyltranspeptidase. The cysteinyl-glycine conjugate is converted to premercapturic acid by cysteinyl glycine dipeptidase. In the last step, premercapturic acid is converted to mercapturic acid by acetylation catalyzed by *N*-acetyltransferase.

GSTs are dimeric proteins formed from two similar or different subunits [23]. The cytosolic GSTs are divided in to seven classes designated as alpha (GST A), mu (GST M), pi (GST P), sigma (GST S), theta (GST T), zeta (GST Z) and omega (GST O). Each family of GSTs has several isoforms and they are designed depending upon the dimers, which form the active enzyme (for e.g., GSTA1-1, GSTA2-2 etc.). Apart from the cytosolic GSTs, a distinct form of mitochondrial GST, which seems to have evolved separately, is classified as the kappa class (GST K).

Because it is one of the primary cellular defenses in the cell against oxidative stress, GSH conjugation plays a critical role in cellular homeostasis. Depletion of GSH or inhibition of GSTs is associated with acute hepatotoxicity of many compounds including acetaminophen and also with chemical-induced tumorigenesis including chemicals like aflatoxin B1. GSH conjugation plays a critical role in pathogenesis of acetaminophen-induced acute liver failure because GSH conjugation of NAPQI, the reactive metabolite of acetaminophen, is the primary cellular defense mechanism against acetaminophen overdose. The current therapeutic use of *N*-acetyl cysteine (NAC) to treat acute liver failure is based on the evidence that NAC provides essential cysteine moieties necessary for biosynthesis and replenishment of GSH.

Acetylation

Many chemicals especially those containing aromatic amine or hydrazine groups are substrates for N-acetylation [26], a conjugation reaction catalyzed by a cytosolic enzyme called *N*-acetyl transferase (NAT). The cofactor used for acetylation reaction is acetyl Co-A, which is conjugated to the substrates by NATs via a two-step mechanism dubbed as "Ping Pong Bi Bi" mechanism.

In humans, two, highly polymorphic genes produce two, distinct isoforms of NATs designated as NAT1 and NAT2. Depending on genetic polymorphism humans can be distributed into "slow acetylators" (mainly in Middle-Eastern populations), and "fast acetylators" (mainly Asian populations).

The substrates that undergo N-acetylation generally first undergo CYP450-mediated metabolism. The substrates include metabolites of caffeine, nitrozepam, and isoniazid.

Methylation

Methylation, a relatively minor, but unique form of conjugation reaction, results in decreased water solubility of the substrates. Methylation is catalyzed by a large group of enzymes called methyl transferases using a cofactor called *S*-adenosylmethionine (SAM or SAMe) [2]. Depending on the heteroatom of the substrate involved, methylation reactions are categorized into O-, N-, and S-methylation. Methylation is much more important and common with endobiotic substrates than xenobiotic substrates and plays a critical role in cellular homeostasis. The classic example of methyl transferase enzyme is catechol-*O*-methyl transferase (COMT), an enzyme present in both cytosol and endoplasmic reticulum. The substrates of COMT include neurotrasmitters such as dopamine, epinephrine, and norepinephrine, and various catechol drugs such as L-dopa. Other examples of methyl transferase enzymes include histamine *N*-methyl transferase (HNMT), phenylethanolamine *N*-methyl transferase (PNMT), thiopurine methyl transferase (TPMT), and thiol methyl transferase (TPT).

Amino Acid Conjugation

Amino acid conjugation reactions can be divided into two groups depending upon the chemistry of the substrate and the amino acids used in conjugation [2]. The conjugation reactions in the first group involve xenobiotics containing carboxylic acid groups and are conjugated with amino acids such as glycine, glutamine, and taurine. In the second group, xenobiotics containing a hydroxylamine group are conjugated with amino acids such as serine and proline. Amino acid conjugation is of immense importance in bile acid biology where bile acids are conjugated with glycine and taurine, increasing their ability to be secreted in the bile. It also plays a critical role in the metabolism of NSAIDs such as ibuprofen.

Several enzymes are involved in amino acid conjugation, and it has been postulated that a separate enzyme specific to each amino acid catalyzes their conjugation. In general, amino acid conjugation is a two step process where the initial step is formation of an acyl Co-A thioester and is catalyzed by an enzyme called ATP dependent acid:CoA ligase. The second step involves transferring the acyl moiety of the xenobiotic to the amino group of the accepting amino acid and is catalyzed by an enzyme called acyl:CoA amino acid *N*-acyltransferase.

Role of Transporters as a Detoxification System in the Liver

Unbound compounds in the sinusoidal blood are taken up by the hepatocytes typically by a transporter-mediated mechanism or by diffusion across the basolateral membrane. Further, Phase II conjugates formed inside the hepatocytes are typically too hydrophilic to passively diffuse across the canalicular membrane into bile or across the hepatic basolateral membrane into the sinusoidal blood. Therefore carrier-mediated transport is required to cross this diffusional barrier. Thus, the extent of drug movement across the hepatocyte membranes is generally affected by the membrane transporters, which have a significant role in facilitating or preventing drug movement. Although, numerous transport proteins are present on the hepatic sinusoidal and canalicular membrane, only a few of these play a major role in the hepatic sinusoidal uptake as well as biliary and basolateral excretion. Therefore, this chapter will focus on reported interactions between the major detoxification transport proteins (Fig. 11.2). Other transport proteins, which play a minor role in hepatic excretion of Phase II metabolites, will be mentioned only briefly.

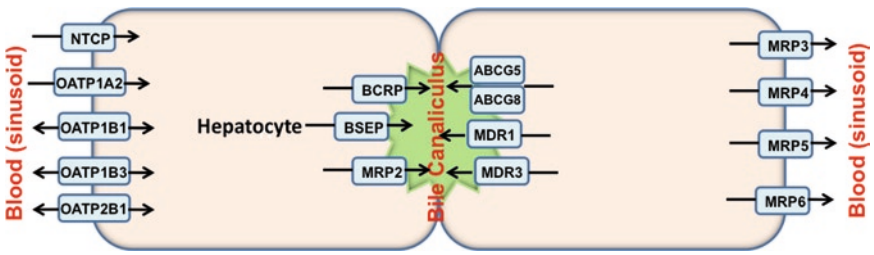

Fig. 11.2 Expression and localization of detoxification transporters in the liver. See text for details

Basolateral (Sinusoidal) Excretion

Excretion of drugs and/or metabolites from the hepatocyte across the basolateral membrane is primarily mediated by members of the ATP-driven multidrug-related protein (MRP) family. In the liver, ABCC3 (MRP3), ABCC4 (MRP4), ABCC5 (MRP5), and ABCC6 (MRP6) reside on the basolateral membrane and pump organic anions and bile acids from the hepatocyte into sinusoidal blood [27]. Of these four hepatic basolateral efflux transporters, only MRP3 and MRP4 appear to play a major role in the basolateral excretion of sulfate, glucuronide, and glutathione metabolites.

ABCC3 (MRP3)

MRP3 is a 1,527 amino acid protein that localizes to the basolateral membrane of hepatocytes [28–31]. MRP3 is a typical organic anion transporter able to transport organic compounds and bile acids into sinusoidal blood. MRP3 is responsible for the transport of conjugated (sulfate, or glucuronate) bile acids including bilirubin-glucuronides [28, 32–37]. Although MRP3 can efficiently transport sulfated bile acids, transport of sulfated xenobiotics and steroids appear to be weak. Transport of glutathione-conjugated xenobiotics (such as dinitrophenyl-*S*-glutathione and acetaminophen glutathione) by MRP3 appears to be controversial [38]. A list of substrates for human MRP3 is presented in Table 11.3.

MRP3 in the liver is highly inducible and is upregulated during cholestasis and in the absence of functional MRP2, as seen in patients with Dubin–Johnson syndrome (DJS) [39]. This is thought to be a compensatory response to enhance excretion of potentially toxic anions into sinusoidal blood for clearance. Further, MRP3 expression has been shown to be regulated by nuclear receptors (RXRα, PXR, PPARα, and Nrf2) [40, 41].

ABCC4 (MRP4)

MRP4 encodes a 1,325 amino acid protein [42]. In the liver, MRP4 localizes to the basolateral membrane of the hepatocytes [42, 43]. MRP4 has high affinity to the sulfated bile

Table 11.3 Human MRP substrates

Human MRP2 substrates		
Sulfate conjugates	*Glucuronide conjugates*	*Glutathione conjugates*
Benzo[a]pyrene-1 [121]	Acetaminophen [122]	Hydroxynonenal [123]
Benzo[a]pyrene-3 [121]	Ethinylestradiol [85]	*N*-ethylmaleimide [83]
Ethinylestradiol [85]	Nicotine-N [124]	Methylfluorescein [125]
Sulfobromophthalein [126]	Cotinine-N [124]	Curcumin [127]
Estrone [128, 129]	Hydroxycotinine-O [124]	Leokotriene C4 [88, 126]
Dehydroepiandrosterone [45, 126, 129, 130]	Estradiol-17β [84]	Dinitrophenol
Taurolithocholate [126, 130]		GSH [127, 131]
		GSSG [122]
Human MRP3 substrates		
Sulfate conjugates	*Glucuronide conjugates*	*Glutathione conjugates*
Lithocholate [37, 132]	Estradiol-17-β [35, 37, 84, 124, 133]	GSH [28, 124, 133]
Glycolithocholate [132]	4-Methylumbelliferone [37, 132]	Leukotriene C4 [35, 37, 134]
Taurolithocholate [37, 132]	Etoposide [133]	Deoxyprostaglandin [135]
Dehydroepiandrosterone [45, 134, 136]	Ethinylestradiol [85]	Dinitrophenol [28, 35, 37, 133, 137]
Ethinylestradiol [85]	Bilirubin-mono [134]	
4-Methylumbelliferone [132]	Bilirubin-bis [134]	
	Acetaminophen [122]	
	Morphine-3 [138]	
	Morphine-6 [138]	
	Nicotine-N [124]	
	Cotinine-N [124]	
	Hydroxycotinine-O [124]	
Human MRP4 substrates		
Sulfate conjugates	*Glucuronide conjugates*	*Glutathione conjugates*
Dehydroepiandrosterone [43, 45]	Estradiol-17β [45, 47, 139–141]	GSH [43, 140, 142]
Lithocholate [45]	Dehydroepiandrosterone [45]	GSSG [43]
Taruolithocholate [45]	Estradiol-3 [45]	Leukotriene C4 [139]
Glycolithocholate [45]	Napththol [139]	Bimane [143]
Estrone-3 [45]	Nitrophenol [139]	Dinitrophenol [139, 143]
Estradiol-3,17-di [45]		Dinitrophenol-mercapturate [139]

acids and steroids [44, 45]. Although, glucuronide metabolites can interact with MRP4, they appear to have a low affinity. MRP4 can transport and be inhibited by glutathione conjugates, and thus might play a role in hepatic basolateral efflux of the glutathione conjugates [43, 46]. A class of endogenous substrates of MRP4 that is structurally related to the bile salts is represented by the conjugated steroids such as E2-17-β glucuronide and dehydroepiandrosterone-3 sulfate (DHEAS), prostaglandin E1, and prostaglandin E2, and other prostanoids [45, 47]. A list of MRP4 substrates is presented in Table 11.3.

Although MRP4 protein levels are relatively low in the liver, they increase under hepatic stress. MRP4 expression is induced with reduced levels of the major canalicular bile salt pump, BSEP and also in the absence of functional MRP2 [44, 45, 48]. Similar increase in MRP4 expression is seen during obstructive jaundice [49, 50]. As with MRP3, this increase appears to be a compensatory response to enhance detoxification of potential toxins into the sinusoidal blood for clearance. MRP4 expression has been shown to be induced by nuclear receptors (CAR and PPARα) [44, 51].

ABCC5 (MRP5) and ABCC6 (MRP6)

MRP5 encodes a 1,437 amino acid protein with an estimated molecular weight of 160 kDa that is expressed at low levels in healthy liver, but is upregulated under cholestatic conditions, suggesting that MRP5 protein levels may be increased as a part of hepatic response to cholestatic conditions [52]. MRP5 has similar substrate specificity as MRP4, including glutathione conjugates, and could play a role in the hepatic basolateral excretion of anionic conjugates [30, 53].

MRP6 gene encodes for a 1,503 amino acid protein with a molecular weight of 165 kDa. MRP6 has been shown to localize to the basolateral/lateral surface of hepatocytes [54, 55]. It transports glutathione conjugates [55, 56]. Relatively little is known about the contribution of MRP5 and MRP6 to the hepatic basolateral excretion of conjugated organic anions, and further studies are needed to evaluate their potential contribution.

Canalicular (Biliary) Excretion

ABCB1 (MDR1 or *P*-Glycoprotein or Pgp)

The multidrug resistance protein 1 (MDR1) is probably the most studied ATP binding cassette transporter due to its notorious role in limiting the oral bioavailability of bulky lipophilic and organic cations [57, 58]. MDR1 is highly expressed at the canalicular membrane of the hepatocytes. As an export transporter capable of efflux against concentration gradient, the predicted function for MDR1 is the extrusion of its substrates from the hepatocyte into the bile. MDR1 transports an extremely wide variety of chemically and structurally diverse compounds including a large number of cytotoxic drugs and their metabolites. MDR1 substrates are usually organic molecules ranging in size from about 200 Da to over 1,000 Da. The list of substrates and inhibitors for MDR1 is growing and the reader is directed to the various reviews for further information [59–64].

In the liver, Pgp contributes to the first-pass elimination of drugs after oral administration, and/or forms an essential pathway for drug biliary excretion after parenteral administration [59]. Nevertheless, the hepatic expression and function of this transporter are highly interindividually and intraindividually variable, which can produce unpredictable changes in drug plasma concentrations and therapeutic effects.

ABCB4/MDR3

ABCB4 codes for a 140 kDa membrane protein that is localized to the canalicular membrane of hepatocytes. MDR3 functions as a flippase, capable of flipping phosphatidyl choline molecules from the inner leaflet of a bilayer to the outer leaflet [65]. ABCB4 appears to play very little role in the direct transport of Phase II conjugates.

Defect in ABCB4 function causes the production of bile with low phospholipid content, increased lithogenicity and high detergent properties leading to cholestasis, referred to as progressive intrahepatic cholestasis type 3 (PFIC III) [66, 67].

ABCB11 (BSEP)

BSEP is a 1,321 amino acid glycol-protein with an apparent molecular mass of 160 kDa [68]. BSEP is expressed at the canalicular plasma membrane of hepatocytes and is responsible for the biliary excretion of unconjugated and conjugated bile acids [68, 69]. While BSEP has not been shown to transport Phase II conjugated drugs, several xenobiotics are known to inhibit BSEP function leading to drug-induced cholestasis and hepatotoxicity [70–72].

Absence of BSEP or nonfunctional mutations in the BSEP gene leads to severe forms of intrahepatic cholestasis [68, 73, 74]. Clinically, cyclosporine A as well as glibenclamide, rifampicin, and rifamycin are competitive inhibitors of BSEP [70, 72, 75, 76]. Other drugs that have been shown to inhibit

BSEP are bosentan, troglitazone, and fluvastatin [70]. Such drugs may precipitate cholestasis in susceptible population, which rapidly resolves after the withdrawal of the drug. Progesterone-sulfate, a key metabolite that increases during pregnancy can result in BSEP dependent cholestasis [76].

BSEP mRNA levels are reduced in individuals with poor drainage as compared to controls (in patients undergoing biliary drainage) [77]. Similarly, in primary biliary cirrhosis, BSEP expression is reduced [68, 78]. In experimental septic models, BSEP mRNA and protein levels were reduced, suggesting that sepsis in patients with gram-negative infections may be associated with BSEP mediated cholestasis [79]. Ursodeoxycholic acid, which is frequently used to treat cholestatic liver disease, was recently demonstrated to stimulate BSEP expression. BSEP expression is also regulated by nuclear receptors; AhR and PXR down regulate BSEP expression while CAR and FXR upregulate BSEP expression [80].

ABCC2 (MRP2)

Human MRP2 is a 190 kDa transmembrane glycoprotein (1,545 amino acid residues) and localizes to the bile canalicular membrane of hepatocytes [81, 82]. MRP2 transports glucuronide, sulfated and glutathione conjugates of both endobiotics and xenobiotics [27, 82–87]. A detailed list of well characterized MRP2 substrates is presented in Table 11.3.

Loss of function mutations in MRP2 is the cause of DJS in humans, a condition which is characterized by conjugated hyperbilirubinemia [88, 89]. This syndrome is an autosomal, recessively inherited disorder characterized by increased concentrations of bilirubin glucuronosides in blood (hyperbilirubinemia) and deposition of a dark pigment in hepatocytes [90, 91]. Many sequence variants in the MRP2 gene have been identified in the human population, but only some of them cause DJS. Among these are sequence variants that result in the absence of a functionally active MRP2 protein. It should be noted, however, that this loss of MRP2 function is usually well tolerated and compensated by the upregulation of other membrane transporters.

In MRP2-deficient rats, the bile flow rate is reduced approximately 50% to that of normal rats, due to the complete absence of bile acid independent bile flow [77]. Accordingly, it is possible that inhibition of MRP2 function results in reduced bile flow rate, which might be clinically significant in diseases such as sclerosing cholangitis, hepatitis C infection, and cholestasis, where MRP2 expression has been demonstrated to be reduced [92].

MRP2 is also expressed in hepatocellular carcinoma. Although the significance of this overexpression is not clear, based on cell culture models, this has been attributed to the acquisition of drug resistance against antitumor drugs [93].

The mechanism of induction of MRP2 has been studied in vitro in cell culture models using cultured human, mouse and rat hepatocytes and in vivo in animal models. In these studies, MRP2 was shown to be regulated by cytokines (IL-1β, TNF-α, and IL-6) and nuclear receptors (AhR, CAR, PXR and FXR) [40, 51, 79, 94]. These results suggest that SNPs in the promoter/enhancer region of MRP2 should also be taken into consideration in accounting for inter-individual differences in MRP2 inter-individual expression levels.

ABCG2/BCRP

BCRP is a 75 kDa protein that localizes to the canalicular membrane of hepatocytes [95]. BCRP transports a wide variety of chemically and structurally diverse compounds including a large number of cytotoxic drugs and their metabolites (Table 11.4). At the canalicular membrane, BCRP is capable of transporting sulfated conjugates of steroids and xenobiotics [95]. The importance of BCRP in biliary excretion is exemplified by the fact that in MRP2 loss of function phenotypes, BCRP inhibitors can significantly alter the biliary excretion of sulfated conjugates [95–99].

The normal function of BCRP is probably to prevent the accumulation of both intracellular and extracellular toxins in cells. Overexpression of this transporter in carcinomas gives rise to the phenomenon of multidrug resistance.

Multiple SNPs have been identified in BCRP, which result in the loss of function phenotypes. Although the role of this

Table 11.4 Human BCRP substrates

Sulfate conjugates	Glucuronide conjugates	Glutathione conjugates
Aminomethylphenylimidazopy-ridine [144]	Estradiol-17β [98, 128, 145]	Leukotriene C4 [98]
Estrone [145, 146]	Estrone-3 [145]	Dinitrophenol [98]
Estradiol [98, 145]	Estradiol-3 [145]	
Dehydroeipandrosterone [98, 145]	4-Methylumbelliferone [98]	
Taurolithocholate [98]	SN-38 [97, 147]	
4-Methylumbelliferone [98]	Aminomethylphenylimidazopyridine [144]	
p-Nitrophenol [98]		

loss of function mutants has been well characterized in multidrug resistance, little has been done with reference to their role in biliary excretion of endogenous conjugates.

ABCG5 and ABCG8

ABCG5 and ABCG8 encode for proteins of 75 and 62 kDa molecular weights, respectively. These proteins function as a single unit [100], as they dimerize to function in transport [100, 101]. They are localized to the canalicular membrane of the liver, and are involved in the transport of cholesterol and other sterols from the liver into bile [101]. Cholesterol and nuclear receptors such as LXR have a significant effect on the regulation of these transporters while deoxycholic acid represses their expression [102, 103]. Mutation in both these genes leads to a disorder characterized by defective transport of plant and fish sterols, and cholesterol (sitosterolemia) [104].

Drug Uptake Transporters in Detoxification

Although drug uptake transporters such as OATs and OATPs can mediate transport across the basolateral membrane into sinusoidal blood, because of their bidirectional nature and their dependence on ion exchange as the driving force for transport, they most likely function as uptake transporters under physiological conditions.

Most of these uptake transporters belong to the solute carrier family (SLC), which currently includes 43 families and 298 transporter genes. Among the SLC family members, only the major liver detoxification related members will be discussed in this chapter. A detailed description of transporters of other organs is beyond the scope of this chapter.

SLC10A1; Na+/Taurocholate Co-Transporting Polypeptide

The human NTCP is a 349-amino acid polytopic membrane glycoprotein with an apparent molecular mass of approximately 56 kDa [105]. NTCP is expressed at the basolateral (sinusoidal) plasma membrane of human hepatocytes, and transports conjugated (taurocholate and taurochenodeoxycholate) and unconjugated bile salts from the portal blood into the hepatocytes [105]. Thus NTCP promotes efficient extraction of bile salts from portal blood to keep plasma concentrations at a minimum and plays an important role in enterohepatic circulation. The driving force for NTCP-mediated transport is provided by the inwardly directed Na^+ gradient maintained by the basolateral Na^+, K^+-ATPase, as well as the negative intracellular potential [105].

An inherited defect in NTCP has not yet been reported. However, NTCP mRNA and protein expression is decreased in various animal models of cholestasis and liver disease [106, 107]. In addition, recent studies have also shown similar decreased NTCP expression levels in percutaneous liver biopsies of patients with cholestatic liver disease [78].

Pharmacologically, NTCP is ideally suited to target drugs to the liver, because its expression is restricted to hepatocytes. Thus, any kind of liver-specific disease is a potential target for treatment with drugs that are conjugated to bile salts, but which, in an unconjugated form, do not home efficiently to the liver. The feasibility of such an approach has been shown for several compounds such as antisense oligonucleotides, amino acids and peptides as well as for cytostatic drugs, which keep their potency even after conjugation to bile salts [108, 109].

Among the nuclear receptors, AhR, CAR, and PXR all downregulate the expression of NTCP [80, 94].

Organic Anion Transporting Polypeptides (SLC21/SLCO/OATP)

The family of organic anion transporting polypeptides contains over 52 members that mediate the sodium independent transport of a wide range of amphipathic organic compounds including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides, numerous drugs, and other xenobiotic substances. Among the members of this family OATP1A2, OATP1B1, OATP1B3, and OATP2B1 are expressed in the liver and thus appear to play an important role in liver detoxification.

The human Organic anion transporting polypeptide 1A2 is a glycoprotein of 670 amino acids [110]. In the liver, OATP1A2 is expressed at the basolateral (sinusoidal) plasma membrane of human hepatocytes. OATP1A2 transports a wide range of amphipathic organic compounds including bile salts and BSP, steroid conjugates and thyroid hormones [111]. OATP1A2 mediated transport is Na+-independent and the driving force for transport appears to be coupled to cellular efflux of bicarbonate, glutathione, and/or glutathione conjugates [111].

The human OATP1B1 encodes a 691-amino acid glycoprotein with an apparent molecular mass of 84 kDa [111–113]. OATP1B1 expression appears to be restricted to the basolateral (sinusoidal) plasma membrane of hepatocytes and transports a spectrum of compounds including bile salts, conjugated and unconjugated bilirubin, BSP, steroid conjugates, and thyroid hormone [112–116]. Thus, OATP1B1 exhibits a similar wide-substrate spectra as the OATP1A-subfamily members. The apparent exclusive expression of OATP1B1 in human liver suggests that it plays a crucial role in the hepatic clearance of amphipathic organic compounds.

Transcriptional expression of OATP1B1 in primary sclerosing cholangitis patients is about 50% lower than the healthy control livers [92]. In addition, basal expression of human OATP1B1 is dependent on the liver-enriched transcription factor HNF-1α [117].

OATP1B3 encodes a glycoprotein of 702 amino acids with an apparent molecular mass of 120 kDa [118, 119]. OATP1B3 shares 80% amino acid identity to OATP1B1. Like OATP1B1, OATP1B3 is predominantly, if not exclusively, expressed at the basolateral plasma membrane of hepatocytes. The spectrum of transport substrates of OATP1B3 is similar to OATP1B1 and includes bile salts, monoglucuronosyl bilirubin, BSP, steroid conjugates, and thyroid hormone [111]. OATP1B3 has been shown to be expressed in various human cancer tissues as well as in different tumor cell lines derived from gastric, colon, pancreas, gallbladder, lung, and brain cancers [118]. The pathobiological significance of this expression remains to be investigated. Hepatic expression of OATP1B3 appears to depend on the nuclear transcription factors HNF-1α, as well as on the bile acid nuclear receptor FXR [117]. These findings indicate that induction of OATP1B3 gene expression by bile acids could serve to maintain hepatic extraction of xenobiotics and peptides under cholestatic conditions.

OATP2B1 is a 709 amino acid protein that localizes to the basolateral membrane of hepatocytes [120]. OATP2B1 substrates include BSP, estron-3-sulfate and dehydroepiandrosterone sulfate [111, 112, 114]. At low pH, OTAB2B1 is also known to transport taurocholate, fexofenadine, statins, glibenclamide, and the loop diuretic M17055 [111].

Conclusion

Detoxification function of the liver is highly complex involving hundreds of different enzymes with unique substrate specificity, regulation, and activity. The drug metabolism function of the liver is also extremely intertwined with metabolism of endobiotics, which provides insights into evolutionary mechanisms involved in fine-tuning the detoxification functions of the liver.

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Chapter 12
Bile Acid Metabolism

John Y.L. Chiang

Introduction

Bile acids are physiological agents that facilitate biliary secretion of lipids and metabolites, and intestinal absorption of fat and nutrients. Bile acids are also signaling molecules that activate nuclear receptors and cell signaling pathways to regulate hepatic lipid metabolism and homeostasis. Bile acids are synthesized from cholesterol in the liver, stored in the gallbladder, secreted to the intestine and reabsorbed in the ileum, and transported back to the liver. This physiological process of enterohepatic circulation of bile acids is regulated by a complex network of membrane transport systems in the hepatocytes, cholangiocytes, and enterocytes. Bile acid-activated nuclear receptors, farnesoid X receptor (FXR), pregnane X receptor (PXR), and vitamin D receptor (VDR), play critical roles in regulation of key regulatory genes involved in bile acid metabolism in the liver and intestine. The bile acid receptors also regulate lipid, glucose, drugs, and energy metabolism. Inborn errors of bile acid synthesis cause neonatal liver diseases. Disruption of bile flow causes cholestatic liver diseases. Bile acids are therapeutic agents that have great potential for treating cholestasis, gallstone, fatty liver, cardiovascular diseases, obesity, and diabetes in humans.

Bile acids are the end products of cholesterol catabolism [1–3]. Please see Chap. 10 for an independent discussion on fat metabolism in the liver. Conversion of cholesterol to bile acids accounts for daily turnover of more than 90% cholesterol. Bile acid synthesis generates bile flow and provides the driving force for biliary excretion of bile acids, phospholipids, cholesterol, and other metabolites. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the major primary bile acids synthesized in human livers. These bile acids are conjugated with taurine and glycine, and secreted into bile. Highly complex and sophisticated membrane transport systems located in the polarized membranes of hepatocytes and enterocytes, control the rate of bile acid synthesis and absorption, distribution of nutrients, and disposal of toxic metabolites and xenobiotics [4].

Recent discovery of bile acid-activated receptors has revealed the molecular mechanisms of bile acid feedback regulation and metabolic control in the enterohepatic system (review in [5–7]). Bile acids are endocrine hormones that activate FXR to regulate bile acids, lipoprotein, and triglyceride metabolisms [8–10]. Recent studies have also uncovered an important role for FXR in regulation of glucose and energy metabolism [9, 10]. Bile acids also have nongenomic function of activating cell-signaling pathways in the liver and other tissues [11–13]. These cell-signaling pathways crosstalk with nuclear receptor signaling to regulate bile acid synthesis [11], lipid and glucose metabolism [12, 14], and apoptosis [15]. An important autocrine function of bile acids is activation of a G protein-coupled receptor TGR5 in brown adipose tissues to stimulate energy metabolism [9]. This chapter will cover bile acid metabolism, its regulation by nuclear receptor and cell signaling; the role of bile acids in regulation of lipid, glucose, and energy metabolism; the molecular pathology of the enterohepatic system; and bile acids as therapeutic agents for human liver diseases.

Bile Acid Metabolism

Bile Acid Structures

Bile acids (or bile salts) are derived from cholesterol. Figure 12.1 shows the structures of cholesterol and CA. Cholesterol is a C_{27}-sterol consisting of the steroid nucleus with four fused carbon rings; three six-carbon rings (A, B, and C) and a five carbon ring (D); and an eight-carbon side-chain (Fig. 12.1). The A ring has a 3β-hydroxyl group and the B ring has a double bond between carbon 5 and 6. The 8-carbon side chain is attached to the D ring. All "modern" bile acids have 24 carbon atoms. C_{27} bile alcohols and C_{27} bile acids exist in the lower vertebrates [16]. Conversion of cholesterol to bile

J.Y.L. Chiang (✉)
Department of Integrative Medical Sciences, Northeastern Ohio Universities Colleges of Medicine and Pharmacy,
Rootstown, OH, USA
e-mail: jchiang@neoucom.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_12, © Springer Science+Business Media, LLC 2011

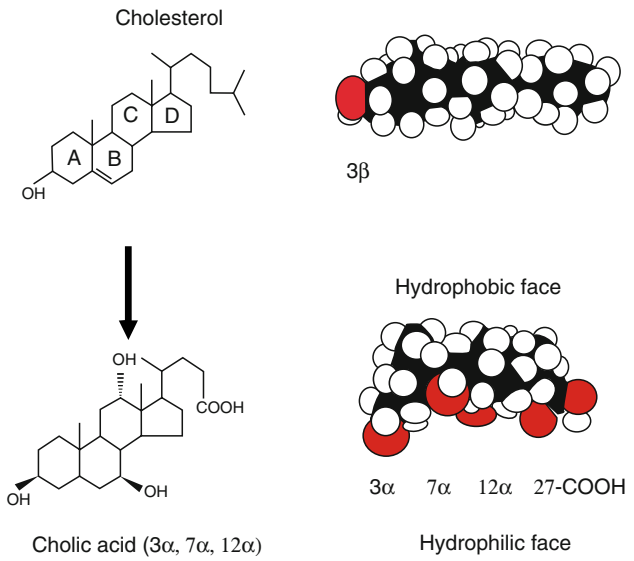

Fig. 12.1 Cholesterol and bile acid structure. The chemical structures of cholesterol and cholic acids are illustrated on the *left*, and the space filling models of cholesterol and cholic acid on the *right*. Oxygen atoms in hydroxyl groups are colored in *red*. The 5β-hydrogen is in a cis-configuration at the A/B junction, and introduces a kink in the planar steroid nucleus in cholesterol to a concaved bile acid structure that has all hydroxyl groups facing in one direction to form a highly hydrophilic face opposing the highly hydrophobic face consisting of carbon skeleton

acids involves hydroxylation reactions, saturation of the double bond, epimerization of the 3-hydroxyl group, and oxidative cleavage of a 3-carbon unit from the side chain. The 3-hydroxyl group in all bile acids has a α-configuration. Most bile acids have a 5β-hydrogen group and a kink along the A and B junction (cis-configuration) in the steroid nucleus (Fig. 12.1). All hydroxyl groups and the carboxyl group in bile acids are faced to one side of the molecule forming a hydrophilic face opposing the highly hydrophobic face consisting of the carbon skeleton. Thus bile acids are amphipathic molecules with detergent properties.

Bile Acid Synthesis in the Liver

An average 70 kg man synthesizes about 600–900 mg cholesterol per day and uptakes about 300–500 mg cholesterol from the diet [17]. This input of about 1–1.5 g cholesterol contributes to a total cholesterol pool of about 100 g. About 500–600 mg of cholesterol is converted to bile acids and another 600 mg of cholesterol is excreted into bile daily. Only about 85 mg cholesterol is incorporated into cell membranes and 50 mg cholesterol is converted to steroid hormones. A constant cholesterol pool is maintained by these input and output mechanisms. Thus bile acid synthesis is the predominant pathway for cholesterol catabolism and plays a critical role in maintaining cholesterol homeostasis in our body. The liver is the only organ that has all enzymes needed for the synthesis of bile acids. There are two major bile acid biosynthetic pathways [1]. The neutral bile acid pathway (or classic pathway) is initiated by cholesterol 7α-hydroxylase (CYP7A1), a cytochrome P450 enzyme located in the endoplasmic reticulum of the liver. The acidic pathway (or alternative pathway) is initiated by sterol 27-hydroxylase (CYP27A1), a cytochrome P450 enzyme located in the mitochondria of most tissues. The neutral pathway is the major pathway that synthesizes two primary bile acids, CA and CDCA in human livers (Fig. 12.2). The acidic pathway contributes up to 9% of total bile acid synthesis in human livers [18], but may be quantitatively important in liver diseases and the neonates. Cholesterol can be hydroxylated to form oxysterols in liver and other tissues. Three hydroxylases may be involved in oxysterol synthesis. In macrophages and other tissues and organs, mitochondrial CYP27A1 converts cholesterol to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. In the liver, a microsomal sterol 25-hydroxylase converts cholesterol to 25-hydroxycholesterol. In the brain, a specific microsomal sterol 24-hydroxylase (CYP46A1) converts cholesterol to 24-hydroxycholesterol, an abundant cerebral-sterol in the brain. A nonspecific oxysterol, 7α-hydroxylase (CYP7B1) then converts 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid to 3β, 7α-dihydroxy-5-cholestenoic acid, and 25-hydroxycholesterol to 5-cholesten-3β, 7α, 25-triol. A brain-specific sterol 7α-hydroxylase (CYP39A1) converts 24-hydroxycholesterol to 5-cholesten-3β, 7α, 24(S)-triol. These oxysterols may be transferred to the liver and converted to CDCA. It has been suggested that transfer of oxysterols from macrophages to the liver to convert to bile acids may be a reverse cholesterol transfer system to protect against atherosclerosis in humans.

The microsomal 3β-Δ^5-C_{27} steroid oxidoreductase (HSD3B7) then catalyzes isomerization of the Δ5, 6 double bond to Δ4, 5 double bond, and dehydrogenation of a 3α-hydroxyl group to a keto group, to form 7α-hydroxy-4-cholesten-3-one (C4), a common precursor for CA and CDCA. The serum C4 levels parallel the CYP7A1 activity in the liver and have been used as a marker for bile acid synthesis [19]. C4 undergoes isomerization and saturation by two reductases. First Δ^4-oxysteroid-5β reductase (aldos-keto reductase 1D1, AKR1D1) reduces the Δ^{4-5} double bond by insertion of a hydrogen at 5β-position, which change the trans- to cis-confirmation at the A/B junction of the steroid nucleus in C4. Then AKR1C4 (3α-hydroxyl steroid dehydrogenase) reduces the 3-keto group to a 3α-hydroxyl group to form 5β-cholestan-3α, 7α-diol. CYP27A1 then oxidizes the C27-carbon of the steroid side chain to a carboxyl group to form 3α, 7α-dihydroxy-5β-cholestanoic acid (DHCA), a precursor of CDCA. A microsomal cytochrome P450 enzyme, sterol 12α-hydroxylase (CYP8B1) hydroxylates C4

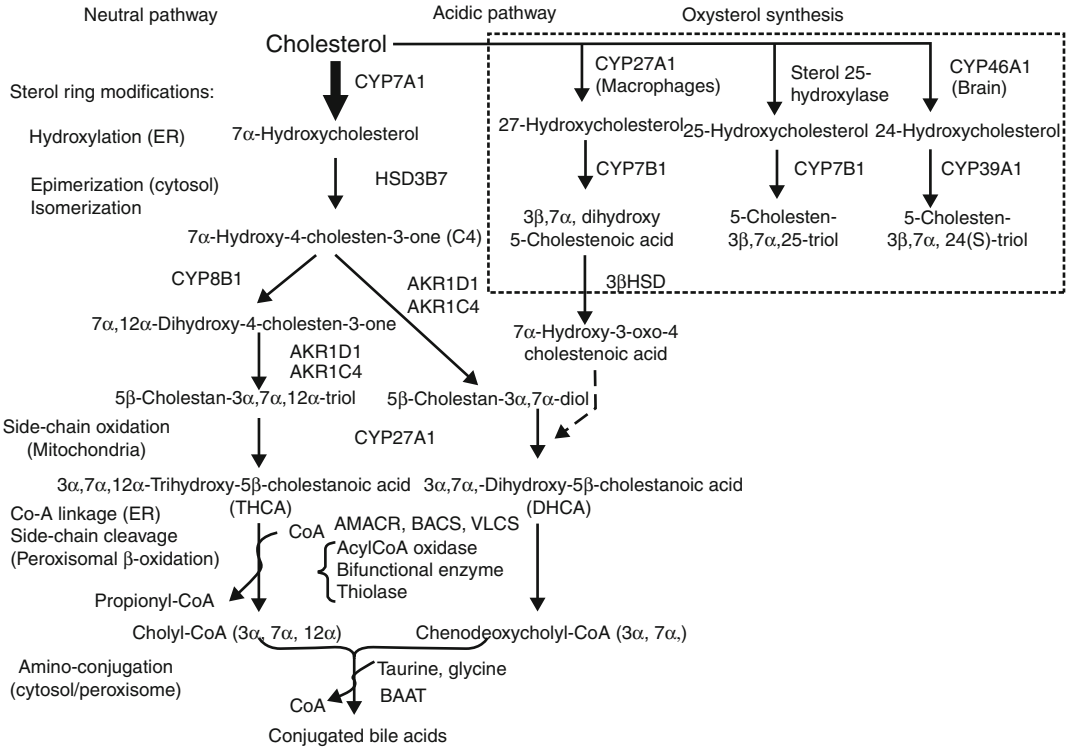

Fig. 12.2 Bile acid biosynthetic pathways. Two bile acid biosynthetic pathways are shown. The neutral (or classic) pathway is initiated by cholesterol 7α-hydroxylase (CYP7A1) located in the endoplasmic reticulum of the liver, whereas the acidic (or alternative) pathway is initiated by mitochondrial sterol 27-hydroxylase (CYP27A1). There are three hydroxylases that convert cholesterol to oxysterols. In macrophages and other tissues and organ, CYP27A converts cholesterol to 27-hydroxycholesterol. In the liver, microsomal sterol 25-hydroxylase converts cholesterol to 25-hydroxycholesterol. In the brain, sterol 24-hydroxylase (CYP24) converts cholesterol to 24-hydroxycholesterol. A non-specific oxysterol 7α-hydroxylase (CYP7B1), in most tissues hydroxylate 27 and 25-hydroxycholesterol, and a brain-specific oxysterol 7α-hydroxylase (CYP39A1) hydroxylate 24-hydroxycholesterol. These oxysterols could be converted to CDCA if transported to the liver. In the liver, sterol 12α-hydroxylase (CYP8B1) is involved in the synthesis of cholic acid (CA). 3β-hydoxysteroid dehydrogenase (3βHSD), aldos-keto reductase 1D1 (AKR1D1), and AKR1C1 catalyze isomerization and saturation of the steroid ring. Then CYP27A1 catalyzes steroid side-chain oxidation to form cholestanoic acids, THCA and DHCA. Bile acid-Co-A synthase (BACS) in ER, or very long-chain Co-A synthase (VLCS) in ER, or peroxisomes, ligate Coenzyme A to the carboxyl groups. Bile acid thioesters are transported into peroxisomes, where α-methylacyl-CoA racemase (AMACR) converts the methyl group from 25 (R) to 25(S) conformation, and three peroxisomal β-oxidation enzymes, branched-chain acyl-CoA oxidase, D-bifunctional enzyme, and thiolase (or sterol carrier protein x) catalyze oxidative cleavage of a propionyl group from the steroid side-chain to form cholyl-CoA and chenodeoxycholyl-CoA. Cytosolic or peroxisomal bile acid: amino-acid transferase (BAAT) catalyzes conjugation of amino acids, glycine or taurine to the carboxyl group of Cholyl-CoA and chenodeoxycholyl-CoA to form conjugated bile acids and release a propionyl-CoA

to 7α, 12α-dihydroxy-4-cholesten-3-one, leading to the synthesis of 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid (THCA), a precursor of CA. A microsomal bile acid, Coenzyme-A synthase (BACS) or peroxisomal (or ER) very long-chain CoA synthase (VLCS) ligates a Co-A group to THCA and DHCA. These two CoA thioesters enter the peroxisomes, where a α-methylacyl-CoA racemase (AMACR) converts the methyl group from 25 (R) to 25(S) conformation, and three peroxisomal β-oxidation enzymes, branched-chain acyl-CoA oxidase, D-bifunctional enzyme, and thiolase (or sterol carrier protein x) catalyze oxidative cleavage of a propionyl group from the steroid side-chain to form cholyl-CoA and chenodeoxycholyl-CoA. Cytosolic or peroxisomal bile acid: Amino-acid transferase (BAAT) catalyzes conjugation of amino acids, glycine or taurine to the carboxyl group of Cholyl-CoA and chenodeoxycholyl-CoA to form conjugated bile acids, and release a propionyl-CoA.

Bile Acid Biotransformation

In humans, most bile acids are amino-conjugated at the carboxyl group (amidation) with the ratio of glycine- to taurine-conjugates of about 3:1. Conjugation of bile acids increases ionization and solubility at acidic pH, prevents Ca^{+} precipitation, minimizes passive absorption, and prevents cleavage by pancreatic enzymes in the intestine. Bile acids are sulfated at the 3α-position by sulfotransferase 2A1 (SULT2A1), amidated at the carboxyl group by BACS and

BAAT, and glucuronidated at 3α, 6- and/or 24-positions by UDP-glucuronosyl *N* -transferases, (UGT1A1, 2B4 and 2B7). In the intestine, conjugated-CA and CDCA are first deconjugated. Then, bacterial 7α-dehydroxylase converts CA and CDCA to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. DCA and LCA are the secondary bile acids (damaged bile acids) generated in the intestine. LCA is highly toxic. Small amounts (1%) of LCA circulated to the liver are rapidly conjugated by sulfation and amidation to form di-conjugated-LCA, which is rapidly excreted into bile. Sulfation is the major pathway for detoxification of hydrophobic bile acids in humans [20]. Mice, but not humans, are able to re-hydroxylate LCA at 7α-position to form CDCA, and hydroxylate at 6α-position to form hyodeoxycholic acid (HDCA, 3α, 6α). CDCA can be metabolized to other soluble bile acids in different species. CDCA is hydroxylated at 6-carbon position by Cyp3a11 to form hyocholic acid (3α, 6α, and 7α) in humans and pigs. CDCA also can be epimerized to UDCA (3α, 7β) in humans and bears. Cyp3a11 and epimerase converts CDCA to 6α- and 6β-muricholic acids (3α, 6α/β, 7β), the predominant hydrophilic bile acids in mice. Hydroxylation at the 6α/β or 7β-position renders bile acid highly soluble and nontoxic.

Enterohepatic Circulation of Bile Acids

Bile acids synthesized in the liver are immediately secreted into bile, reabsorbed in the intestine and transported back to the liver. The enterohepatic circulation of bile acids not only plays an important role in absorption and transport of nutrients and disposal of toxic metabolites and xenobiotics, but also serves as a feedback control of bile acid synthesis. Biliary secretion of bile acids generates bile flow to secrete conjugated-bilirubin and other metabolites. Small amounts of bile acids may spill over into sinusoidal blood, reabsorbed when passing through the renal tubules in the kidney, and are circulated back to the liver through systemic circulation. Some bile acids secreted in the bile duct are reabsorbed in the cholangiocytes (bile duct epithelial cells) and recycled back to hepatoytes (the cholangiohepatic shunt). Bile acids are stored in the gallbladder. After each meal, cholecystokinin secreted from the intestine stimulates gallbladder contraction to empty bile acids into the intestinal tract. When passing down the intestinal tract, small amounts of unconjugated bile acids are reabsorbed in the upper intestine by passive diffusion. Most bile acids (95%) are reabsorbed in the brush border membrane of the terminal ileum, trans-diffused across the enterocyte to the basolateral membrane, secreted into portal blood circulation to liver sinusoid, and taken up into hepatocytes. In the colon, DCA is reabsorbed and recycled with CA and CDCA to the liver. A bile acid pool of ~3 g consisting of ~ 40% CA, 40% CDCA, 20% DCA, and trace amount of LCA (1–4%), is recycled 4–12 times a day. Bile acids lost in the feces (~0.5 g/day) are replenished by *de novo* synthesis in the liver to maintain a constant bile acid pool. Interruption of the enterohepatic circulation of bile acids causes malnutrition and cholestatic liver diseases in humans.

Regulation of Bile Acid Metabolism

Bile acid synthesis is inhibited by bile acids returning to the liver via enterohepatic circulation by inhibiting CYP7A1, the first and the only rate-limiting enzyme of the classic pathway. Bile acids inhibit CYP7A1 gene transcription and mRNA stability [1]. Bile acids also inhibit CYP8B1, which determines the ratio of CA to CDCA, and thus the hydrophobicity index of bile acids in human bile. The promoters of CYP7A1 and CYP8B1 genes consist of several AGGTCA-like repeating sequences that are recognized by nuclear receptors [1, 6]. Nuclear receptors are ligand-activated transcription factors that play important roles in embryogenesis, development, and metabolic regulation. These highly conserved sequences confer bile acid inhibition, and are referred as the bile acid response elements (BARE) in the CYP7A1 and CYP8B1 gene promoters [11]. The mouse BARE-I sequence (-73 to -55) contains a direct repeat separated by four nucleotides (DR4), which binds a nuclear receptor liver X receptor (LXR) to induce *Cyp7a1* gene transcription [21]. Feeding a high cholesterol diet to wild type mice stimulates bile acid synthesis to excrete excess dietary cholesterol, but causes massive accumulation of cholesterol in the liver of *Lxr* knockout mice [22]. However, the DR4 sequence is altered in the human *CYP7A1* promoter and LXRα does not bind to the human *CYP7A1* gene [23]. Thus mice and rats are able to convert excess dietary cholesterol to bile acids, whereas humans cannot. The BARE-II (-149 to -118) has an 18-nucleotide sequence that is completely conserved in different species. The BARE-II contains a DR1 sequence, which binds an orphan nuclear receptor, hepatocyte nuclear factors 4α (HNF4 α, NR2A1). HNF4α interacts with a nuclear receptor coactivator, peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α), and trans-activates the *CYP7A1* gene.

Bile acids activate three nuclear receptors, FXR (NR1H4) [24], PXR (NR1I2), and VDR (NR1I1) [25]. FXR is activated by free and conjugated-bile acids; the hydrophobic bile acid CDCA is the most efficacious bile acid ligand of FXR (EC50 = ~10 μM), followed by LCA, DCA, and CA, while UDCA and MCA do not activate FXR. LCA and its metabolite 3-keto-LCA are the most efficacious bile acid ligands for both VDR and PXR (EC50 = ~100 nM). Bile acids also indirectly activate the constitutive androstane receptor (CAR). These nuclear receptors coordinately regulate bile

acids, lipoproteins, drugs, glucose, and energy metabolism [7, 9]. Bile acids also have been shown to activate several cell-signaling pathways, which regulate bile acid synthesis, glucose, and energy metabolism [12]. Thus, bile acid activation of nuclear receptors and cell signaling pathways converge to coordinately regulate a complex network of cellular metabolisms (see recent reviews in [8–10, 26]).

Regulation by Bile Acid Receptors

FXR is a master regulator of bile acid metabolism: Bile acid-activated FXR plays a critical role in regulation of bile acid synthesis in the liver, biliary bile acid secretion, intestinal bile acid absorption, and hepatic uptake of bile acids (Fig. 12.3). Thus FXR regulates bile acid metabolism and the enterohepatic circulation of bile acids. FXR activates target gene transcription mainly by binding to an inverted repeat with one-base spacing (IR1) in the gene promoter. *Fxr* knockout mice have increased bile acid synthesis and Cyp7a1 expression suggesting FXR mediating bile acid inhibition of Cyp7a1 [27]. FXR does not bind to the CYP7A1 and CYP8B1 gene promoter. The negative effect of FXR is through an indirect mechanism to be described below. FXR induces BACS and BAAT gene transcription by binding to the FXR response elements in the genes. FXR induces a bile salt export pump (BSEP, an ATP binding cassette transporter, ABCB11) located in the canalicular membrane of hepatocytes. BSEP is the principal bile acid efflux pump, which utilizes ATP hydrolysis to excrete conjugated-bile acids against a biliary bile acid concentration about 1,000-fold higher than in hepatocytes. FXR also induces a multidrug resistant protein 3 (MDR3), which effluxes phosphatidylcholine

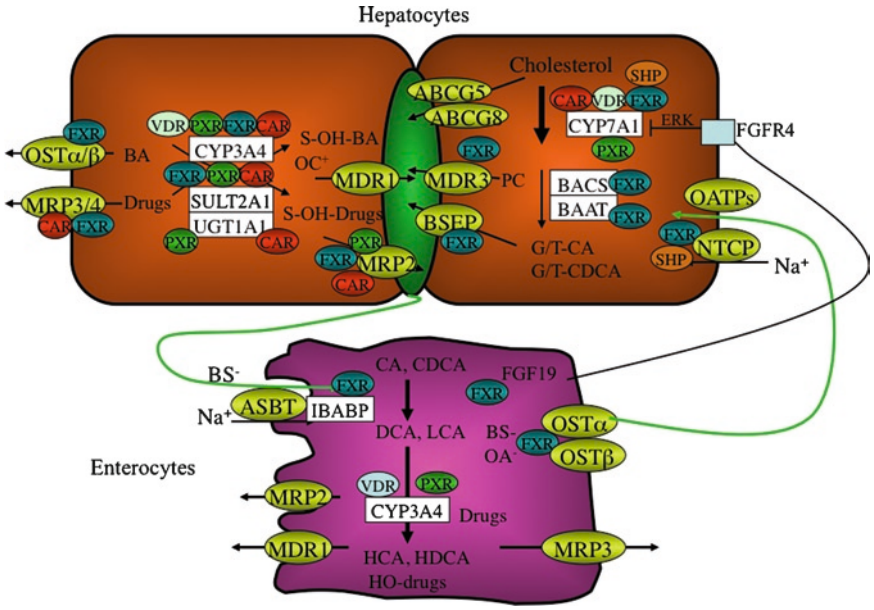

Fig. 12.3 Bile acid-activated nuclear receptors regulate enterohepatic circulation of bile acids. Major bile acid transporters in human hepatocytes and enterocytes are shown. Enzymes and transporters regulated by FXR, SHP, PXR, VDR, and CAR are indicated. In hepatocytes, bile acid activates FXR, VDR, PXR, and CAR to inhibit *CYP7A1* gene transcription. FXR induces SHP to inhibit CYP7A1. In enterocytes, FXR induces intestinal hormone FGF19, which is circulated to hepatocytes to activate FGFR4 signaling to inhibit CYP7A1 via activation of the ERK1/2 pathway. FXR induces bile acid-Co-A synthase (BACS), which links a Co-A to cholestanoic acid for peroxisomal oxidative cleavage of a three carbon-unit to form C24 bile acids. FXR induces bile acid: amino acid transferase (BAAT) to conjugate glycine or taurine to form conjugated bile acids. Conjugated CA and CDCA are excreted into the bile via BSEP. FXR coordinately induces bile salt expert pump (BSEP) to efflux bile acids and MDR3 to efflux phosphatidylcholine to bile. Bile acids also facilitate cholesterol efflux by ABCG5/ABCG8. FXR, PXR, CAR, and VDR induce phase I drug oxidation enzyme CYP3A4, the major drug-metabolizing enzyme in liver and intestine. These receptors also induce phase II drug conjugation enzymes, UDP-glucuronosyl transferase 1A1 (UGT1A1) and sulfotransferase 2A1 (SUL2A1). This drug-metabolizing system detoxifies bile acids and metabolites in the liver and intestine. FXR, PXR, and CAR also induce phase III drug transporter multidrug resistant protein 2 (MRP2), which effluxes glucuronidated- and sulfated-bile acids, organic anions, and drugs into bile. In the sinusoidal membrane, FXR, PXR, and CAR induce MRP3/4 to efflux bile acids and metabolites as an adaptive response to cholestasis. FXR also induces organic solute transporter α/β dimmer (OSTα/β) to efflux bile acids into sinusoidal blood. In enterocytes, bile acids are reabsorbed by apical sodium-dependent bile salt transporter (ASBT) in the apical membrane. FXR induces IBABP, which binds bile acids. In the basolateral membrane, OSTα/β effluxes bile acids into portal circulation. MRP3 may be induced by FXR as an adaptive response to cholestasis. Bile acids returned to hepatic sinusoid are taken up mainly by Na+-taurocholate cotransport peptide (NTCP), and also by Na+-independent organic anion transport proteins (OATPs). FXR inhibits NTCP via SHP to protect hepatocytes as a feedback control of bile acid influx. Many of these membrane transporters (ASBT, OSTα/β, MRP2/3) also are present in cholengiocytes for reabsorption of bile acids, and in renal proximal tubule cells for reabsorption of bile acids from blood circulation and excretion of hydrophilic bile acids

(Fig. 12.3). FXR also induces multidrug resistance related protein 2 (MRP2, ABCC2), which effluxes glucuronidated and sulfated bile acids, organic anions and drugs [28]. ABC-half transporters, ABCG5 and ABCG8 heterodimer may be responsible for biliary excretion of cholesterol. In the gallbladder, bile acids form mixed micelles with phosphatidylcholine and cholesterol. This allows storage of bile acids in high concentrations (mM) and prevents cholesterol from precipitation in the gallbladder. In the terminal ileum, conjugated bile acids are reabsorbed by apical sodium-dependent bile salt transporter (ASBT) located in the brush border membrane of the enterocytes (Fig. 12.3). Once inside the enterocytes, bile acids bind to the ileum bile acid binding protein (IBABP), which is highly induced by FXR [29]. Bile acids are excreted into portal circulation by the organic solute transporter α and β dimer (OSTα/β) located in the basolateral membrane [30, 31]. OSTα/β appears to be the major bile acid efflux transporter in the intestine [32]. FXR induces OSTα/β gene transcription [33]. Bile acids are circulated via portal blood to hepatocytes, where a sinusoidal Na^+-dependent taurocholate cotransport peptide (NTCP) uptakes bile acids into hepatocytes. FXR inhibits NTCP gene transcription [34]. Thus, FXR plays a critical role in coordinated regulation of bile acid synthesis, biliary bile acid secretion, intestinal bile acid reabsorption and secretion, and bile acid uptake into hepatocytes. Defective regulation of these FXR target genes impairs enterohepatic circulation of bile acids, and causes cholestatic liver diseases (see Sect. "Molecular Pathology of the Enterohepatic System", below) [35–38].

The liver initiated-FXR/SHP/LRH-1 pathway. FXR inhibits *CYP7A1* gene transcription by indirect mechanisms. It is thought that bile acid-activated FXR inducts the small heterodimer partner (SHP) to inhibit *CYP7A1* and *CYP8B1* gene transcription [39, 40]. SHP is a negative nuclear repressor that has no DNA-binding domain. SHP inhibits the transactivating activity of liver related homologue-1 (LRH-1, also known as α-fetoprotein transcription factor, NR5A2), and results in inhibiting CYP7A1 gene transcription [39, 40]. SHP also interacts with HNF4α to block HNF4α interaction with PGC-1α and results in inhibiting *CYP7A1* and *CYP8B1* transcription [41, 42]. The FXR/SHP mechanism is supported by the finding that SHP and CYP7A1 mRNA expression levels show an inverse relationship, and CYP7A1 expression and bile acid synthesis is induced in *Shp* knockout mice. Paradoxically, bile acid feeding to *Shp* knockout mice inhibits CYP7A1 expression and bile acid synthesis [43, 44]. More recent studies of conditional liver- or intestine- *Lrh-1* knockout mice shows that CYP7A1 expression is not affected and a FXR agonist GW4064 inhibits CYP7A1 mRNA expression in *Lrh-1* knockout mice [45, 46]. These surprising results suggest that LRH-1 is not required for FXR inhibition of *Cyp7a1* gene transcription. In contrast, *Cyp8b1* gene expression and CA synthesis is abolished in *Lrh-1* knockout mice indicating that the FXR/SHP/LRH-1 mechanism is involved in bile acid inhibition of CYP8B1 and CA synthesis.

The intestine initiated FXR/FGF19/FGFR4 pathway. Another FXR-dependent mechanism is based on the observation that GW4064 induces an intestine hormone, fibroblast growth factor 19 (FGF19), which activates a hepatic FGF receptor 4 (FGFR4) signaling to inhibit CYP7A1 mRNA expression in hepatocytes [47]. A subsequent study shows that FXR induces FGF15, a mouse orthologue of human FGF19 in mouse intestine, and the expression of FGF15 mRNA is inversely correlated to the CYP7A1 mRNA expression levels in mouse livers [48]. Both *Fgfr4−/−* and *Fgf15−/−* mice have increased bile acid pool, fecal bile acid secretion, and CYP7A1 expression [49]. Overexpressing of a constitutively active FGFR4 represses CYP7A1 expression and decreases bile acid pool size in wild type mice [50]. These results are consistent with the role of FGF15/FGFR4 in mediating bile acid inhibition of bile acid synthesis. It appears that FGF15 functions as an enterohepatic signal to activate FGFR4 signaling to inhibit CYP7A1 [48]. Furthermore, GW4064 represses CYP7A1 expression in liver *Fxr* knockout mice, but not in intestine *Fxr* knockout mice [51]. Interestingly, FXR-mediated repression of CYP8B1 expression is more dependent on liver FXR than intestine FXR, and FGF15 represses CYP7A1, but not CYP8B1 expression. These results provide convincing evidence that the intestine FXR, but not liver FXR is required for bile acid inhibition of *CYP7A1* gene transcription. FGF19 activation of FGFR4 signaling requires a membrane-bound glycosidase β-Klotho [52]. In *β-Klotho* knockout mice, bile acid synthesis and secretion and CYP7A1 expression was increased, but CYP8B1 expression was not altered [53]. All these studies support the differential regulation of CYP7A1 by the FXR/FGF19/FGFR4 pathway and CYP8B1 by the FXR/SHP pathway.

Serum FGF19 levels exhibit a diurnal rhythm with two major peaks at 3 and 9 PM, which are ~90–120 min following the peaks of serum bile acids and C4 [54]. These observations are consistent with the concept that FGF19 is secreted from the intestine to blood circulation in response to postprandial efflux of bile acids to inhibit bile acid synthesis in the liver. However, GW4064 and bile acids also are able to induce FGF19 synthesis and secretion in human hepatocytes [55]. The FGF19/FGFR4 signal mainly activates the MAPK/ERK1/2 pathway to inhibit *CYP7A1* gene transcription.

Regulation by Cell Signaling Pathways

Bile acids are signaling molecules that have been shown to activate several cell signaling pathways to regulate bile acid synthesis, lipoprotein, and glucose metabolism in the liver, and energy metabolism in brown adipose tissue and skeletal muscle [11, 12, 14].

PKC

It has been reported that bile acids mimic phorbol esters to activate several protein kinase C isoforms (PKCα, PKCβ, PKCδ, PKCε, PKCξ) in different tissues and cells, and either stimulate or inhibit cAMP production via activation of GPCR, G protein, and adenylyl cyclase (review in [14]). Bile acids may play a role in regulation of glucagon receptor via PKC. Some bile acids induce translocation of phospholipase C (PLC)δ, PKCα and PKCβ1 to cell membranes, and PKCδ to the nucleus to activate cell signaling. Earlier studies show that bile acid (TCA) activated-PKCα activates the MAPK/JNK pathway to inhibit *CYP7A1* gene transcription in rat hepatocytes [56, 57]. Recent studies have shown that the JNK pathway activates and phosphorylates cJun, which phosphorylates HNF4α to inhibit HNF4α trans-activation of the *CYP7A1* and *CYP8B1* genes [6].

Proinflammatory Cytokines

Bile acids are known to activate proinflammatory cytokine (TNFα and IL-1β) release from Kupffer cells [58]. These cytokines may cross sinusoid to hepatocyte to activate cytokine receptors, Toll-like receptors signaling to activate the Rac/MEK4/7/JNK1/2 signal cascade, which phosphorylates HNF4α and reduces its binding to the BAREs, and results in inhibiting *CYP7A1* and *CYP8B1* gene transcription [59, 60].

Growth Factor and Insulin Signaling

It has been reported that conjugated-bile acids activate both AKT and the MAPK/ERK1/2 pathway. Taurocholic acid activation of tyrosine phosphorylation of epidermal growth factor receptors (EGFRs) is blocked by pertussis toxin suggesting that Gαi protein-coupled receptors mediate these bile acid effects [61]. However, there is no direct evidence that TCA binds and activate these GPCRs. Unconjugated bile acids may also activate the AKT pathway through mitochondrial reactive oxidizing species (ROS), which activate EGFRs [62]. AKT phosphorylates and inhibits glycogen synthase kinase 3, and activates glycogen synthesis in rat primary hepatocytes [63]. This implies that bile acids may mimic the insulin action in regulating glucose metabolism by stimulating glycogen synthesis and inhibiting gluconeogenesis. AKT is known to phosphorylate forkhead box transcription factor O1 (FoxO1), which is excluded from the nucleus and results in inhibiting phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase).

TGFβ1 secreted from Kupffer cells activates the Toll-like receptor 4 in hepatic stellate cells (HSC), which also secrets TGFβ1 to activate the TGFβ1 receptor and the Smad signaling pathway in hepatocytes. Bile acids stimulate TGFβ1 expression in hepatocytes and activate the latent TGFβ1 in HSC, and activate HSC. Smad3 recruits histone deacetylase (HDAC) and a repressor mSin3A to inhibit HNF4α activation of CYP7A1 gene transcription [64]. TGFβ1 and bile acids activate the Ras/MAPK/JNK pathway to interact and phosphorylate a tumor suppressor p53 [65, 66], which is known to interact with HNF4α [67] and inhibit HNF4α transactivation of CYP7A1. Another cytokine, hepatocyte growth factor (HGF) has been shown to inhibit bile acid synthesis in primary human hepatocytes [68]. HGF secreted from HSCs activates a tyrosine receptor kinase cMet and rapidly inhibited CYP7A1 expression. HGF induces cJun and SHP, and the ERK/1/2 and JNK pathways. This leads to recruitment of cJun and SHP, but inhibits coactivators PGC-1α and CBP binding to CYP7A1 chromatin, and results in inhibiting CYP7A1 gene transcription.

Role of Bile Acids in Metabolic Regulation

Bile Acids Regulate Lipid Metabolism

It has been known for long time that bile acid sequestrants or interruption of enterohepatic bile acid circulation increases serum triglyceride and VLDP levels, and bile acid treatment reduces serum triglycerides in hyperlipoproteinemic patients [69]. The finding that FXR null mice have increases hepatic bile acids, cholesterol, triglycerides, and pro-atherogenic serum lipoprotein profiles provides the first evidence that bile acid-activated FXR plays a role in regulation of lipid metabolism [27]. It has been shown that FXR induces human apolipoprotein C-II expression [70]. ApoC-II activates lipoprotein lipase involved in lipolysis in adipocytes and muscles. FXR also regulates several other genes involved in lipoprotein and triglyceride metabolism, including ApoA-V, ApoC-III, apoE, PPARα, syndecan-1 [8, 26]. It has been reported that FXR mRNA expression is induced by PPARγ and HNF4α during fasting to reduce serum triglyceride levels by improving triglyceride clearance [71]. The role of FXR in triglyceride metabolism is also supported by the finding that overexpression of FXR by adenovirus-mediated transduction or treatment with GW4064 reduces serum triglyceride levels in wild type and diabetic mice [72]. Another study shows that the FXR/SHP pathway inhibits steroid response element binding protein-1c (SREBP-1c) and other lipogenic genes, and results in reducing serum triglycerides and VLDL production in mice [73].

Bile Acids Regulate Glucose Metabolism

It has been reported that bile acids inhibit PEPCK suggesting that bile acid synthesis and gluconeogenesis are coordinately regulated and linked to the fasting-to-refeeding cycle in mice [74]. This study shows that during starvation CYP7A1 expression is stimulated in parallel to PEPCK, and bile acids inhibit both CYP7A1 and PEPCK gene expression. Another study shows that FXR inhibits PEPCK by SHP-dependent inhibition of HNF4α and FoxO1 [75]. Several recent studies of wild type, FXR null mice and diabetic mice have consistently shown that FXR regulates glucose metabolism and insulin resistance. FXR expression is reduced in diabetic mice and overexpression of FXR or treatment with GW4064 reduces serum glucose and improves insulin sensitivity in wild type and diabetic mice [72]. This is consistent with elevated serum glucose and impaired glucose and insulin tolerance in *Fxr* null mice [76]. Furthermore, activation of FXR increases glycogen levels and induces L-pyruvate kinase, ACC and spot 14 involved in glucose metabolism during fasting-refeeding transition [77]. It should be mentioned that the reported effects of bile acids and FXR on glucose homeostasis in mice are small, and may be limited to the fasting-refeeding cycle [8]. It has been observed that CYP7A1 expression is increased in starvation and parallels the increase of PGC-1α, suggesting that PGC-1α is responsible for fasting and diabetes dependent induction of CYP7A1 in mouse livers [78]. This is in contrast to reduction of bile acid synthesis in fasting and increase of bile acid synthesis during the postprandial period in humans [79]. Also, mice and humans have different diurnal rhythm in bile acid synthesis. In mice, diurnal rhythm of bile acid synthesis shows a peak at midnight and reduces to minimal in mid-day. In contrast, human bile acid synthesis increases in the morning and exhibits two peaks at 1 PM and 9 PM, and decreases at night, regardless of feeding. Glucagon and cAMP induce CYP7A1 in mice, but inhibit CYP7A1 in human hepatocytes [80].

Bile Acids Regulate Energy Metabolism

Fatty acids and glucose are two major fuel molecules in the body. By controlling lipid and glucose metabolism, bile acids play a central role in energy metabolism. However, the mechanism of bile acid regulation of energy metabolism is not clear. The role of FXR in energy metabolism is implicated by the finding that FGF19 increases metabolic rate and reduces adipocity, and reverses diet-induce and leptin-deficient diabetic mice [81, 82]. FGF19 induces hepatic leptin receptor, reduces acetyl-CoA carboxylase 2 expression, and increases fatty acid oxidation. FXR deficiency in mice increases susceptibility to torpor supporting the role that FXR plays in energy metabolism [83]. It has been reported recently that FGF19 suppress insulin-induced fatty acid synthesis in hepatocytes by inhibiting lipogenetic genes and PGC-1α, and inducing SHP [84]. It has been reported that *Shp* null mice show increased energy expenditure, PGC-1α expression, and diet-induced obesity suggesting that SHP is involved in energy production in brown adipocyte tissue by inhibiting PGC-1α expression [85].

Recently, TGR5 has been identified as a bile acid-activated Gαs protein coupled receptor [86, 87]. Conjugated and free bile acids bind and activate TGR5 in the order of TLCA>LCA>DCA>CDCA>CA. TGR5 is expressed in many tissues including gallbladder, spleen, liver, intestine, kidney, skeletal muscle, pancreas, and adipocytes. TGR5 is not expressed in hepatocytes, but has been detected in liver sinusoidal endothelial cells and gallbladder epithelial cells [88, 89]. TGR5 signaling stimulates cAMP, which activates PKA and target gene expression. It has been reported recently that bile acids increases energy expenditure in brown adipose tissue and TGR5-dependent cAMP activation of a type 2 iodothyronine deiodinase (D2) is required for bile acid effect on energy metabolism. D2 converts thyroxine T_4 to the biologically active hormone T_3, which is known to regulate oxygen consumption in mice [90]. In *D2 −/−* mice, bile acids are unable to increase energy production. Knockout of the *Tgr5* gene reduced bile acid pool size by 25%, and female *Tgr5* null mice showed significant weight gain and fat accumulation when fed a high fat diet [91]. These phenotypes are consistent with the role of TGR5 in energy metabolism. However, adult humans have very little brown adipose tissues and the role of TGR5 in energy metabolism in men is not clear. TGR5 regulates energy metabolism in human muscle cells. However, TGR5 levels in human skeletal muscle, adipose tissues, and intestine are very low [87]. The role of TGR5 in energy metabolism in humans remains unclear.

It has been reported that bile acids and TGR5 stimulate glucagon like peptide-1 (GLP-1) secretion in an enteroendocrine cell line STC-1. Knockdown of TGR5 mRNA expression by siRNA reduced GLP-1 secretion suggesting that bile acid induces GLP-1 secretion by TGR5-dependent cAMP production [92]. GLP-1 plays a critical role in regulating glucose homeostasis, appetite, insulin and glucagon secretion in pancreas, and diabetes. Interestingly, *Tgr5* null mice did not develop gallstones when fed a lithogenic diet [93]. These mice have impaired bile acid feedback, and upregulation of CYP7A1 may prevent gallstone formation in *Tgr5* null mice. A study of human gallstone patients reports that TGR5 mRNA and protein are expressed in all patients, and TGR5 mRNA but not protein expression levels are increased in gallstone patients [89]. This study shows localization of TGR5 in the apical membrane and recycling of endosome in gallbladder epithelial cells. TGR5 is colocalized with cystic fibrosis transducer regulator

(CFTR) and ASBT suggesting the coupling of TGR5 in bile acid uptake and chloride secretion.

Molecular Pathology of the Enterohepatic System

Bile acids increase cell proliferation and apoptosis in the liver and intestine [13, 15]. Accumulation of toxic endobiotics and xenobiotics causes damage to cells and organs in the digestive tract. Inborn errors in bile acid metabolism have been identified by analysis of abnormal bile acid metabolites in human patients [94]. Disruption of the enterohepatic circulation of bile acids by obstruction of bile duct causes cholestatic liver diseases [95].

Metabolic Defects in Bile Acid Metabolism

Decrease of bile acid synthesis can be caused by a primary defect in the enzymes involved in the bile acid biosynthetic pathways or secondary to the diseases of the digestive system. Five enzyme defects in bile acid synthesis have been identified.

Defects in Bile Acid Synthesis

A decrease in bile acid synthesis and reduced CYP7A1 activity has been reported in patients with gallstone diseases (review in [96]). Recently, a family of patients with a mutation in the *CYP7A1* gene has been reported [97]. These patients have marked reduction of fecal bile acids (94% lower), hypercholesterolemia, premature atherosclerosis, and gallstone disease supporting the critical role of CYP7A1 in regulating lipid homeostasis. Deletion of double T (1302–1302delTT) in exon 6 causes a frame shift and early termination. The mutant CYP7A1 expressed in 293 cells has no enzymatic activity due to truncation of the C-terminal heme-binding region. Analysis of liver biopsy of one patient showed 70% decrease of CYP7A1 activity and twofold increase of CYP27A1 activity. Analysis of the bile acid composition revealed reduced cholic acid plus deoxycholic acid to CDCA plus LCA ratio. These data are consistent with upregulation of the alternative bile acid biosynthetic pathway as compensation to CYP7A1 deficiency. It is possible that other 7α-hydroxylase activities may be involved in bile acid synthesis in these patients.

An infant patient with a mutation in the *CYP7B1* gene has been reported [98]. This infant has severe neonatal cholestasis, cirrhosis, and liver failure. Analysis of bile acids showed increased urinary sulfate and glycosulfate conjugates of mono-hydroxy-bile acids, 3β-hydroxy-5-cholenoic, and 3β-hydroxy-5-cholestenoic acids indicating a defect in 7α-hydroxylation. Hepatic CYP7B1 activity was undetectable. Sequence analysis revealed a C to T transition resulting in the conversion of R388 in exon 5 to a stop codon. The truncated enzyme was inactive when expressed in 293 cells. The cholestasis caused by CYP7B1 deficiency in this infant suggests that the acidic pathway is quantitatively important in synthesis of bile acid in neonate, and accumulation of hepatotoxic mono-hydroxy bile acids causes cholestatic liver diseases.

The defect in 3β-Δ^5-C_{27} steroid oxidoreductase (HSD3B7) activity is the most common inborn error in bile acid synthesis [99]. Infant patients have hepatitis, cholestatic jaundice, and accumulation of monosulfate and glycine conjugates of 3β, 7 α-dihydroxy-and 3β, 7α, 12α-trihydroxy-5-cholenoic acids (DHCA and THCA). A two-base mutation (Δ1057–1058) in exon 6 of the HSD3B7 gene was identified in an infant with progressive intrahepatic cholestasis [100]. Additional patients with HSD3B7 mutations have been identified more recently [101].

Defects in Δ^4–3-oxysteroid 5β-reductase (AKR) activity have been identified in infants with neonatal hepatitis, cholestasis, and hemochromatosis [102, 103]. Predominant bile acids in these patients are 7α – hydroxy-3-oxo-4-cholenoic and 7α, 12α-dihydroxy-3-oxo-4- cholenoic acids, consistent with a defect in AKR. More recent case studies have identified several patients with AKR1D1 mutations [104, 105].

Mutations of the *CYP27A1* gene cause cerebrotendinous xanthomatosis (CTX), a sterol storage disease [106, 107]. Patients have progressive neurologic dysfunction, xanthoma, and accumulation of cholesterol in the tissues, premature coronary heart disease, and cholesterol gallstones. CTX patients have decreased bile acids, particularly CDCA. The defect in CYP27A1 leads to excessive accumulation of 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one (C4), 5β-cholestane, 3α, 7α, 12α-triol, cholesterol and C27 bile alcohol, cholestanol, which is derived from C4. The reduced bile acid synthesis in CTX patients may upregulate CYP7A1 activity and leads to the accumulation of these intermediates. Since bile acid feedback also inhibits HMG-CoA reductase, lacking bile acids in CTX patients' increases de novo cholesterol synthesis and causes accumulation of cholesterol in serum. CDCA and UDCA have been used to replace bile acids, and inhibit CYP7A1 to reduce bile acid intermediates in CTX patients. Surprisingly, *Cyp27a1* null mice do not develop CTX phenotypes. This is because mouse liver CYP3a11 is able to metabolize 5β-cholestane-3α, 7α, 12α-triol by 25-hydoxylation to form 5β-cholestane-3α, 7α, 12α, 25-tetrol is, which a potent mouse PXR ligand and induces Cyp3a11 expression in mice, but not in humans [108].

Defects in Bile Acid Conjugation

Oxidative cleavage of the steroid side chain by β-oxidation occurs in peroxisomes. A general defect in peroxisome biogenesis impairs bile acid synthesis and accumulation of C27-bile acid intermediates, DHCA and THCA [109]. Zellweger syndrome and related peroxisomal diseases are characterized by neurological disorder, and defective bile acid synthesis [110, 111]. C27-bile acid intermediates are cytotoxic and cause mitochondria dysfunction by inhibiting oxidative phosphorylation, and the respiratory chain [112]. Bile acid therapy has been used to treat peroxisomal diseases [113].

Cholestatic Liver Diseases

Cholestasis is caused by a disruption of bile flow, which results in a lack of bile in the intestine, accumulation of toxic bile acids and other metabolites in the liver, and increased bile acids in the systemic circulation [36]. A detailed account on the molecular basis of this pathology is available in Chap. 32. Obstruction of the bile ducts by tumors or stones, genetic mutations of bile acid transporter genes, and acquired dysregulation of bile transport system by drugs, pregnancy and pathophysiological conditions causes intra- and extrahepatic cholestasis. A detailed review of the molecular mechanisms of cholestasis has been published recently [95].

Hereditary Cholestatic Diseases

The hereditary defects of ABC transporters in the hepatobiliary system in the neonates and children have been identified. The progressive familial intrahepatic cholestasis (PFIC) and benign recurrent intrahepatic cholestasis (BRIC) are autosomal recessive diseases linked to mutations in ATP8B1 (Type 1, PFIC1), BSEP (Type 2, PFIC2), and MDR3 (Type 3, PFIC3). PFIC1 (also known as Byler disease) is linked to mutations in ATP8B1, which codes an aminophospholipid flippase that maintains membrane asymmetry by inward flipping of phosphatidylserine. The mechanism of ATP8B1 in pathogenesis of PFIC1 is unknown. Several studies have shown reduced hepatic FXR expression levels in PFIC1 patients [114, 115]. It has been suggested that a membrane signaling involving PKCξ may regulate FXR activity by phosphorylation and nuclear translocation [116]. In contrast, another recent study reports that hepatic FXR mRNA expression is not altered and ATP8B1 deficiency may disrupt the bile canalicular membrane structure and cause cholestasis [117].

PFIC2 is linked to BSEP mutations [118]. Bile acids are accumulated in the liver and leaked into systemic circulation. This causes hepatitis and liver damage requiring liver transplant in pediatric patients. BSEP mutations and polymorphisms have been linked to intrahepatic cholestasis of pregnancy (ICP) [119–121] and drug-induced liver injury [122].

PFIC3 is linked to mutation of MDR3, a phospholipid flippase in the canalicular membrane [119, 123]. PFIC3 patients have high levels of γ-glutamyl transpeptidase activity (GGT), progressive cholestasis, bile duct damage, and may require liver transplant. Patients have low phospholipids in bile, which are required for mixed micelles formation with bile acids and cholesterol. Without forming mixed micelles, bile acids damage canalicular membrane and cholangiocytes. Mutations of MDR3 may cause cholesterol gallstone diseases and has been linked to ICP [123, 124].

Mutations in MRP2 has been linked to Dubin-Johnson syndrome, a disease characterized by chronic hyperbilirubinemia [125]. MRP2 excretes conjugated bile acids, bilirubin, and other organic anions. Patients have elevated bile acids and cholestasis. MRP2 mutations have been associated with ICP.

Acquired Cholestasis

Genetic polymorphisms and heterozygote mutations of the PFIC1, PFIC2, and PFIC3 genes may increase susceptibility to the acquired cholestasis in adults including ICP, drug-induced liver injury, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC).

Obstructive Cholestasis

In obstructive cholestasis, bile flow is decreased due to blockage of bile duct by gallstones or tumors, and bile acids are accumulated in the liver and are absent in the intestine. A study of obstructive cholestasis in human patients shows that bile acid synthesis is suppressed, but CYP7A1 expression is not altered [126]. Based on the FXR/SHP mechanism, accumulation of bile acids in the liver in cholestatic patients should activate FXR to inhibit CYP7A1 expression. However, based on the FXR/FGF19/FGFR4 pathway, reduced intestinal bile acids should reduce FGF19 in obstructive cholestasis and result in increasing CYP7A1 expression and bile acid synthesis. A recent study reports that CYP7A1 mRNA expression is repressed in human patients with obstructive extrahepatic cholestasis [127]. These investigators found that plasma FGF19 levels were increased, instead of decreased, and FGF19 mRNA are detected in human livers [127]. These observations suggest that bile acids may induce FGF19 in hepatocytes to inhibit CYP7A1 by an autocrine pathway [55].

Activation of FXR protects cholestatic injury by bile acids, drugs, and other xenobiotics. Bile acids induce FXR to

inhibit CYP7A1 and reduce bile acid synthesis. FXR inhibits NTCP and OATPs to inhibit sinusoidal uptake of bile acids. FXR also may upregulate MRP3, MRP4, and OSTα/β in the sinusoidal membrane as an adaptive response to efflux bile acids into systemic circulation in obstructive cholestasis in mice. It remains to be verified that this adaptive response to cholestasis exists in cholestasis patients. Bile acids also play a protective role in controlling bacterial overgrowth in the intestine [128]. Obstruction of bile flow or knockout of the *Fxr* gene in mice increases bacterial growth and mucosal injury in the intestine, and bile acid administration reduces bacterial growth in obstructive cholestasis.

Bile Acids as Therapeutic Agents

The therapeutic potential of bile acids and derivatives for treating liver diseases and metabolic diseases are now well recognized [9, 28, 129].

Bile Acid Displacement and Replacement

CDCA and UDCA have been used for effective gallstone dissolution for many years. However, CDCA and CA have toxic effect in humans, and are difficult to manage. CDCA has been used to treat bile acid deficient patients as a replacement of bile acids in the bile acid pool. CA is less toxic than CDCA, but is converted to DCA, which is more toxic than CA and a colon cancer promoter. CA is more efficient in intestinal absorption of cholesterol than other bile acids, and may cause gallstone formation and hypercholesterolemia in human patients [130]. UDCA is a highly soluble, nontoxic bile acid, and has been approved for gallstone dissolution and PBC. UDCA has been used in traditional Chinese medicine for treating digestive disease for several centuries. UDCA reduces the cytotoxicity of circulating bile acid pool, protects cholangiocytes, stimulates hepatobiliary secretion, and inhibits liver cell apoptosis [15]. UDCA may also activate PXR and induce PXR target genes, CYP3A4, SULTs, UGTs, BSEP, MDR3, and MRP4. Bile acid sequestrants, cholestyramine, and cholestipol have been used for gallstone dissolution and lipid lowering in humans. These drugs bind bile acids in the intestine and interrupt enterohepatic circulation of bile acids, and result in stimulating bile acid synthesis, increasing LDL receptors, and in reducing serum cholesterol levels. Cholestyramine and colesevelam also have glucose-lowering effect [131]. Colesevelam has been approved for improving glycemic control for type 2 diabetes [132].

FXR Agonists for Cholestasis, Gallstone, Fatty Liver and Cardiovascular Disease

The therapeutic potential of FXR agonists for metabolic diseases has been recognized. A specific FXR agonist GW4064 has been widely used to study the function of FXR and to identify FXR target genes. GW4064 reduces serum triglycerides and cholestasis in mice. However, this FXR agonist has poor bioavailability and is not a suitable therapeutic drug. A potent synthetic bile acid derivative, 6-ethyl-CDCA (INT-747) is effective in protection against estrogen-induced cholesterol in mice [133]. INT-747 is in the second phase of clinical trials for primary biliary cholestasis. Another bile acid derivative 6α-ethyl-23(S)-methyl-CDCA is a selective TGR5 agonist [134], which is in the first phase clinical trials for diabetes and obesity. Fatty acid: Bile acid conjugates have potential for treating nonalcoholic fatty liver disease [135, 136], gallstone disease, [137–139] and cardiovascular diseases [140, 141].

Acknowledgment This research is supported by NIH grants DK44442 and DK58379

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Part II
Molecular Basis of Liver Development, Growth, and Senescence

Chapter 13
Liver Development

Klaus H. Kaestner

Introduction

About 5% of the body mass of mammals is made up by the liver, our largest internal organ. Absence of the liver is not compatible with life, due to the multiple essential metabolic functions of the organ. In addition, multiple diseases are caused wholly or in part by impaired liver function. Examples of the impressive functional diversity of the liver, which are discussed in detail elsewhere in this volume, are the secretion of serum components and clotting factors, the regulation of glucose, protein and lipid metabolism, and the detoxification of xenobiotics, drugs, and other chemicals.

The development of the vertebrate liver has served as a paradigm for understanding fundamental mechanisms of organogenesis in general. Multiple approaches have been employed over the years to address this important problem: from embryonic explant cultures to inducible and tissue-specific gene ablation, from gene expression profiling to lineage tracing. While, like all developmental processes, liver development represents a continuum, for didactic purposes it is useful to divide this into several stages, from the formation of the earliest hepatic bud, or "Anlage" from the embryonic endoderm, to the final functional maturation of hepatocytes and cholangiocytes that are the epithelial cells that constitute the bulk of the liver. In this chapter, I will focus on the earliest events of liver formation, those which take place in the first 48 h of hepatogenesis in the mouse embryo, and that span the induction of the first hepatogenic progenitors, the delineation of the hepatic domain from neighboring organ systems, and the migration of the first hepatoblasts out of the liver diverticulum.

K.H. Kaestner (✉)
Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA
e-mail: kaestner@mail.med.upenn.edu

Inducing the Hepatic Domain in Foregut Endoderm

Fate Mapping of Liver Precursors: Where to Hepatoblasts Come from?

During gastrulation of the early vertebrate embryo, three germ layers are established: the ectoderm, mesoderm, and endoderm. During subsequent organogenesis, the ectoderm gives rise to the nervous system and skin; the mesoderm to heart, kidneys, and blood; and the endoderm to the alimentary tract with its associated organs including salivary glands, thyroid, lungs, pancreas, and liver.

Initially, the endoderm is comprised of a single-cell layer epithelial sheet lining the ventral surface of the embryo. Shortly after the definitive endoderm emerges from the embryonic node, the important organizing center of gastrulation, this epithelium invaginates anteriorly to form the foregut tube, which will later fuse with the oral cavity. The ventral foregut gives rise to liver, lung, thyroid, and ventral pancreas, while the dorsal endoderm develops into the intestine and the dorsal pancreatic bud [1–3]. Central questions in the field of organogenesis are: How is the positional information established that differentiates anterior structures such as salivary glands from posterior structures such as the colon? How do certain cells in the endoderm, which begins as a simple epithelial sheet, know that they will become hepatoblasts, the precursors of hepatocytes and cholangiocytes?

Fate mapping studies have recently established which cells in the endoderm become liver progenitors. For these studies, very early mouse embryos, at a stage when there are neither morphological nor molecular markers of the liver primordium evident, where injected in individual or clusters of cells of the endoderm with a nondiffusible dye. The embryos were then cultured to allow for further development, and assessed for whether dye-injected cells had become part of the liver primordium [4]. Surprisingly, it was found that there is not a single domain of hepatic precursors, but rather three spatially separated areas. As shown in Fig. 13.1, there are two paired lateral domains at the one to three somite

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_13, © Springer Science+Business Media, LLC 2011

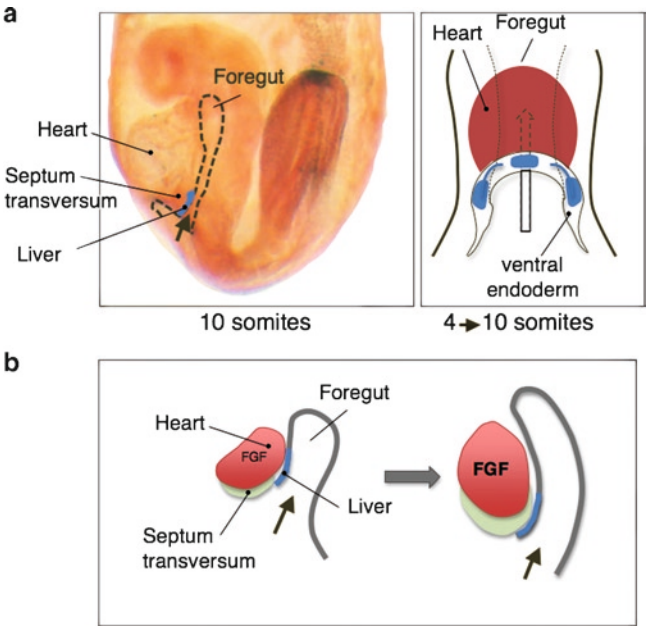

Fig. 13.1 Initiation of liver development in the mouse embryo. (**a**) The *left panel* shows a mouse embryo at E8.25 (equivalent to ten somites). The *dashed line* indicates the foregut delineated by the endoderm. The foregut still has not closed posteriorly at this stage. The *arrow* indicates the anterior intestinal portal. The *right panel* schematizes a slightly younger embryo with a ventral view of the anterior intestinal portal. The *blue areas* correspond to the hepatic progenitor domains in the ventral endoderm as defined by lineage tracing. (**b**) Schematic representation (*lateral view*) of the positions of liver, heart, and septum transversum at two stages of liver development. As Fgf production by the heart increases, the liver moves away from the heart and becomes adjacent to the septum transversum to ensure that the liver cells are exposed to the appropriate Fgf concentration. From Lemaigre [67] used with permission

stage, which migrate anteriorly and medially to fuse with a third, medial endoderm domain when hepatoblasts are eventually specified.

From Endoderm to Hepatoblasts: Inductive Signals

These fate-mapping studies raise an important question: Are these liver-fated cells already specified irreversibly in the endoderm at the early foregut stage, or do they need instructive signals to activate a hepatogenic gene expression program? Multiple studies, beginning as long as 45 years ago, have addressed this question and strongly favor the latter model. In the 1960s and 1970s, LeDourain used tissue explant studies with chicken embryos to demonstrate that isolated endoderm by itself will not develop into a hepatic primordium [5–8]. Rather, only when foregut endoderm was cultured together with cardiogenic mesoderm was the early liver fate induced. Thus, it is clear that signaling from the early heart field to the adjacent foregut endoderm is instructive for liver development.

So what is the nature of the signaling molecules(s) that are derived from the cardiogenic mesoderm? It was only 10 years ago that some of these inducers were discovered. Again using cultured endoderm explants, though this time from the mouse, Zaret et al. showed that blocking fibroblast growth factor (Fgf) signaling from the cardiac mesoderm to the co-cultured foregut endoderm prevented induction of early liver-specific gene expression [9]. Conversely, when endoderm was cultured without cardiogenic mesoderm, but in the presence of Fgf, the hepatic program was activated. Thus, Fgf signaling is one of the important inducers of hepatic fate.

These initial studies have since been confirmed and extended in multiple systems and species [10–16]. Fgfs can signal both via mitogen-activated protein kinase (MAPK) and the phosphatidyl inositol 3 kinase (PI3 kinase) pathway. Using specific inhibitors, Calmont et al. established that the MAPK pathway is essential for hepatic induction. Interestingly, Fgf signaling simultaneously suppresses the ventral pancreatic program, thus taking part in the differentiation of closely related fates that will be discussed in detail below [10, 15, 16].

Fibroblast growth factors are not the only signaling molecules that have been shown to be involved in early liver development. Adjacent to the heart is the septum transversum mesenchyme, which will later develop into the diaphragm and ventral mesentery. Bone morphogenetic proteins (Bmps), secreted from the septum transversum mesenchyme, have been shown to act coordinately as inductive signals mediating both the hepatic and ventral pancreatic fates [15–17]. Interestingly, suppression of other signaling events is also required for the early stages of endoderm specification. Wnt signaling, for instance, must initially be inhibited to allow proper hepatic specification, although its re-activation is required at later stages to support expansion of the liver bud and liver organogenesis [18–22].

From Endoderm to Hepatoblasts: Intrinsic Factors

It is now believed that the signaling pathways described above impact cell-autonomous factors such as transcriptional regulators to initiate highly-specific gene expression programs. However, it has also been suggested that these inductive signals alone are not sufficient to elicit the desired impact on their target cells in the developing endoderm. Rather, specific molecular events within the receiving cells must first occur before the tissue can become competent to respond to the instruction of the signal [23]. This model is based on the observation that dorsal endoderm, which does not normally

differentiate into liver cells, can be induced to express the liver marker albumin if dissected between E8.5 and E11.5 and cultured in the presence of Fgf [24]. This competence is lost, however, if the dorsal endoderm is isolated at E13.5 or later, suggesting that factors required for competence are no longer present at this stage or have ceased to disseminate the directive of the signal. Interestingly, there is a direct correlation between the ability of Fgf to induce hepatic gene expression in the dorsal endoderm, and the binding activity of Foxa and Gata proteins to an albumin gene enhancer region. Thus, the loss of competence was accompanied by the loss of Foxa and Gata binding in the more mature dorsal endoderm [24]. In support of this model, earlier studies from the Zaret lab had demonstrated selective binding of Foxa and Gata transcription factors to this enhancer in the endoderm, even prior to activation of the albumin gene, further implicating Foxa and Gata proteins as important factors involved in the establishment of developmental competence [25, 26].

The intrinsic or cell-autonomous transcription factors important in hepatogenesis were first identified from the analysis of the regulatory elements that govern the expression of the earliest liver-specific transcripts, among them albumin, alpha-fetoprotein, and transthyretin. In addition, expression studies of early mouse embryos established that these transcriptional regulators are expressed in the right place and at the right time, that is, in the endoderm before liver induction. The first Foxa gene to be activated in vertebrate embryos is Foxa2, whose mRNA and protein gene expression are first detectable in the mouse at embryonic day 6.5 (E6.5) in the "node" and the anterior primitive streak [27–30]. The node of the mammalian embryo is critical to gastrulation, the process that first establishes the three germ layers mentioned above. Indeed, embryos deficient for Foxa2 lack anterior and medial endoderm, and thus all derivate structures [31]. However, because of the failure to develop foregut endoderm, the Foxa2-null embryos were not informative with regard to the question of whether Foxa factors are required for liver induction; the answer to this question required the application of cell-type-specific gene ablation, which is discussed in detail below. The Foxa2 gene is not only active in the early embryo, but also in adults, including the endoderm derivatives such as liver, pancreas, lung, thyroid, and prostate [30, 32–35].

Foxa1, a close relative of Foxa2, is expressed in a very similar pattern in the early embryo and into adulthood; however, Foxa1 is activated about 12 h later than Foxa2 [27–30]. While genetic experiments (see below) have shown Foxa1 and Foxa2 to have overlapping functions in many developmental processes, there is a time when the only Foxa gene expressed in the embryo is Foxa2. This explains why Foxa2-null embryos have a severe early embryonic phenotype, while Foxa1- or Foxa3-null embryos develop almost normally until birth [36–42]. The third gene in this subclass of Foxa transcription factors is Foxa3, expression of which does not initiate in the endoderm until E8.5, and which is not present in the primitive streak or axial mesoderm [28]. For this reason, the Foxa3 regulatory elements have been used successfully to drive the expression of specific transgenes into the endoderm, including a Cre recombinase used to delete loxP-flanked target genes in the early gut tube (see below [43, 44]).

The Foxa factors have been shown through in vitro studies to differ in important functional properties from other sequence-specific DNA binding proteins and transcriptional regulators. Most transcription factors are thought to promote gene activation via the recruitment of cofactors that interact with the basal transcriptional machinery or that covalently modify certain histone residues, thereby altering the chromatin structure. The Foxa proteins are different in that they appear to be able to directly modify chromatin structure. Such a mechanism of action was first suggested when the crystal structure for the Foxa DNA-binding domain complexed with DNA was solved. Strikingly, the Foxa DNA-binding domain is similar to that of the linker histone H5, where both are configured in a so-called winged helix motif [45, 46]. Linker histones play a role in the compaction of chromatin, which renders the DNA inaccessible to most sequence-specific DNA binding proteins. The structural similarity of the Foxa proteins and linker histones raised the possibility that the Foxa factors could directly disrupt nucleosome assembly by displacement of linker histones. Elegant in vitro studies from the Zaret lab showed that Foxa proteins could indeed bind their target sequences in DNA templates that are fully covered by nucleosomes, and even alter local nucleosome occupancy [47–49]. A nucleosome-covered template containing the part of the albumin enhancer could not be bound by transcription factors such as NF-1 and C/EBP unless Foxa protein was added first [48, 50]. In combination with its capacity to bind target sequences in advance of gene activation, it is this unique ability to engage target sites compacted in nucleosomal DNA and to enable the subsequent binding of additional regulators that has led to the designation of Foxa proteins as "pioneer" factors [23]. Interestingly, members of the GATA family of transcription factors, which are also expressed in the foregut endoderm, have similar properties in vitro, although their binding to nucleosome compacted targets in the albumin enhancer is weaker than binding by Foxa proteins [48].

In addition to sites in the albumin enhancer, the Foxa proteins bind to and/or regulate numerous genes in the liver [51–54]. In fact, the Foxa proteins were initially purified as "Hepatocyte nuclear factor 3" because of their ability to bind to regulatory regions of the liver-expressed transthyretin (Ttr) and alpha-1-antitrypsin genes [55]. Their many target genes in the liver, the aforementioned properties as pioneer factors that can bind to compacted chromatin targets, and the

activation in the hepatogenic region of the foregut endoderm have made the Foxa proteins as prime candidates to be essential intrinsic regulators of the hepatic fate. However, the genetic analysis of their contribution to liver development was complicated by: (a) partial functional redundancy between closely related proteins, and (b) important roles of Foxa2 in gastrulation. Specifically, the early lethality of Foxa2$^{-/-}$ embryos, owing mostly to node and notochord defects [36, 42], prevented the analysis of liver development in these mice. To overcome these limitations, conditional ablation using the Cre/loxP system was employed.

The Cre/loxP system is an elegant technology that allows for the derivation of "designer mosaic mice," that is, animals that contain wild type amounts of the gene product of interest in most cells of the body, but are deleted in the cell type(s) of interest. The system was first employed in mice about 15 years ago by Rajewsky et al. and has revolutionized mouse genetics, not only in how it applies to liver development, but all aspects of biology and biomedicine [56]. The system consists of a site-specific DNA recombinase enzyme termed Cre (for "causes recombination") that recognizes only one specific DNA target, the 34-bp loxP (for "locus of crossing over") sequence. Thus, when loxP sites are placed strategically surrounding an essential exon of a gene of interest, but without interrupting gene function, then the gene can be deleted in all cell types that express the Cre recombinase enzyme – in the liver, for instance, under the control of the albumin promoter.

Using this approach, mice homozygous for a "floxed" (surrounded by loxP sites) Foxa2 allele were derived [57]. As expected, these mice were phenotypically wild type, as they expressed normal levels of Foxa2 protein in all cells. Surprisingly at first, when these mice were crossed to mice carrying a Foxa3-driven Cre recombinase, effecting deletion in the endoderm and its derivatives beginning at day 8.5 of gestation, the resulting *Foxa2*$^{loxP/loxP}$; *Foxa3*-Cre mice were viable, and showed a histologically normal liver, even though they lacked Foxa2 protein in all hepatocytes [43]. As mentioned above, Foxa1$^{-/-}$and Foxa3$^{-/-}$mice also have normal liver histology [39, 40]. So are the Foxa proteins not required for hepatogenesis? The answer came when mice lacking both Foxa1 and Foxa2 in foregut endoderm were obtained. In a complex mating scheme, Foxa1$^{-/-}$; Foxa2$^{loxP/loxP}$; Foxa3-Cre embryos were produced, and were found to lack all histological and molecular markers of hepatogenesis [58]. Thus, mice deficient for Foxa1 and Foxa2 are "liverless" [59]. Furthermore, when foregut endoderm from these embryos was placed in culture and incubated with the hepatogenic inducer Fgf2 mentioned above, none of the early liver genes were activated [58]. What these data also imply, incidentally, is that the third member of the Foxa class, Foxa3, is not able to compensate for the absence of its two relatives in liver formation. This observation suggests that the in vivo targets of Foxa3, at least in foregut endoderm, differ from those of Foxa1 and Foxa2.

Strikingly, a similar model applies to Gata4 and Gata6. Genetic ablation or mutation combined with siRNA-mediated suppression of the two genes in mice and zebrafish, respectively, showed that these two factors are required redundantly for liver development [60–62]. These genetic data clearly demonstrate that the Foxa and Gata factors are intrinsic mediators of liver development, and without them, no amount of inductive signal can induce the hepatic gene expression program. However, at present, it is not certain that their mechanism of action in vivo is truly that of pioneer factors: opening up the compacted chromatin to allow for access of additional transcriptional regulators. Future work will have to determine whether the absence of either the Foxa or Gata proteins converts endoderm chromatin to an inaccessible state.

Defining the Liver Field: Lineage Segregation Within the Foregut Endoderm

The region of the foregut endoderm from which the liver develops is actually a crowded place. Within the space of a few cell diameters, the lungs, thyroid, liver, hepatobiliary tract, ventral pancreas, stomach, and duodenum all need to be specified. In particular, the liver, ventral pancreas, and the extrahepatobiliary system comprising the common duct, gall bladder, and cystic duct; are very close together. How then are these different lineages separated, and how is the liver field specified? Interestingly, gene ablation and lineage tracing studies suggest that the extrahepatobiliary system has a common origin with the ventral pancreatic bud, while the intrahepatic biliary system is derived from hepatic progenitors.

Two systems have been shown to be involved in lineage segregation in the foregut endoderm. Mice lacking the Notch signaling effector Hes1 exhibit defects in lineage specification. These mice lack the gall bladder, and instead elaborate pancreatic fates in the common duct [63, 64]. One of the effector targets of Hes1 is likely the transcriptional regulator Sox17, because Hes1 mutants elaborate more Sox17^{+} cells in the foregut than wild type control embryos. Sox17 itself is initially expressed broadly in the foregut endoderm, but is then successively downregulated as the organ anlagen of liver, and ventral pancreas are established [65]. If Sox17 is ablated in foregut endoderm using the Cre/loxP system, pancreatic fates are activated in both the liver and extrahepatobiliary system. In a complex feedback loop, Sox17 is required for expression of Hes1, and the Sox17-dependent specification of the biliary and pancreatic fields is dependent on Hes1 [65].

Constructing the Liver Bud

A Transcription Factor Network Controls the Formation of the Hepatic Primordium

Once the first hepatoblasts have been specified through the mechanisms outlined above, they have to expand in number and grow into a true liver bud. This process requires both a dramatic increase in cell number, and the migration of nascent hepatoblasts away from the confines of the foregut epithelium into the mesenchyme of the septum transversum (Fig. 13.2). Liver bud formation is initiated on day 22 of human embryonic development, corresponding to day 9 in the mouse, with the formation of a liver diverticulum, or pouch, by the nascent hepatoblasts. These endoderm-derived cells first adopt a columnar shape, leading to a thickening of the foregut tube at the site of liver formation. Soon thereafter, these cells transition into a thickened, pseudostratified epithelium [66]. This conversion is the result of interkinetic movement of nuclei during mitosis, that is, the repositioning of nascent nuclei to the apex of the dividing cell.

In order to expand in mass and to begin to attain their final position in an organ separate from the gut tube, nascent hepatoblasts have to leave the gut epithelium and migrate through its basement membrane. During this process, hepatoblasts transiently adopt mesenchymal-like morphology, and show decreased expression of the epithelial adhesion protein E-cadherin. Thus, hepatoblasts undergo a partial and reversible epithelial transition in order to form the liver primordium; however, they retain expression of many hepatoblast markers during this time of migration.

This complex behavior of nascent hepatoblasts is controlled by a second set of transcriptional regulators, chiefly among them the homeobox transcription factors Hex and Prox-1, the T-box factor Tbx3, the "onecut" factors OC-2 and HNF6 (also known as OC-1), and the aforementioned factor Gata6 (recently reviewed in [67]). The contribution of the members of this network to liver development was again delineated though gene ablation studies in mice. For instance, Tbx3 promotes liver bud expansion by preventing premature differentiation of hepatoblasts into cholangiocytes, and is necessary for hepatoblast migration via activation of its target Prox-1 [68]. Prox-1, in turn, is required for delamination of hepatoblasts from the early liver bud. Consequently, in absence of Prox-1, hepatoblasts stay in a tight cluster in the hepatic bud [69]. The homeobox gene Hex plays several roles in liver bud formation, both in the nuclear migration mentioned above and in hepatoblast proliferation [66, 70]. Finally, without Gata6, hepatoblasts lose their differentiation markers over time. The overall transcription factor network active in hepatic bud formation is schematized in Fig. 13.2.

Just as is the case for the initial induction of the hepatogenic program from foregut endoderm in the previous

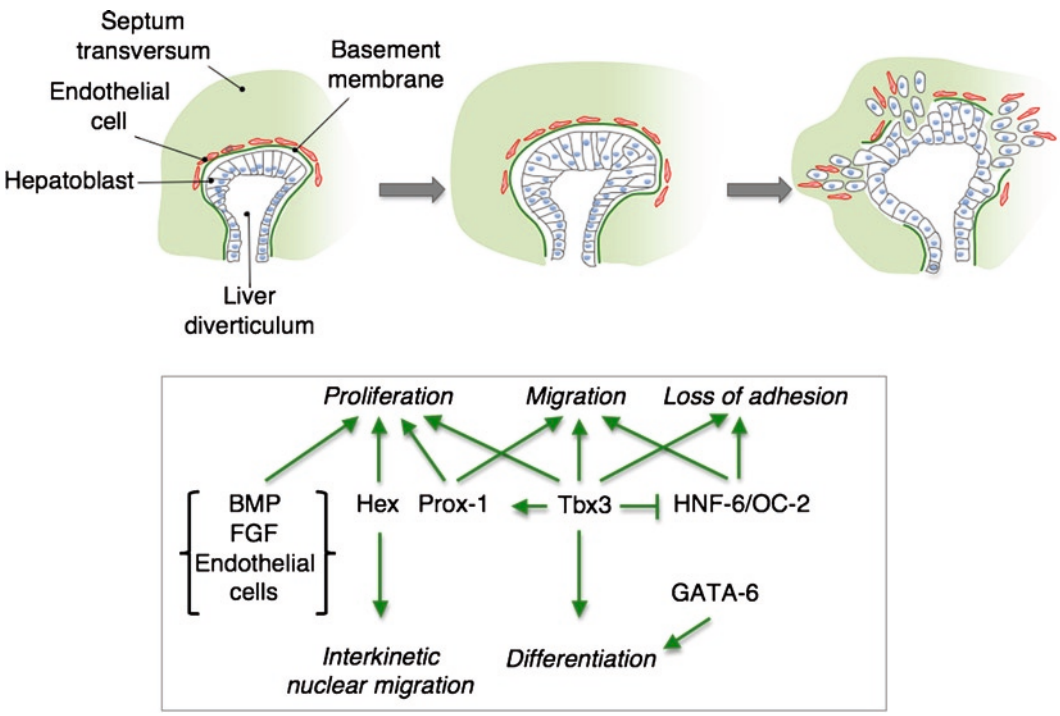

Fig. 13.2 Budding of the liver from the foregut endoderm. The *upper panel* schematizes the changes in morphology of the liver when it emanates from the endoderm. The *lower panel* summarizes the control of key regulatory events by transcription factors and extracellular signaling. From Lemaigre [67] used with permission

section, extrinsic factors are essential in addition to the cell-autonomously acting transcription factor network outlined above. Thus far, three such extrinsic systems have been described. First, when embryos lacking all endothelial cells due to absence of the Flk1 gene, which encodes the receptor for the vascular endothelial growth factor 2, were analyzed, liver development was blocked after the induction of the early hepatogenic program [71]. In fact, using a tissue explant system, Matsumoto et al. could show that without endothelial cells hepatic outgrowth is blocked. Teleologically, it appears to make sense that the growth of the liver is coordinated with that of the vascular system.

A second source of growth factors is the septum transversum mesenchyme, which is in close apposition to the foregut endoderm. Septum transversum mesenchyme is the source of bone morphogenetic proteins 2 and 4 (Bmp2 and 4), which are members of the TGFβ family of signaling molecules. Bmp4 null-embryos show a growth delay in the formation of the liver bud [17]. However, when Bmp4-null tissue explants were placed in culture, liver outgrowth proceeded normally, suggesting the action of a redundant factor, which is likely Bmp2. Thus, when Bmp signaling was impeded further through the addition of the Bmp-inhibitor noggin, liver outgrowth was blocked. Thus, Bmps from the septum transversum mesenchyme are additional factors essential for the growth of the hepatic primordium.

So how do nascent hepatoblasts cross the basement membrane of the foregut? After hepatic induction, nascent hepatoblasts express the matrix metalloproteases MMP14, while the septum transversum mesenchyme secretes MMP2 [72]. Together, these enzymes partially degrade the basement membrane, enabling hepatoblast migration. In fact, when metalloproteases were inhibited pharmacologically in an explant system, hepatoblast migration was blocked [72].

Expanding the Liver Primodium

Once formed, the growth of the liver primordium is extraordinary, so that by mid-gestation it occupies about half of the peritoneal cavity of the embryo. How is this dramatic expansion achieved? Multiple growth factors coordinate this process, each employing an elaborate signal transduction cascade (reviewed in [73]).

One of the major players in liver expansion is the Wnt/β-catenin signaling pathway. May be best known for its role in colorectal cancer, where aberrant β-catenin activation is the initiating step in tumor formation, Wnt/β-catenin signaling is highly complex in the liver, and some of the details are still being worked out. Expression analysis in the mouse found evidence for the involvement of eleven different Wnt ligands and eight of their receptors, termed Frizzled (Frz; [74]). Wnt signaling is also a prominent example of a novel theme emerging in vertebrate organ development, namely that the same signaling pathways can have opposite effects on the same system at different stages of development. Specifically, in the developing *Xenopus laevis* embryo, an antagonist of Wnt signaling, the secreted protein Frp5 (for frizzled related protein 5) is expressed in foregut endoderm during liver induction [19]. Frizzled related proteins act as competitive inhibitors of Wnt signaling, by binding to the ligand in the extracellular space, preventing it from binding to the Frz receptors on the cell surface. Thus, a gradient of active Wnt signaling is established, with high activity favoring posterior endoderm fates, i.e., the intestine, while low Wnt signaling is required for liver (and pancreas) initiation. In fact, McLin et al. could link high Wnt signaling activity to repression of Hex, the transcription factor introduced above as promoting hepatic bud expansion. Thus, only when Wnt/β-catenin activity is low, can the Hex gene be activated and liver expansion proceed [19].

However, just a short developmental time later, the situation is reversed, and Wnt/β-catenin signaling is in fact required for hepatoblast expansion. Thus, when the central mediator of canonical Wnt signaling, that is β-catenin, is conditionally ablated from hepatoblasts using the aforementioned Cre/loxP system, proliferation is blocked. The resulting embryo displays a severely hypoplastic liver, due to both reduced proliferation and increased cell death [22]. Wnt/β-catenin is not only required for the expansion of hepatoblast cell number, but also the acquisition of a fully differentiated hepatocyte phenotype, as β-catenin deficient hepatoblasts exhibit reduced expression of the important hepatocyte transcription factors, C/EBPα and HNF4α [22] (See also the following chapters).

In addition to responding to canonical Wnt signaling, β-catenin also integrates several additional regulatory pathways. for instance, β-catenin can bind to the receptor for hepatocyte growth factor (Hgf), termed c-myc, and become activated in response to Hgf stimulation [75]. In addition, Fgf10 secreted from myofibroblasts also stimulates hepatocyte proliferation via β-catenin [76]. These complex pro-proliferative interactions important for the expansion of the hepatic primordium are schematized in Fig. 13.3.

What about additional growth factors? Prominent among them is the already mentioned hepatocyte growth factor (Hgf). In addition to promoting β-catenin activation, Hgf signals via its cognate receptor, the tyrosine kinase receptor c-met. Once HGF-bound, c-met initiates a signaling kinase including mitogen-activated protein kinase kinase SEK1 (also called MKK4; official name MAP2K4), the mitogen-activated protein kinase p38, and their downstream effectors including ATF2 and ATF7 [77–81] (Fig. 13.3). Mutations in any of these components leads to dramatically reduced liver size, indicative of the importance of this pathway.

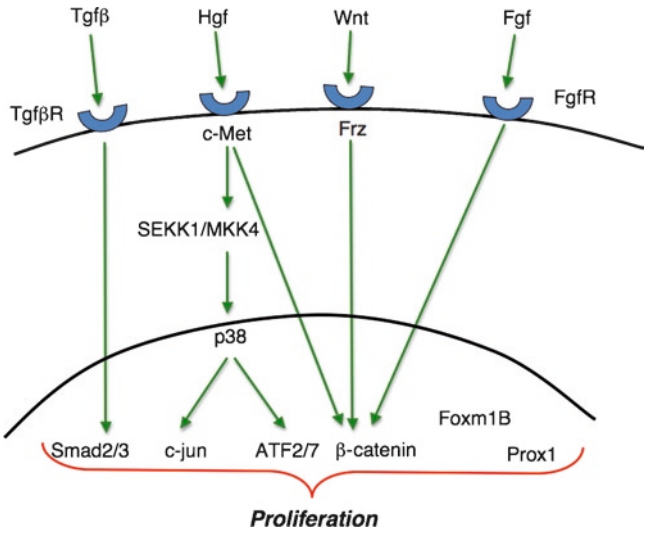

Fig. 13.3 Growth regulation in the hepatic primordium. The signaling factors and downstream cascades that control proliferation of hepatoblasts are illustrated. Liver growth depends on the coordinated control of these biological processes. Modified from Lemaigre [67] used with permission

The Hgf signaling pathway interacts with yet another growth factor system, in this case, TGFβ signaling. Mice heterozygous for two of the downstream signaling mediators, Smad2 and Smad3, develop a hypoplastic liver [82]. The main target in this case is possibly β1 integrin. Through the analysis of chimeric embryos, that is, embryos composed of both wild type and β1 integrin null cells, it had been shown 15 years ago that β1 integrin-deficient endoderm cells are excluded from the developing liver, though the exact mechanism of this defect remains to be elucidated [83]. Regardless, expression of β1 integrin is reduced in liver explants heterozygous for Smad2 and Smad3, but restored by addition of Hgf, thus demonstrating a link between the two pathways [82] (Fig. 13.3).

As before, intrinsic or cell autonomous factors are also essential for continued hepatoblast mass expansion. Using isolated fetal hepatoblasts in culture, Kamiya et al. showed that overexpression of Prox-1 (the same factor shown by gene ablation studies to be required for the migration of nascent hepatoblast out of the foregut endoderm) causes sustained proliferation of hepatoblasts. This proliferative effect is antagonized by Lrh1 (for liver receptor homologue 1), another liver-enriched transcription factor [84]. These findings suggest that the role of Prox-1 is not limited to its function in the delamination of hepatoblasts from foregut endoderm as described above, but also promotes continued hepatoblast proliferation. In the future, it would be valuable to confirm these novel functions of Prox-1 in liver bud expansion using conditional gene ablation.

Another crucial contributor for hepatoblast expansion is the winged helix transcription factor Foxm1B, a relative of the Foxa gene mentioned above. Foxm1B is an important activator of the cell cycle machinery in many settings, specifically the G2 and M phases of the cell cycle. When Foxm1B is removed from hepatoblasts using total or conditional gene ablation, hepatoblast mitosis is dramatically reduced, and hypoplastic livers result [85]. The entire network of signaling pathways and their intracellular effectors operative in the hepatic bud are schematized in Fig. 13.3. It is likely that future discoveries will expand on this already complex system. For those interested in learning about this process in more detail, several recent reviews are recommended [2, 67].

Summary

In the short span of 96 h of development in the mouse, a group of unremarkable cells in the epithelial sheet of endoderm has acquired a hepatogenic program, migrated though the basement membrane to its future location, and expanded dramatically in number and mass. This nascent organ built up of hepatoblasts now forms the substrate for further differentiation events to establish the two major cell types in the liver: the hepatocyte, with its multitude of metabolic and biosynthetic functions, and the cholangiocyte, which sets up a remarkable network, the biliary tree, which allows for the secretion of essential, yet hepatotoxic bile safely out of the liver. The events that lead to the differentiation and expansion of these two cell types are discussed in the following chapters.

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Chapter 14
Transcriptional Control of Hepatocyte Differentiation

Joseph Locker

Introduction

The unique gene expression that defines the hepatocyte conforms to a set of general regulatory principles. The genome encodes the programs of mature gene expression and of the preceding developmental stages. Transcription factors execute these programs by binding to specific DNA sequence motifs grouped together as promoters and enhancers. Expression of each gene therefore reflects the synergistic integration of separate regulations via individual factors. Each cell type expresses a distinctive mixture of hundreds of transcription factors. This mixture, superimposed on the epigenetic history of the cell, determines its phenotype.

As the regulator of energy storage and metabolic processing, and the major source of serum proteins, the mature liver maintains a very high level of transcription. Its function as a transcription factory, along with the size and relative homogeneity of the liver, lead to the initial discovery of important transcription factor families by liver – HNF1, FOXA (HNF3), HNF4, HNF6 (Onecut1), and C/EBP [1–7]. Once thought to be liver-specific, *liver-enriched* is a better description of these transcription factors, since all are important in other tissues.

From the beginning, it was apparent that the liver-enriched factors integrate into a hepatocyte-defining network of mutual regulation [4, 8]. Development of this concept has revealed a robust regulatory network around a central core of three factors, FOXA2, HNF4α, and C/EBP (Fig. 14.1) [9, 10]. The network factors appear sequentially during development and their persistent constitutive expression controls the adult liver phenotype. Some are required for development, while others are necessary only for expression of specific genes. Several other transcription factors function in liver development (HEX, PROX1, TBX3, XBP1, and GATA4/6) and persist in adult liver, but there is limited information about their regulation and target genes.

Evolution of Studies on Transcriptional Regulation In Liver

Research on hepatic transcription control has evolved over decades. Studies in the 1970s defined regulation of individual phenotypic genes. Research in the 1980s focused on elements from these genes, and led to characterization of most liver-enriched transcription factors. The research of the 1990s turned to mouse genetic models, progressing through transgenics, global knockouts, and liver-targeted conditional knockout. These showed the importance of regulation, but did not distinguish direct from indirect gene regulation, or direct effects on hepatocytes (cell autonomous) from those due to extracellular factors (noncell autonomous). Research in the 2000s stressed global characterization of gene expression, first by microarray characterization of mRNA abundance. This focus has now shifted to gene-specific analysis of chromatin by chromatin immunoprecipitation (ChIP), first gene-by-gene, then global detection via microarray (ChIP-on chip), and most recently, unbiased global sequencing (ChIP-Seq). Despite the exponential increase in information about gene regulation, there are still fundamental unanswered questions, particularly how individual interactions integrate into tissue specific gene control.

Binding sites for individual factors are ambiguous. This is not simply due to incomplete information. Rather, the weakened binding at sites that deviate from an optimum motif is an essential property. Weak sites are optimum for dynamic gene regulation, because they are highly sensitive to the level and affinity of transcription factors [11]. Binding sites can only be understood by their contextual relationship to neighboring sites, because the factors interact to exert *combinatorial control* of gene expression, best characterized for the β-interferon *enhanceosome* [12]. Complex transcriptional regulatory elements have been defined for several liver

J. Locker (✉)
Department of Pathology, Albert Einstein College of Medicine, Bronx, New York, NY, USA
e-mail: locker@aecom.yu.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_14, © Springer Science+Business Media, LLC 2011

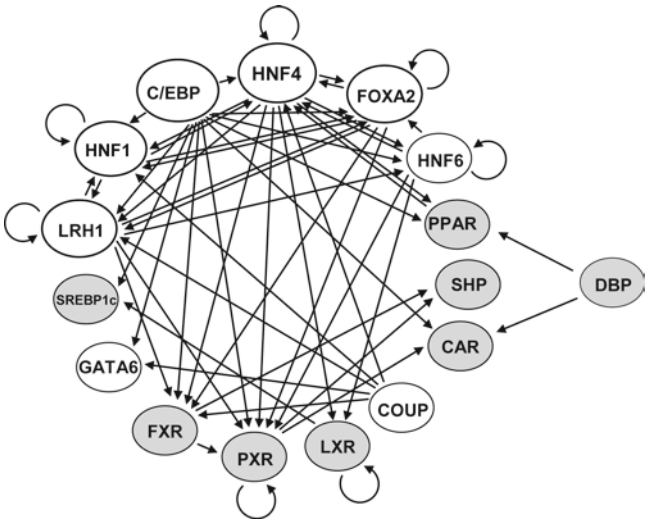

Fig. 14.1 The regulatory network of liver-enriched transcription factors in the mature hepatocyte. The figure is drawn from ChIP data, mostly from Kyrmizi et al. [9] and Odom et al. [10] and represents transcription factor binding to gene regions, not activation or repression. Factors that appear late and do not affect development are marked in *gray*. SHP and DBP are activated by the circadian factor BMAL1. The numerous potential repressive targets of SHP are not marked since no ChIP data are available (see text)

genes, notably albumin, α-fetoprotein (AFP), transthyretin, HNF4α, and phosphoenolpyruvate carboxykinase (PEPCK) [13–18]. All of these regulatory elements are bigger than the β-interferon enhanceosome – none is fully resolved.

The Transcriptional Environment in the Adult Liver

Liver Enriched Factors

Microarray studies have demonstrated that liver expresses several hundred transcription factors and the vast majority of strong detections represent hepatocytes (Fig. 14.2). Basic leucine zipper (bZIP) and nuclear receptor factors dominate the list (Tables 14.1 and 14.2).

bZIP factors combine a characteristic basic DNA-binding domain with a leucine-zipper dimerization domain. They always bind DNA as dimers, commonly as heterodimers of different members of a subfamily. C/EBP and DBP factors dimerize only within their own subfamilies, but are both abundant phenotypic regulators that frequently regulate the same genes through the common binding sites. The ATF/CREB and AP1 families generally regulate housekeeping functions through rapidly activated transcriptional responses. ATF5 and CREBH are liver-enriched, and liver regeneration strongly induces ATF3, otherwise expressed at very low levels. ATF/CREB and AP1 factors also heterodimerize with each other.

The nuclear receptors are dynamic regulators, either constitutively active or ligand dependent. Very strong expression of liver-enriched HNF4α, CAR, EAR2, PPARα, RXRα, FXR, SHP, LRH1, and PXR, dominate the mature hepatic phenotype. Other nuclear receptors prominently, but not selectively, expressed in liver are retinoic acid receptor-related orphan receptors (ROR), estrogen receptor related receptors (ERR), REV-ERB, the thyroid hormone receptors (TR), and the glucocorticoid receptor (GR). The REV-ERBs are heme receptors, but ligands for ROR and ERR are unknown. Other well-known nuclear receptors: Estrogen receptor (ER), vitamin D receptor (VDR), and retinoic acid receptors (RAR), may also be important in hepatocytes, but their expression is significantly lower.

Liver also expresses a diverse set of widely-expressed *housekeeping* factors. Some represent nonphenotypic processes like cell division or apoptosis, but many others collaborate with liver-enriched transcription factors to regulate phenotypic genes. Since various cell types express the general factors at different levels, their abundance in liver is a specific feature of hepatocyte gene regulation.

Transcriptional regulation of the liver develops through four phases:

1. Establishment of *developmental competence* before liver specification.
2. Establishment of definitive hepatic structures.
3. Constitutive regulation of the hepatocyte phenotype.
4. Dynamic gene regulation in mature liver.

Although these phases lack discrete boundaries, they provide a conceptual framework for considering individual transcription factors.

Phase 1: Prehepatic Expression and Developmental Competence

FOXA. The three *Forkhead Box A* proteins (FOXA1, A2, and A3) appear sequentially in visceral endoderm, foregut, and liver (A2, A1, then A3) with persistent expression in adult hepatocytes. They contribute to the regulation of many genes in mature liver, frequently by binding to distant enhancers. FOXA proteins are nearly global regulators in liver, because ChIP studies showed binding to thousands of genes [19, 20]. In mouse knockouts, ablation of individual FOXA genes produced only mild liver pathology with moderate changes in gene expression. However, combined deletion of FOXA1 and FOXA2 in late foregut endoderm prevented formation of the liver, while deletion after liver formation distorted genesis of the bile ducts [21]. The three FOXA proteins have subtle

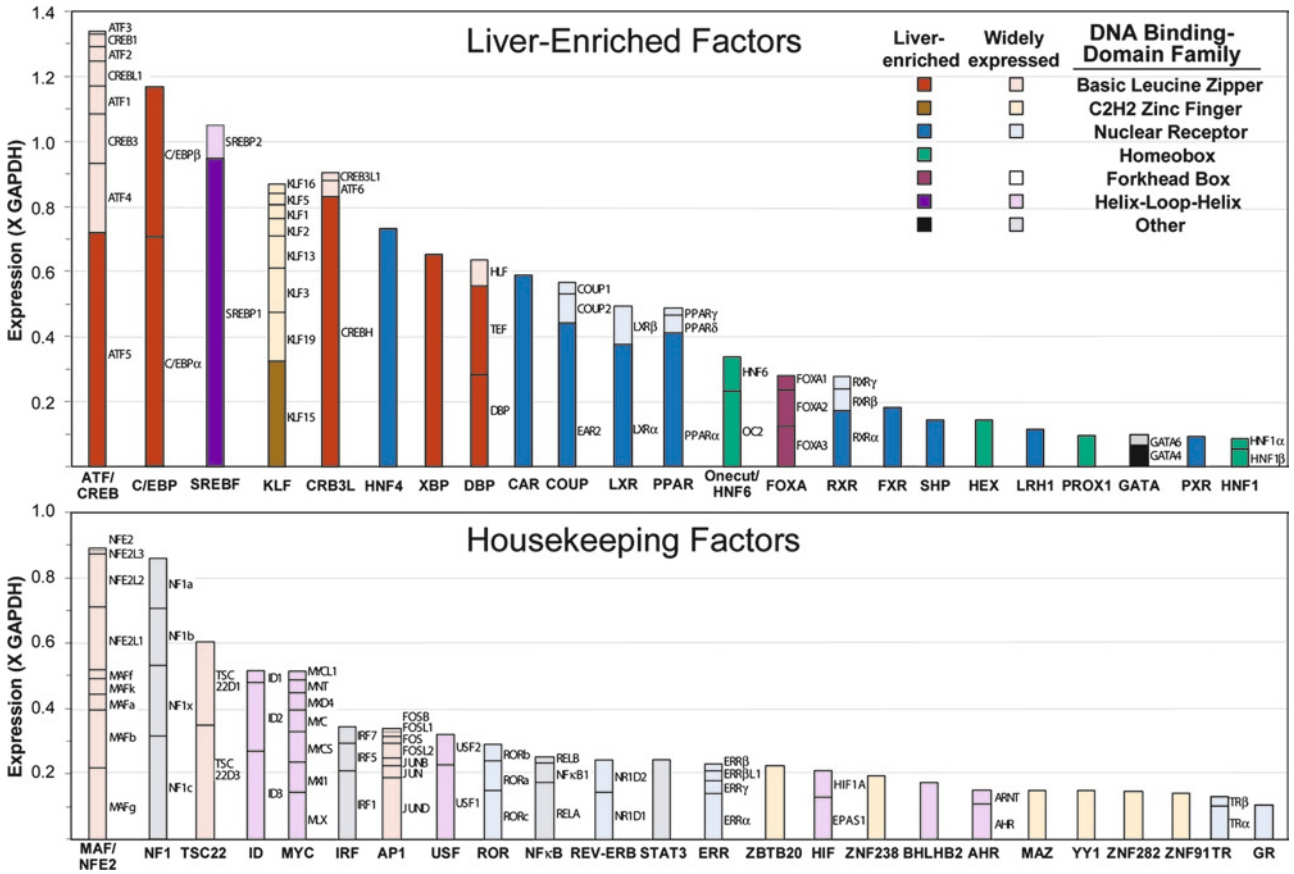

Fig. 14.2 Abundant transcription factor subfamilies in the mature liver. mRNA levels, averaged from microarrays of normal mouse liver RNA, are displayed relative to the expression of GAPDH (unpublished data). The averaging has normalized the levels of factors that show circadian variation. The arrays detect about 450 transcription factors. The 113 expressions displayed in the figure have been verified in expression databases obtained using a different array platform (www.genecards.org). Abundant factors are grouped as subfamilies with detected relatives that heterodimerize or bind equivalently to the same DNA motifs. Three factors activated by posttranslational cleavage (CREBH, ATF6, and CREB3l1) are grouped separately from other ATF/CREB factors. The liver-enriched transcription factors (above) are discussed individually in this chapter. Abundant housekeeping factors (below) are also major components of the transcriptional regulatory milieu of liver

differences in transcriptional function [22]. Such differences occur in virtually every group of seemingly equivalent factors. Nevertheless, the total expression of three similar factors provides the level of FOXA protein necessary for optimum gene regulation.

Molecular studies of the albumin gene enhancer first demonstrated distinctive transcriptional function for FOXA2 as a *pioneer factor* that binds native chromatin and reorganizes nucleosomes. Surprisingly, FOXA2 first binds to the albumin enhancer in endoderm, several cell generations before the liver formation or albumin gene expression. Indeed, the same genomic sites bind FOXD3 in embryonic stem cell, which is then replaced by FOXA2 when the cells differentiate to endoderm. FOX factors therefore establish *developmental competence* – they configure transcriptional regulatory elements for later activation during development [21, 23, 24].

HEX. In mouse, the hepatic primordium appears as a specialized region of the anterior foregut on E8.5, with hepatocyte specification marked by the appearance of albumin mRNA. Hepatoblasts then rapidly grow outward so that a distinct liver bud is apparent by E9.5. HEX, a regulatory target of FOXA2, appears at E8.5 and marks hepatic specification. Knockout models confirm the developmental importance of HEX. Global ablation selectively blocks formation of the liver [25], while targeted ablation shortly after liver bud formation arrests further development. Ablation at a later developmental stage interferes with bile duct formation [26]. Expression of HEX in the liver persists throughout life, but with few known regulatory targets, the relationship of HEX to the mature liver phenotype is unclear [27, 28]. HEX is a multifunctional protein, so it could affect development in many ways. In addition to its action as a DNA-binding transcription factor, HEX is also a coactivator or corepressor of other transcription factors, and an mRNA-binding protein that regulates their transport from the nucleus [29, 30].

GATA4/6. Two closely related zinc-finger proteins, GATA4 and 6, transcriptional activators, also appear in anterior

Table 14.1 Molecular properties of hepatocyte transcription factors

Factor	Family	Complex	Binding motif[a]	Ligand	Coactivators	Corepressors
FOXA1 (HNF3α), FOXA2 (HNF3β), FOXA3 (HNF3γ) [19, 20, 22]	Fox	Monomer	TRTTKRYNY	–	–	GRG3
HEX (HHEX, PRH) [30]	Hom	Dimer, oligomer	YWATTAAR	–	–	GROUCHO
GATA4, GATA6 [147]	GATA ZnF	Monomer	WGATAR	–	CBP/p300	FOG/ZPFM
TBX3 [148]	T-box	Dimer	TCACACC	–	–	–
PROX1 [149]	Hom	Monomer?	YAAGNNR	–	–	–
HNF6 (Onecut1, OC1), Onecut2 (OC2) [46]	Cut and Hom	Monomer	DHWATTGAYTWWD AAGTCAATA ATCGAT	–	CBP/p300	–
HNF4α [49–51]	NR	Homodimer	DR1:AGGTCA N AGGTCA KGCWA R GKYCAY	Linoleic acid (nonactivating)	p160, PGC1α	SHP, NCOR, SMRT, PROX1, Hes6
HNF1α (TCF1, MODY3), HNF1β (vHNF1, TCF2, MODY5) [62]	POU and Hom	Dimer	GTTAAT N ATTAAC	–	CBP/p300, p160	–
C/EBPα, C/EBPβ [69, 71]	bZIP	Homo- and Heterodimers	RTTAY GTTAY	–	CBP/p300	–
XBP1 (hXBP1, TREB5) [72]	bZIP	Dimer	GNTGAC GTGK TGAC GTGG	–	–	–
ATF1, ATF2, ATF3, ATF4, ATF5, ATF6, CREB1, CREB3, CREB1l1, CREB3l1, CREBH (CREB3l3) [74, 81]	bZIP	Homo- and Heterodimers	TGAC GTCA	–	CBP/p300	–
JUN, JUNB, JUND, FOS, FOSl1 (Fra1), FOSl2 (Fra2), FOSB [75]	bZIP	Heterodimers	TGACTCA	–	CBP/p300	–
KLF15 (KKLF) [85]	C2H2 ZnF	Monomer	CACCC	–	–	–
SREBP1a, SREBP1c, SREBP2 [87, 88]	bHLH	Homo- and heterodimers	TCAC CCCA	–	PGC1 α	–
DBP, TEF (VBP), HLF [69]	bHLH and PAR	Homo- and heterodimers	RTTAY GTAAY	–	CBP/p300	–
LRH1 (FTF, NR5A2) [100, 103–105]	NR	Monomer	YCAAGGYCR RNTCAAGGNCW	Phospholipid	p160	SHP, NCOR, SMRT, PROX1
EAR2 (NR2F6),COUP-TF2 (ARP, NR2F2), COUP-TF1 (EAR3, NR2F1) [107]	NR	Homodimer Heterodimers? Heterodimers with RXR?	DR1:RGKTCA N RGKTCA	Retinoids?	p160	NCOR, SMRT
SHP [104, 111, 112]	NR	Heterodimer with LRH1	–	?	p160	NCOR, SMRT
FXR (NR1H4) [114, 115, 117]	NR	Heterodimer with RXR	IR1:RGGTCA N TGACCY	Bile acids	p160	NCOR, SMRT
LXRα, LXRβ [119]	NR	Heterodimers with RXR	DR4: RGKTYA NNNN RGKTYA YGMCCS NNNN TAACCC	Oxysterols	p160	SHP, NCOR, SMRT

PPAR α (NR1C1) [121, 125]	NR	Heterodimers with RXR	5-base extension + DR1: RAACT AGGNCA A AGGTCA	Unsaturated fatty acids, eicosanoids, clofibrate drugs	p160, PGC1α	NCOR, SMRT
PXR (NR1I2) [126, 129]	NR	Heterodimer with RXR	DR4:AGNTCA NGNN AGTTCA	XenobioticsLithocholate	p160	NCOR, SMRT
CAR (NR1I3) [132–134]	NR		DR4:RGGYCN NNNN AGYNCN	Xenobiotics (agonists) Phenobarbital (nonligand activator) Androstanol (inverse agonist)	p160	–
RXR α [138]	NR	Homodimer Heterodimer with other NRs	DR1:RGGYCA N RGGYCA	9-cis retinoic acid	p160	–
ZHX2 [142]	C2H2 ZnF and Hom	Homodimer	NA	–	–	NA
ZBTB20 [145, 146]	C2H2 ZnF	Homodimer	NA	–	–	NA

Fox forkhead box; *Hom* homeobox; *ZnF* Zinc finger; *NR* nuclear receptor; *bZIP* basic leucine zipper; *bHLH* basic helix-loop-helix; *DR* direct repeat; *IR* inverted repeat

[a]Extended DNA code: Y = C,T; W = A, T; R = A, G; S = G, C; K = G, T; M = A, C; D = C, T, G; H = A, T, C

Table 14.2 Ontogeny of hepatocyte transcription factors

Factor	Onset [1]	Peak[a]	Global knockout	Liver-specific knockout	Target genes and effects
FOXA2	Early gastrula E6.5	Adult	Embryonic lethal at E10	Mild changes in gene expression; reduced induction of gluconeogenesis	Serum proteins, metabolic enzymes; regulate enhancers [21]
FOXA1	Late gastrula E7	Adult	Early postnatal lethal; normal liver; hypoglycemia		
FOXA3	Hepatic bud, E9.5	Adult	Normal liver; mild metabolic changes		
HEX	Foregut E8.5	Adult	Embryonic lethal, fails to form liver bud	Ablation in the hepatic diverticulum blocks further liver development; ablation in fetal liver leads to defective genesis of bile ducts	HNF4α and HNF6?; coactivator, corepressor; mRNA transporter [25, 29, 35]
GATA4, GATA6	Late gastrula E7	Gastrula, early liver	Embryonic lethal, blocks expansion of hepatic bud	–	HNF4α, HEX, albumin, fatty acid binding protein [31–34]
TBX3	Liver bud, E9	Fetal liver	Embryonic lethal, forms small hepatic bud with few hepatoblasts	–	Repressor of p19ARF and other genes [36, 40]
PROX1	Hepatic bud E9	Adult	Embryonic lethal at E14.5 with small liver	–	Represses LRH1- and HNF4α-activated genes [42, 43]
HNF6, Onecut2	Hepatic bud E9	Adult	Single knockouts have little effect on liver development, but die as neonates from biliary abnormalities; double knockout is embryonic lethal with failure to expand the hepatic bud	–	Stimulates hepatoblast proliferation and migration; regulates some genes of metabolism, serum proteins; regulates biliary differentiation [37]
HNF4α	Late gastrula E7	Adult	Knockout dies during gastrulation, from defects in visceral endoderm	Knockout targeted to midgestation liver impairs growth, hepatocyte differentiation; knockout targeted to postnatal liver cause fatty liver, abolishes zonal gene expression	Metabolic regulators; HNF1α, other liver-enriched transcription factors [38, 49, 51, 52, 56]
HNF1β	Visceral endoderm E6; liver bud E9	Visceral endoderm, early liver	Early embryonic death from failure to form visceral endoderm; tetraploid rescue extends development and allows partial formation of liver bud	–	Serum proteins, metabolic enzymes [39]
HNF1α	Liver bud E9	Early liver	Normal development, with early postnatal death from metabolic defects	–	Serum proteins, metabolic enzymes, e.g., albumin, phenylalanine hydroxylase [64]
C/EBPα	Hepatic bud E9	Adult	Normal liver development; Neonates die from hypoglycemia	–	Global regulation of serum proteins, metabolic enzymes [66, 70]
C/EBPβ	Hepatic bud E9	Adult	Viable without liver pathology; impaired liver regeneration	–	
XBP1	Constitutive	Constitutive	Late embryonic death with hypoplastic liver	–	Lipogenesis, steroid synthesis, unfolded protein response [72]

CREBH	Liver bud E9	Adult	Normal liver development; impaired gluconeogenesis	–	Acute phase and ER stress response [79]
KLF15	Liver, E16	Adult	Normal liver development; impaired gluconeogenesis	–	Gluconeogenesis [86]
SREBP1c	Liver bud E9,or earlier	Adult	Normal liver development, increased cholesterol stores, high lethality	Altered feeding-fasting responses; loss of lipogenic responses mediated by LXR	Synthesis of fatty acids, triglycerides and phospholipids [87]
DBP, TEF, HLF	Postnatal day 7Fetal liver, E17 Postnatal day 0	Adult	Single knockouts have normal liver development, dampened circadian expression. Triple knockouts lose circadian expression of many genes, especially those of xenobiotic metabolism	–	Circadian expression of serum proteins, metabolic enzymes, and xenobiotic pathways [90]
LRH1 (FTF)	Fetal liver, E14	Adult	Early embryonic death from endodermal defects	Morphologically normal liver; alterations in cholesterol metabolism and bile acid homeostasis	Cholesterol transport and metabolism, and bile acid homeostasis; transcription factors SHP and FXR [101]
COUP-TF1	Embryonic liver, E11 or earlier	Liver, E11 or earlier	Embryonic death before E10; ± is viable, with altered liver expression of genes controlling lipid homeostasis	–	Lipid homeostasis; antagonizes gene activation by HNF4α and PPAR [106, 108, 109]
COUP-TF2	Embryonic liver, E11 or earlier	Liver, E11 or earlier	Perinatal lethality; liver development unremarkable; no characterization of liver gene expression	–	
EAR2	Embryonic liver, E11 or earlier	Adult	Liver unremarkable, without characterization of gene expression	–	
SHP	Postnatal day 0	Adult	Normal liver with some changes in gene expression	–	Circadian regulator of metabolic genes; antagonizes activation by HNF4α, LRH1, PPAR, CAR, PXR, LXR, FXR and possibly other nuclear receptors [111–113]
FXR	Fetal liver, E17	Adult	Normal liver with increased fat, hyperlipedemia, and reduced bile acid transport	–	Bile acid transport and metabolism, cholesterol catabolism, and lipoproteins [115, 116]
LXRα, LXRβ	Fetal liver, E18	Adult	LXRα –/– has normal liver development, hepatomegaly, fatty liver; phenotype is exacerbated in LXRα–/– LXRβ–/– double knockout	–	Genes of fatty acid and triglyceride synthesis, cholesterol uptake and its conversion to bile acids [119]

(continued)

Table 14.2 (continued)

Factor	Onset [1]	Peak[a]	Global knockout	Liver-specific knockout	Target genes and effects
PPARα	Fetal liver, E18	Adult	Normal liver, but without peroxisome proliferator response to inducing drugs; elevated serum cholesterol and lipoproteins	–	Genes of fatty acid uptake and β-oxidation; peroxisomes [121, 123]
PXR	Postnatal day 0	Adult	Normal liver, with altered drug-induced metabolic responses, and subtle changes in liver regeneration	–	CYP3A4/Cyp3a1/Cyp3a11; drug transport, Phase I, and Phase II modification [130]
CAR	Fetal liver, E16	Adult	Normal liver, with altered drug-induced metabolic and growth responses	–	CYP2B6/Cyp2b1/Cyp2b10; drug transport, Phase 1, and Phase II modification; hepatocyte proliferation [133, 134, 137]
RXRα	Preimplantation embryo	Adult	Embryonic lethal at E13.5 to E 15.5 due to cardiac defects; liver forms normally but shows reduced growth	Normal liver development; develops massive fatty liver and hyperlipedmia	A wide variety of metabolic enzymes [141]
ZHX2	Postnatal	Adult	BALBc/J has null mutation; they have normal development without liver changes	–	Partially represses AFP, GPC3, H19, and lipoprotein lipase [142]
ZBTB20	Postnatal	Adult	Normal liver; impaired glucose metabolism due to nonhepatic effects	–	Partially represses AFP [145, 146]

[a]Data from Li et al. [87] or individual sources

foregut endoderm prior to liver specification. Ablation allows hepatic specification, but blocks subsequent cell expansion. Their function is redundant, although knockout of GATA6 has a milder effect [31]. Both HNF4 and HEX are important transcriptional targets [32, 33]. Liver expression of GATA4 and 6 persist throughout life, and contribute to the expression of several proteins, including albumin and fatty acid binding protein [34].

Phase 2: Establishment of Definitive Hepatic Structures

Hepatic specification, local differentiation of single-layer foregut endoderm into hepatic progenitors occurs at the 18-somite stage embryo (mouse E 8.5), early outgrowth (psuedostratification) at 20 somites (E9.0), and a liver bud of hepatoblasts and stroma is apparent by 23 somites (E9.5) [35, 36]. Several transcription factors that appear during this interval play important roles. Loss of PROX1, TBX3, or HNF6 + OC2 blocks early liver growth [37]. HNF4α appears at about the same time, and its ablation reduces liver bud expansion [38]. HNF1β expression precedes liver specification, but is also required for expansion of the liver bud [39]. It is difficult to define an exact temporal sequence of these factors.

TBX3 knockout leads to a much smaller hepatic bud that is almost devoid of epithelial cells, with decreased expression of HNF4α and C/EBPα, and increased expression of HNF6 and HNF1β [36, 40]. However, it is not clear whether these are direct transcriptional targets of TBX3. With a T-box DNA binding domain, TBX3 binds DNA as a dimer, and acts as a transcriptional repressor. One possible mechanism of hepatic bud expansion is that TBX3 suppresses p19ARF, which is a negative regulator of cell proliferation. Only weak expression of TBX3 persists in adult liver [41].

PROX1. Effects of the PROX1-null mutation are quite similar to those of TBX3 mutation – failure of early expansion of the hepatic primordium [42, 43]. PROX1 is an activating transcription factor that persists in adult liver [44]. PROX1 also acts as a transcriptional corepressor of two liver-enriched factors, HNF4 and LRH1 [45]. As for TBX3, there is little direct knowledge of PROX1 regulatory targets, or whether its main developmental function is activation or repression.

HNF6. Like PROX1, expression of HNF6 (Onecut1, OC1) and its homologue OC2 appear in the liver bud shortly after specification. They are functionally redundant transcription factors in the Onecut family, characterized by a cut domain and an atypical homeodomain [46]. Separate inactivation of HNF6 or OC2 has little effect, but combined inactivation attenuates growth of the early liver bud [37]. High-level expression of both factors persists in mature liver, where they have been detected in the chromatin of more than 200 genes [47]. They are also critical transcriptional regulators of bile duct epithelium, and required for normal biliary differentiation [48].

HNF4α, a central regulator in the network of liver transcription factors [9, 10], has two critical developmental roles, first in formation of visceral endoderm, later in differentiation of early hepatocytes and enlargement of the liver bud [38]. High level expression persists throughout life and HNF4α regulates more than ~900 genes in mature liver [47, 49]. HNF4 is an ancient factor, highly conserved throughout metazoans, and it binds genes as a ligand-independent constitutively active homodimer [50]. Most other liver-enriched nuclear receptors evolved more recently, and they bind DNA as ligand-activated heterodimers with RXR. HNF4α is thus more fundamental and has a broader but less dynamic role in shaping the hepatic phenotype.

Long considered an *orphan* nuclear receptor, the possible ligand regulation of HNF4α has been controversial. A recent study has demonstrated that its ligand-binding domain is occupied in vivo by linoleic acid [50], an appropriate regulator since HNF4α regulates several aspects of fatty acid metabolism [51]. Even so, the function of this "ligand" is problematic, since it does not affect transcriptional activation. The binding might therefore be vestigial, left over from an earlier HNF4 that was dynamically controlled by ligand. Alternatively, other molecules related to linoleic acid, drugs or endogenous molecules, might act as *inverse agonists* that lead to repression of HNF4α targets.

HNF4α directly regulates expression of other liver-enriched transcription factors, and a broad range of metabolic proteins, mediating "reciprocal expression of fatty acid transport and metabolizing enzymes during feeding and fasting" [51]. Thus a liver-targeted HNF4α knockout has fatty liver, and HNF4α is upregulated in fasting, largely due to stimulation by PPARα.

Zonation is a well-known feature of mature liver. Centrilobular hepatocytes selectively express genes of ammonia metabolism, detoxification, glycolysis, and the TCA cycle. In contrast, genes of gluconeogenesis and urea formation show periportal expression. Many zonal genes are direct regulatory targets of HNF4α and its knockout causes periportal gene expressions to spread over the whole lobule [52]. β-catenin signaling also controls zonal expression, activated via Wnt peptides that originate from vascular endothelium of the central vein [53]. While β-catenin itself is not a transcription factor, it functions instead as a latent transcriptional coactivator. Wnt signaling induces dephosphorylation and release from cytoplasmic complexes, allowing β-catenin to translocate to the hepatocyte nucleus [54]. In the nucleus, β-catenin binds and coactivates the four TCF/LEF transcription factors to mediate canonical Wnt responses [55].

Liver expresses low, constitutive levels of all four factors: LEF1 (TCF1α), TCF1 (TCF7), TCF3 (TCF7L1), and TCF4 (TCF7L2). The zonal relationship between Wnt and HNF4α is complex. Where Wnt signaling is strong, β-catenin-LEF1 displaces HNF4α from selected binding sites near the promoters of zonally regulated genes [56]. In the context of these genes, weakly activating HNF4α is replaced by strongly activating β-catenin-LEF1.

Expressed at high levels in liver throughout development, β-catenin is nevertheless a critical developmental regulator of the liver, since its selective depletion in fetal hepatoblasts is lethal. A specific defect in hepatocyte maturation leads to decreased proliferation and a hypoplastic liver [57, 58]. Wnt/β-catenin signaling also has a major role in bile duct differentiation, which is inhibited by β-catenin depletion and accelerated by exogenous Wnt3a [57, 59]. Thus, Wnt signaling through β-catenin has a multitude of effects on hepatocyte proliferation, differentiation, and zonal gene expression in the mature liver. Most of the specific TCF/LEF target genes that mediate these processes are unresolved.

HNF1. Despite their lower abundance, the HNF1 transcription factors were among the first liver-enriched transcription factors to be discovered [1, 3, 60], presumably because of the high site-specific affinity of these dimeric factors. HNF1α and β have nearly identical DNA binding and dimerization domains, which are a distinctive fusion of a homeodomain and part of a POU domain [61]. Rigorously defined as an inverted repeat dimer, GTTAAT N ATTAAC, the binding site motif accommodates either homo- or heterodimers, and typically occurs near promoter regions of genes encoding serum proteins and metabolic enzymes. Almost 200 target genes are apparent in ChIP-Chip studies [47, 62]. During development, HNF1 expression is regulated by HNF4α, and in mature liver, HNF1α regulates expression of several liver-enriched transcription factors, including HNF4α, FOXA2, LRH1, and FXRα.

Early endoderm expresses HNF1β, well before liver formation, and HNF1α appears with liver specification. HNF1β is more critical, because the knockout fails to form visceral endoderm. Tetraploid rescue allows development to continue, but these embryos form only an attenuated hepatic bud [39]. HNF1α knockout has milder phenotype, with normal liver development, but impaired expression of some hepatic genes. The postnatal absence of phenylalanine hydroxylase results in phenylketonuria and early death [63, 64].

Both HNF1α and β are transcriptional activators, but their activation domains differ. Moreover, studies of the albumin and VDP genes have suggested that HNF1α distinctively regulates interactions between promoters and distant enhancers [13, 65].

Phase 3: Establishment of a Constitutive Phenotype

C/EBP. Two closely related bZIP factors, C/EBPα and β, become highly expressed in the liver bud, although C/EBPβ expression is more widespread [66]. The two C/EBPs, both activators, recognize identical DNA motifs, bind DNA as homo- or heterodimers, and function equivalently to activate a large number of liver genes [67, 68]. C/EBPα and β are among the most abundant of all transcription factors in liver, and contribute to the transcriptional activation of many genes. However, C/EBP has relaxed site specificity so that binding motif occur every 200–300 bp within the genome [69]. This has made identification of functional sites very difficult.

Mice with ablation of either gene have normal liver development, but reduced expression of many genes. C/EBPα–/– neonates die from hypoglycemia due to reduced expression of glycogen synthase, glucose-6-phosphatase, and PEPCK [70]. C/EBPβ–/– mice survive with more subtle metabolic defects, but have impaired liver regeneration [68]. Despite similar activation function, C/EBPα distinctively inhibits cell proliferation, by binding and inhibiting Cdk2 and Cdk4, or by binding E2F [71]. Hepatocytes apparently compensate for this inhibition by reducing C/EBPα and increasing C/EBPβ during liver regeneration [68]. Both proteins have short inhibitory isoforms, resulting from translational initiation at internal AUG codons [67]. Since the short isoforms are present at significantly lower levels than the full-length isoforms, their regulatory function is probably subtle.

XBP1. Although its function overlaps with CREB-ATF and AP1 factors, XBP1 is in a special category because its knockout impairs hepatic development. The so-called *X-box binding protein*, XBP1, is a bZIP transcription factor primarily known for its function in B-cells. However, XBP1–/– fetuses die in late gestation with hypoplastic livers. Consistent strong expression is apparent at all stages, but liver expresses more than most other tissues, and XBP1 directly regulates genes of lipogenesis and steroid synthesis. Like several ATF/CREB transcription factors, XBP1 mediates the unfolded protein response, binding as a homodimer on the unfolded protein response element (UPRE) motif, TGACGTGG. The motif is similar to ATF/CREB binding sites [72].

An unusual posttranscriptional regulation controls XBP1-mediated activation of the unfolded protein response. IRE1, a specialized mRNA splicing enzyme found in endoplasmic reticulum (ER) controls the relative levels of two isoforms [73]. Unspliced mRNA encodes XBP1u, a repressing isoform that binds response elements, but lacks an activation domain. ER stress activates latent IRE1, which splices out a small RNA segment to frame-shift the distal part of XBP1 mRNA. The new translation product is the active transcription factor, XBP1s. It displaces XBP1u from binding sites to stimulate target genes.

Phase 4: Dynamic Gene Regulation in the Mature Liver

Stress-responsive bZIP factors. In addition to XBP1, liver prominently expresses three bZIP families: ATF/CREB [74], AP1 [75], and Maf/NFE2 [76] associated with stress responses, carbohydrate metabolism, and fatty acid oxidation [77–79]. Many are inducible factors, regulated by diverse mechanisms of gene transcription (AP1, ATF3), controlled mRNA splicing (XBP1), controlled translation (ATF5), proteolytic activation of latent protein (CREBH, ATF6), or induced phosphorylation (CREB1, 3). The unusually high level of several mRNAs in this group probably reflects these mechanisms of induction, to provide large pools of mRNA for induced splicing or translation, or of latent protein for induced cleavage. The pools allow repeated induction by weak signals. CREB (*cAMP response element binding*) factors mediate cAMP responses, especially stimulation of gluconeogenesis and fatty acid oxidation in liver. The optimum ATF/CREB binding site is TGAC GTCA, an inverted repeat of 4-base motifs that each contact one subunit of the dimer. Induced high-level expression may activate target genes with weaker binding sites, or modulate responsiveness to cAMP by replacement of CREB with ATF [80]. AP1 factors bind DNA as obligate heterodimers, e.g., JUN + FOS, on a shorter motif, TGACTCA. This motif also has two 4-base half sites, but in this case, the half sites overlap at the central base. AP1 factors also heterodimerize with CREB-ATF factors [81]. Leucine zipper factors in the Maf/NFE2 family are also prominent in liver, but without a known relationship to hepatic phenotypes. Maf factors form heterodimers on a larger motif, TGCTGANYCNGNN, that contains an AP1 site (underlined), and therefore share common regulatory targets with AP1 [76].

Transcription of AP1 factors is induced by mitogens, cytokines, injury, etc. They activate dynamic responses in liver and other tissues, including cell proliferation. Quiescent liver has particularly strong expression of JUND, but the significance of this expression is unclear, since the JUND knockout mouse is viable, without apparent liver abnormality [82].

The high abundance of CREBH mRNA stands out among liver-enriched transcription factors. CREBH is a regulatory target of HNF4, and its expression is prominent from late gestation. However, CREBH−/− mice are viable without liver pathology [79]. Gene regulation by CREBH is controlled posttranslationally by regulated intramembrane proteolysis (RIP). Latent CREBH localizes to ER, and RIP is activated by ER stress, e.g., treatment with tunicamycin. RIP liberates an active transcription factor that relocates to the nucleus, where it activates genes of the unfolded protein and acute phase responses.

ATF5 is another strongly expressed liver-enriched factor. Fasting stimulates transcription of ATF5 mRNA, but the main regulation is posttranscriptional, in response to ER stress, oxidative stress, or proteasome inhibition. ATF5 mRNA contains upstream region that normally blocks translation of the ATF5 reading frame. However, stress induces phosphorylation of translation initiation factor, eIF2, which then selectively activates translation from the ATF5 initiation site [80, 83].

ATF3, a mediator of different stress responses is present at low levels in quiescent liver, but strongly induced by partial hepatectomy or endotoxin treatment. In this case, however, ATF3 is a transcriptional repressor, so induction blocks responsiveness to cAMP, and may dampen activation by factors like ATF5 or CREBH [84].

KLF15. *Kruppel-like factor 15* is a prominent liver-enriched transcription factor that first appears in late gestation. Proteins in this family have C2H2 zinc fingers that bind a CACCC binding site motif [85]. Although KLF15 is the most prominent, liver also expresses several other members of this family that may have redundant or competitive function on the same binding motifs.

Dynamically increased by food deprivation and reduced by feeding, KLF15 regulates genes of glucose transport and gluconeogenesis. The KLF15−/− mouse has no pathological changes in mature liver, but lacks fasting-induced gluconeogenesis. Thus KLF15 is an important metabolic regulator [86].

SREBP1, perhaps the most abundant transcription factor in liver, has two isoforms expressed from different promoters, SREBP1a and SREBP1c. They have different activation domains, but a common basic helix-loop-helix (bHLH) domain that mediates dimerzation and DNA-binding. SREBP1c predominates and has regulated transcription, while SREBP1a is constitutively expressed at low levels [87]. Liver also expresses lower levels of SREBP2. They bind DNA as homo- and heterodimers, but with different effects: SREBP1c stimulates fatty acid synthesis, SREBP2 stimulates cholesterol synthesis, and SREBP1a stimulates both pathways equally [88]. Among gene targets, SREBP1c transcriptionally activates its own gene expression, a *feed-forward* stimulatory mechanism.

Transcription of SREBP1c is directly stimulated by LXR, indirectly stimulated by insulin, and repressed by glucagon [87]. However, most active regulation is via unique post-translational controls that respond to intracellular cholesterol. Latent SREBPs are bound to ER, and their activation is controlled by three proteins, SCAP, S1P, and S2P. With cholesterol depletion, SCAP transports SREBP1c to the Golgi complex, where endopeptidases S1P and S2P progressively release an N-terminal region. This cleaved N-terminal peptide is a complete transcription factor, a nuclear-localizing transcriptional activator. As for CREBH, the requirement for a pool of latent SREBP1c may explain the unusually high transcript levels.

SREBP2–/– germline knockout is embryonic lethal, while SREBP1 knockouts are viable with increased liver cholesterol and decreased fatty acid synthesis. Moreover, liver specific knockouts of SCAP and S1P have reduced synthesis of cholesterol and fatty acids, via strong downregulation of SREBP target genes [89]. In addition to regulation by LXR, SREBP1c integrates into the hepatocyte transcriptional network as a direct repressor of HNF4α.

PAR-domain proteins and circadian gene expression. Three proteins, DBP, TEF, and HLF, comprise a subfamily of bZIP factors with an additional PAR (proline and amino acid rich) domain [90]. PAR-domain factors bind DNA as homo- and heterodimers, and are prominent regulators in mature liver. The prototype, DBP, was first discovered by its binding at the D-site of the albumin promoter, which also strongly binds C/EBP. Indeed, the DNA binding domains of DBP and C/EBP differ by only one amino acid, a substitution that makes DBP binding about 20X more selective [69]. Thus PAR factors generally augment transcriptional stimulation at a subset of C/EBP sites, to regulate high level expression of serum proteins and metabolic enzymes. Because the genes encoding CAR and PPARα are direct regulatory targets, PAR factors preferentially activate genes of xenobiotic metabolism.

DBP and TEF are functionally redundant; simple and compound knockouts show additive losses of gene expression [90]. Both factors appear late with full expression only in mature liver [91, 92], where they control circadian gene expression. Indeed, the daily amplitude of both DBP and TEF mRNA is greater than 100-fold, causing, e.g. a tenfold variation in the levels of nascent albumin mRNA. DBP levels typically begin to rise at 2 p.m., reach maximal levels at 8 p.m., and decline sharply during the night [93, 94].

PAR factor transcription is circadian, in a pattern controlled by neuronal and humeral signals from the suprachiasmatic nucleus of the hypothalamus. In response to daylight, these signals stimulate expression of three PAS-family bHLH transcription factors, BMAL1 (ARNTL), and its heterodimeric partners CLOCK and NPAS2. Their dimers bind to E-box motifs of the DBP gene to stimulate its transcription [95]. The gene activation phase of the circadian cycle is terminated by other targets of BMAL1, corepressive proteins Cryptochrome (CRY1), and Period (PER1 and 3), which form inactivating complexes with BMAL1 heterodimers. Another pair of PAS-bHLH factors, the aryl hydrocarbon receptor (AHR) and its dimeric partner, ARNT, may regulate through the same E-boxes. Thus, xenobiotic compounds that activate AHR can override circadian regulation [96].

Nuclear receptors. In addition to HNF4, several nuclear receptors are important phenotypic regulators of mature liver. They do not have critical roles during development, but function as dynamic ligand-activated regulators. Most form heterodimers with a common partner, RXR. With limited exceptions, they all act through similar mechanisms, binding corepressors NCOR1 and SMRT (NCOR2) when unliganded, and p160 coactivators SRC1 (NCOA1), GRIP1 (NCOA2), and ACTR (NCOA3) in the presence of ligand. Some shuttle between cytoplasm and nucleus, also controlled by ligand. Multiple isoforms are typical and have subtle differences in ligand affinity. Their ligands are either endogenous products of metabolic pathways, or xenobiotic compounds. In addition to phenotypic regulation, three receptors, CAR, TR, and PPARα, stimulate hepatocyte proliferation in rodents. Some receptors, LXR and PPARγ, also function as coregulators of other transcription factors, particularly to suppress inflammatory responses [97], but the following discussion will consider only direct gene regulation in hepatocytes.

The late-appearing nuclear receptors can be divided into three overlapping groups: LXR, FXR, PPAR, and LRH1 regulate lipid, cholesterol, and bile metabolism; PXR and CAR regulate metabolism of xenobiotic compounds; RXR, SHP, and COUP are global regulators, binding partners or modulators of other nuclear receptors. The nuclear receptors transcriptionally regulate each other and thus integrate into the hepatocyte regulatory network of transcription factors [9, 98, 99].

With the exception of LRH1, the nuclear receptors bind DNA as dimers, with each subunit contacting a specific half-site motif. The half sites are usually arranged as imperfect direct repeats (DR) or inverted repeats (IR), separated by 0–5 base pairs. As for all transcription factors, binding tolerates wide variation in the motifs, and some flexible dimers may accommodate variable spacing between the half sites. Thus, the motifs are conventions with limited predictive value. They do not readily discriminate the binding of different receptors. DR4 (e.g., AGGTCA NNNN AGGTCA) describes the binding of RXR-CAR, RXR-PXR, RXR-LXR, and RXR-TR dimers, but there is limited overlap among their regulatory targets.

LRH1 (NR5A2) is an unusual nuclear receptor because it binds DNA as a monomer on an extended motif, YCAAGGYCR [100, 101]. Initially found in liver as a regulator of AFP (*fetoprotein transcription factor*, FTF) [102], LRH1 is well integrated into the hepatic transcription factor regulatory network of mature liver, stimulated by both C/EBP, FOXA2, HNF1 and HNF4α, and a stimulator of FXR, PXR, and HNF1 [9]. Knockout mutant embryos have defective visceral endoderm and die before liver formation. In contrast, liver-targeted knockout has a mild phenotype, morphologically normal liver with impaired expression of genes that regulate cholesterol and bile acid metabolism [101].

Various phospholipids can bind LRH1 within the ligand-binding domain, and stimulate transcriptional activation [103], but their relationship to metabolic regulation is unclear. LRH1 can form heterodimers with the incomplete nuclear receptor, SHP, which leads to repression of LRH1-target genes [104]. PROX1, itself a transcription factor, also acts as a corepressor of LRH1 [105].

COUP. Liver expresses three nuclear receptors in the *COUP/NR2F subfamily:* EAR2 (NR2F6), COUP-TF2 (ARP, NR2F2), and COUP-TF1 (EAR3, NR2F1). EAR2, strongly expressed, is liver enriched [106], although most studies have focused on COUP-TF1 and -TF2. All three are most strongly expressed in early liver, but persist at lower levels throughout life. They bind DNA as homodimers on DR1 sites, but can also heterodimerize with RXR and probably with each other [107]. COUP-TF1–/– mutation causes embryonic death before E10. COUP-TF1+/– heterozygotes have moderate changes in liver gene expression and are resistant to steatosis induced by a high fat diet [108]. The COUP-TF2–/– mouse dies perinatally, and the EAR2 –/– mutant is viable without obvious liver abnormalities, though they lack characterization of liver gene expression or metabolic responses [106, 109].

The physiological ligands are not known [107] and the COUP effects on liver gene expression are generally repressive, suggesting activity of unliganded receptors. COUP dimers inhibit gene expression by competing for DR1 sites with HNF4α, RXR-PPAR, and RXR-RXR dimers [110]. Within the hepatocyte regulatory network, COUP acts directly on regulatory elements of HNF4α, HNF1α, HNF6, and LRH1 genes [9].

SHP (small heterodimeric partner, NR0B2), is an unusual nuclear receptor because it does not have an N-terminal DNA-binding domain [104, 111, 112]. No activating ligands are known, and unliganded SHP acts as a repressor by two different mechanisms. First, SHP is a dimeric partner of LRH1, and the LRH1-SHP dimer is strongly repressive [104]. Second, SHP has LXXLL motifs that typify coactivators, not nuclear receptors. These allow SHP to compete for coactivator binding sites, and leads to repression, since SHP recruits corepressors after binding. By this mechanism, SHP can corepress HNF4α, PPAR, LXR, CAR, and FXR [111, 112]. Moreover, FXR and PXR induce SHP expression, and thus induce negative feed-back to dampen their activation of target genes. In normal liver, SHP shows significant circadian expression as a direct transcriptional target of BMAL1. SHP therefore imposes circadian modulation to both constitutive and inducible genes [113].

FXR. The so-called Farnesoid X receptor (NR1H4) is actually a receptor of bile acids, especially chenodeoxycholic acid and its derivatives. FXR generally binds DNA as a heterodimer with RXR on an IR1 binding motif. Farnesoids and retinoids also activate, but only at high nonphysiological levels [114].

Expression appears in liver near the end of gestation. FXR knockouts have normal liver development, but develop moderate fatty liver and hyperlipedemia [115, 116]. The livers have marked changes in gene expression: reduced expression of bile acid transporters, apolipoproteins, and genes of carbohydrate and amino acid metabolism. FXR is therefore a critical regulator of cholesterol catabolism, controlled by the level of bile acids. Within the hepatocyte transcription factor network, FXR is the regulatory target of HNF1, HNF4, Foxa, and LRH1, and in turn activates expression of SHP and PXR [9, 114, 117].

In addition to activation of target genes, FXR represses other genes, notably cholesterol 7-α-hydroxylase gene (CYP7A1), which encodes the rate-limiting enzyme in bile acid synthesis. In this case, the negative regulation is indirect. LRH1 activates CYP7A1 transcription. FXR stimulates expression of SHP, which binds LRH1 and converts activation to repression [118].

LXR. The *Liver X receptors* — liver-enriched LXRα (NR1H3) and widely-expressed LXRβ (NR1H2) — balance the metabolic effects of FXR [119]. LXR, when liganded by oxidized cholesterol derivatives, directly stimulates genes of fatty acid and triglyceride synthesis, cholesterol uptake and its conversion to bile acids. LXR also suppresses genes of gluconeogenesis by an indirect mechanism. Mice with knockouts of LXRα, LXRβ, and double mutants, are all viable with normal liver development [119]. The LXRα–/– develops an enlarged fatty liver, and double knockout exacerbates this phenotype. LXRα stimulates its own transcription by a feed-forward mechanism [120], and integrates into the hepatocyte transcriptional network via stimulation by HNF4α and HNF6 [10].

PPAR. The *Peroxisome Proliferator Activated Receptor* has three isoforms – PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3). All are present in liver, but PPARα dominates. PPARα was originally characterized as a drug receptor that regulated a striking response of hepatomegaly with massive accumulation of peroxisomes, accompanied by hypolipedemia. Regulatory target genes include acyl CoA oxidase (ACOX), other components of the peroxisomal fatty acid β-oxidation pathway, and genes regulating fatty acid uptake [121]. PPAR have unusually large ligand-binding pockets that bind diverse ligands including various unsaturated fatty acids, eicosanoids, and clofibrate drugs. The ligand specificity of PPARγ, significantly diverged from PPARα, includes the thiazolidinedione drugs [122]. The PPARα–/– mouse completely loses the pathological responses to PPARα-specific ligands [123]. However, PPARβ and γ are functional in liver, because high doses of drugs specific for β or γ can induce peroxisome proliferation in PPARα–/– mice [124]. Thus, the main difference among the three isoforms is ligand specificity, not DNA binding or target gene selection.

The crystal structure of PPARγ-RXRα dimer is distinctive. Its polarity with RXR is reversed compared to other nuclear receptor heterodimers. The PPARγ subunit is 5' to RXR, each sitting on a half site within a DR1 motif. Also, PPARγ has a C-terminal extension of its DNA binding domain that contacts additional bases 5' to the first half site. These features are conserved in PPARα and β [125].

In addition to its effects on phenotypic genes, PPARα also stimulates a proliferative response in rodent liver. The effect is very significant, since prolonged stimulation leads to hepatocellular carcinoma. PPARα ligands are thus *nongenotoxic carcinogens*, but an elegant study showed that the effect is embodied in rodent but not human PPAR. The PPARα–/– mouse was "humanized" by incorporation of a transgene encoding human PPARα. The human and mouse genes vary at only a few amino acids, and activate an identical set of phenotypic target genes in response to activating ligand. However, human PPARα does not induce the proliferative response [123].

PXR. Another nuclear receptor, PXR (NR1I2), functions primarily as a receptor of xenobiotic compounds [126], although the toxic bile acid, lithocholate, is an endogenous ligand [127]. The induced genes metabolize lithocholate and a wide variety of drugs [128]. Liganded PXR dimerizes with RXR on DR4 binding motifs [129], and the prototype target gene in human is *CYP3A4* (= mouse *Cyp3a11*, rat *Cyp3a1*). Other activations include genes expressing Phase I and II drug metabolizers and drug transporters [130]. The DNA binding domain, and hence target gene specificity, is highly conserved. In contrast, the ligand-binding domain has significantly diverged so many activating ligands are species-specific. Thus, the standard experimental ligand, pregnenolone-16α-carbonitrile (PCN) activates mouse, but not human PXR, while rifampicin has the opposite specificity [126]. The PXR–/– mouse is essentially normal, but with slight impairment of regenerative and injury responses [131]. A human PXR transgene has been incorporated into the PXR–/– mouse, to create a valuable model with humanized drug responses [128].

CAR (NR1I3), the so-called *Constitutive Androstane Receptor*, is another xenobiotic receptor that heterodimerizes with RXR on DR4 binding sites [132]. The classical CAR-responsive gene is human *CYP2B6* (= rat *Cyp2b1*, mouse *Cyp2b10*), a relationship revealed by the response to phenobarbital [132, 133]. CAR mediates detoxification responses by activating numerous genes — p450 cytochromes, sulfotransferases, and transporters — in response to a wide range of ligands. As for PXR, there is divergence of affinity for specific ligands among species, while DNA binding is highly conserved. Beyond its spectrum of gene regulation, CAR has distinctive features compared to other nuclear receptors. Due to the altered structure of the Helix 12 segment of its ligand-binding domain, CAR is a constitutive transcriptional activator in the absence of ligand. Nevertheless, gene responses are inducible because CAR, normally cytoplasmic, migrates to the nucleus when liganded. There are added complexities because phenobarbital, the prototype CAR activator, induces translocation by an unknown mechanism without actually binding as a ligand. In addition, certain compounds, for example, androstanol, are *inverse agonists* that bind to CAR and cause it to become repressive [134].

CAR activates a strong proliferative response in hepatocytes, another distinctive feature. As a consequence, CAR activators like phenobarbital or the mouse-specific ligand, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) are powerful tumor promoters. These drugs induce liver hyperplasia via a set of gene responses that differs significantly from the compensatory proliferation of liver regeneration [135, 136]. Despite its cancer-inducing potential, CAR-induced growth is presumably an adaptive response that rapidly increases the liver's ability to detoxify injurious compounds. However, CAR is dispensable for normal development and liver function [137].

RXR. Three *Retinoid X Receptor* isoforms: RXRα (NR2B1), RXRβ (NR2B2), and RXRγ (NR2B3), bind DNA as the heterodimeric partners of many nuclear receptors [138]. Among these, RXRα is a liver-enriched transcription factor, not surprising, because of the important metabolic functions of its partners in the hepatocyte. Despite its abundance, however, there is no obvious regulation of RXRα expression by factors in the hepatocyte transcription factor network.

RXRs also homodimerize on DR1 binding sites, with a specific activating ligand, 9-cis retinoic acid [138]. It is difficult to discriminate the direct the targets of RXR-RXR dimers, because 9-cis retinoic acid also activates RXR within *permissive* heterodimers (e.g., RXR-CAR, RXR-PXR, RXR-LXR, RXR-PPAR, and RXR-TR) to increase stimulation of their target genes [139]. Without ligand, RXR generally acts as a passive partner in heterodimers [140].

RXRα knockout is developmentally lethal because of cardiac defects, but a hepatocyte-specific RXR knockout is viable. This mouse develops extreme fatty liver and hyperlipidemia, indicating the importance of RXR as the dimeric partner of numerous factors that regulate lipid metabolism [141].

Repression and the Oncofetal Paradigm

This chapter has portrayed liver development as the progressive acquisition of activating transcription factors. An opposite paradigm developed from studies of AFP, a serum protein that appears during early liver development. AFP reappears in liver cancer and is thus the prototype *oncofetal protein*. This pattern of expression has led to two influential concepts – that development requires repression of fetal or embryonic properties, and that carcinogenesis is a reawakening of these properties. In the adult phenotype, the repressed AFP gene is different from many other nonexpressed genes because dynamic regulation continues via binding of transcription factors and recruitment of corepressors [15]. Nevertheless, the reason for AFP repression is unknown, since persistent expression has no detrimental effects. Three other genes share

the oncofetal expression pattern – Glypican 3 (GPC3), the microRNA precursor H19, and lipoprotein lipase [142–144]. They have limited developmental or oncogenic activity, and recent global studies of gene expression have not significantly enlarged the set of oncofetal proteins in liver.

The repression of AFP is complex and at least four different repressive mechanisms contribute. The first is repression by ZHX2, previously known as AFP repressor 1 (Afr1). Part of an unusual family of transcription factors that have two C2H2 zinc fingers and five homeodomains, it is unclear whether ZHX2 acts as a DNA-binding repressor, or as a corepressor that binds other transcription factors. Many tissues express low levels, but liver expression of ZHX2 is exclusively postnatal. BALB/cJ mice have moderate persistent expression of AFP and GPC3, because this strain carries an inactive gene due to an insertional mutation. BALB/cJ is therefore a naturally-occurring ZHX2-null mutant. C3H mice carry a permissive allele of a second repressive gene, Afr2, which allows persistent AFP expression after liver regeneration [142]. Neither the ZHX2-null mutant nor the permissive allele of Afr2 are associated with developmental abnormalities or liver pathology.

ZBTB20 (DPZF), a third AFP repressor, is a C2H2 zinc finger transcription factor selectively expressed by mature liver. ZBTB20 binds near the AFP gene promoter and a knockout mouse has clear derepression of AFP, although other changes in liver gene expression are nonphenotypic. The knockout has normal development and no liver pathology, but the mice die by 12 weeks. Introduction of a liver-specific ZBTB20 transgene repressed AFP and reversed some other changes in gene expression, but did not rescue the lethality [145, 146]. The major effects of ZBTB20 are therefore nonhepatic.

A final repressive mechanism targets LRH1. This strong positive regulator of AFP [102] is converted to a transcriptional repressor by binding either SHP [104] or PROX1 [45], both abundantly expressed in adult liver.

Beyond the repressive activities that regulate AFP, many other repressors activities modulate liver gene expression. Phenotype-specific repression centers on nuclear receptors, since many are repressive when unliganded. Moreover, SHP and PROX1 are repressing partners or corepressors. Several dynamic housekeeping factors prominent in liver (Fig. 14.2) modulate between repression and activation. For example, repressing NFκB1 (p50) homodimers alternate with activating NFκB1-RELA (p50-p65) heterodimers. The abundance of several zinc-finger proteins that are homologs of known repressors (ZNF238, ZNF92, Z91) also suggest nonphenotypic repression, although their specific gene targets are unresolved. Finally, liver expresses high levels of ID1–3, incomplete bHLH factors that dimerize with and antagonize many activators in this important transcription factor family. The inability to generalize developmental, phenotypic, or pathological mechanisms from the specialized postnatal regulation of AFP is a major disappointment. Instead, most repression dynamically targets individual genes.

Closing

The hepatocyte is a synthetic factory of serum proteins, and appearance of these proteins is synonymous with the early phenotypic development of the liver. This serum-protein defined phenotype depends on a constitutive network of mutually regulated transcription factors, FOXA, C/EBP, HNF1, HNF4, and HNF6, but these always collaborate with housekeeping transcription factors. Beyond their mutual regulation, the early expression of these factors must be driven by HEX and other direct developmental regulators. After birth, the hepatocyte becomes a primary regulator of lipid, carbohydrate and amino acid metabolism, bile synthesis, and detoxification. The enzymes that regulate these processes gradually appear during late gestation after the liver has formed, or even during postnatal maturation. Expression of these metabolic regulators is largely controlled by a new network of nuclear receptor transcription factors (FXR, LXR, PXR, CAR, PPAR, and RXR) that collaborate with the constitutive network. The essential characteristic of these new regulators is that they are dynamic, modulating from repression to activation in response to hormonal, metabolic, and xenobiotic signals. With full maturity, the dynamic regulation becomes integrated into a circadian physiological pattern by DBP/TEF, factors regulated by signals from the central nervous system.

Although this formidable list of transcription factors explains many aspects of liver development and function, the presentation in this chapter has simplified or omitted a number of important issues. One is the complex function of individual factors. Each selectively recruits coactivators and corepressors after they bind to the DNA. These coregulators are part of the machinery of transcriptional regulation, but few are cell specific.

Transcription factors respond to regulatory information that is encoded in the genome, but progress on deciphering this information is limited. Even for well-studied factors, the deduced binding motifs have considerable ambiguity. More important, even the most efficient binding motifs occur widely, and most do not regulate gene expression. The true regulatory sites, often hard to recognize, are clustered with binding sites for other transcription factors. The clustering enables the factors to synergize. The synergy is mediated by cooperative binding of factors to each, cooperative modification of local DNA structure, and cooperative recruitment of coregulators by multiple transcription factors. New research has also revealed another type of factor interaction, a *scripted*

sequence of bindings. The prototype is FOXA binding as a *pioneer factor* to native chromatin, which causes local remodeling of histones to allow eventual binding by a second factor. These interactions will eventually define the language of liver gene expression. It is this language, not individual transcription factors, that makes the liver unique.

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Chapter 15
Bile Duct Development and Biliary Differentiation

Frederic P. Lemaigre

Introduction

Bile is excreted by the hepatocytes in the bile canaliculi and flows via the canals of Hering into the intrahepatic bile ducts. See Chap. 1 for more details on liver anatomy. The latter drain the bile to the intestine via the extrahepatic biliary tract, which consists of hepatic ducts, cystic duct, gallbladder, and common bile duct. All segments of the biliary tract are delineated by cholangiocytes, a specialized epithelial cell type that modifies the composition of the bile when it transits through the ducts. An independent chapter describing biliary epithelial cells is included in the textbook (see Chap. 4). Within the liver, the biliary tree forms a branched network in which the ducts are classified with respect to the lobular architecture into ductules and interlobular ducts [1]; an alternative view which takes the functional heterogeneity of the cholangiocytes into account classifies the bile ducts according to their size [2–4].

The cholangiocytes that line the intrahepatic and extrahepatic biliary tracts have different embryonic origins: Intrahepatic biliary cells derive from hepatic precursor cells, extrahepatic cholangiocytes derive directly from the endoderm. Development of the intrahepatic ducts has been intensively investigated at the molecular level, leading to significant improvement of our understanding of the molecular circuitry involved in biliary differentiation and morphogenesis. However, no mechanism has yet been identified that differentiates the development of the large vs. small ducts or of subsegments of the intrahepatic biliary tree. Nor is it clear how ramification of the ducts is programmed. Development of the extrahepatic ducts is even less well understood, and due to their proximity with the ventral pancreatic buds, they have mainly been studied in parallel with the development of the pancreas. In this chapter, we will discuss the mechanisms of cholangiocyte differentiation and of intra- and extrahepatic duct formation with the aim to provide a basis for understanding biliary dysgenesis and dysfunction in human disease.

F.P. Lemaigre (✉)
de Duve Institute, Université catholique de Louvain,
Brussels, Belgium
e-mail: frederic.lemaigre@uclouvain.be

Development of the Intrahepatic Bile Ducts

Markers of Cholangiocyte Differentiation

The earliest morphological sign of liver development is a thickening of the endoderm on the ventral wall of the gut. This is detectable in the human embryo around the 18th day of gestation, and in the mouse embryo around embryonic day (E) 8.5. Slightly later, the gut forms a diverticulum at the level of this thickening and so generates a liver bud mainly composed of hepatic precursor cells. For more details on liver development, please see Chap. 13. The hepatic precursor cells sprout from the liver bud, invade the septum transversum mesenchyme and interact with the vasculature to progressively form the liver parenchyma [5, 6]. These cells are also called hepatoblasts and based on their gene expression profile are considered to be the progenitors of the hepatocytes and intrahepatic cholangiocytes [7–9]. Although this view of a common origin of the two cell types dates back more than two decades and has since been further supported by numerous morphological and molecular evidences, a rigorous in vivo genetic lineage tracing has not yet been performed. Despite this limitation, the hypothesis that intrahepatic ducts may be formed by ingrowth of the extrahepatic ducts (reference [10], cited in [9]) is no longer considered and the differentiation of intrahepatic cholangiocytes will be discussed in this chapter as resulting from a lineage choice of hepatoblasts.

Biliary development in human and rodent liver starts with the expression of cholangiocyte markers in hepatoblasts adjacent to the mesenchyme surrounding the branches

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_15, © Springer Science+Business Media, LLC 2011

of the portal vein. The identification of cholangiocytes is based upon the expression of specific marker proteins, among which biliary-specific cytokeratins (CK) were long considered as the most useful. In human liver, the earliest hepatoblasts express CK8, 18, and 19. When they give rise to cholangiocytes, the expression of CK19 increases in cholangiocytes, but becomes extinguished in hepatocytes, whereas the expression of CK8 and 18 persists in both cell types. At a later stage, CK7 becomes specifically expressed in the duct cells [11, 12]. In mouse and rat liver, CK19 is absent from hepatoblasts and is only detectable in cholangiocytes albeit with some technical difficulties in early cholangiocytes owing to antibody and sample preparation procedures [13]. The fact that CK19 is not cholangiocyte-specific in humans and the observation that its detection is sometimes difficult in rodent cholangiocytes [13] has prompted the search for early and more specific biliary markers. The recent identification of SRY-related HMG box transcription factor 9 (Sox9) as a biliary marker, opens new perspectives. This transcription factor is detected in periportal hepatic cells starting at E11.5 and remains restricted to biliary cells, except in CC14-injured liver in which Sox9 is also detected in activated stellate cells [14, 15]. Another cholangiocyte marker, osteopontin, becomes expressed slightly later than Sox9 and is also useful to characterize early biliary development [14]. Several other proteins are used as markers of cholangiocyte differentiation. These include γ-glutamyl transpeptidase IV (rat) or VI (mouse and human) [16], connexin 43 [17], integrin β 4 [18], or binding proteins for *Dolichos biflorus* agglutinin [19]; they are either expressed in hepatoblasts and are less specific to biliary cells than Sox9, or were only described at a late stage of biliary development.

Biliary Cells Differentiate Near the Portal Vein

The observation that cholangiocyte differentiation takes place near the portal mesenchyme prompted the search for localized mechanisms that drive differentiation of hepatoblasts to cholangiocytes. In recent years, Activin/Transforming Growth Factor beta (TGFβ) and Notch signaling were found to match the criteria for local inducers of cholangiocyte differentiation. To learn more about Notch and TGFβ signaling cascade, please see Chap. 20. The TGFβ ligands, ActivinA, TGFβ2, and TGFβ3, are predominantly expressed in the periportal mesenchyme, and they bind to differentiating cholangiocytes. TGFβ signaling is strongly active in the periportal region, as shown by the periportal expression of green fluorescent protein in a mouse line that expresses this protein under control of a TGFβ-responsive promoter. In addition, treating cultured liver explants or hepatoblast lines with TGFβ triggers differentiation of hepatoblasts to cholangiocytes, and perturbation of vtion [14, 20, 21].

Notch signaling has been suspected for a long time to regulate the initiation of biliary differentiation. This assumption was based on genetic studies, which identified mutations in Notch signaling components as the cause of Alagille syndrome, a polymalformative disease in which patients are affected with bile duct paucity (OMIM #118450 and #610205). Indeed, mutations in the ligand Jagged-1 and in the receptor Notch2 were identified [22–24], prompting several teams to investigate the involvement of these two proteins in bile duct development. A first issue was to determine which cell types express Notch signaling components in developing and adult liver, and Loomes et al. summarized the often controversial data on this topic [25]. This set of data, the recent immunostainings using Jagged-1 antibodies and the analysis of mice expressing a reporter gene under control of Notch2 regulatory sequences, suggest that Notch2 is expressed in differentiating cholangiocytes, whereas Jagged-1 is found in the periportal mesenchyme [26, 27]. This explains in part how Notch signaling may restrict biliary development to the periportal area. However, Jagged-1 is also expressed in the developing cholangiocytes [27], indicating that cell-autonomous activities of Notch signaling may contribute to cholangiocyte differentiation. Periportal mesenchymal cells constitute a heterogenous cell population and it is not yet determined with accuracy which mesenchymal cells express Jagged-1, except for a recent report indicating that a subset of mesenchymal cells characterized by expression of the p75 neurotrophin receptor also coexpress Jagged-1 [28].

Beyond the analysis of the expression pattern of Notch signaling components, several animal models were investigated to address the function of this pathway. These include the zebrafish [29] and several mouse models that are deficient in Jagged-1, Notch1, Notch2, or combinations thereof [26, 30, 31]. Although the study of these animal models provided compelling evidence for a role of Notch in duct formation, it did not support the notion that Notch signaling controls the initiation of cholangiocyte differentiation, most likely as a result from redundant functions of the various Notch ligands and receptors. However, this potential redundancy was recently bypassed by inactivating Recombination Signal-Binding Protein 1 for J-κ (RBP-Jκ [(καππα)]), a common mediator of Notch signaling. The results revealed that when Notch signaling is inhibited, the number of hepatoblasts differentiating to the cholangiocyte lineage is strongly reduced (27), thereby demonstrating that Notch signaling is required to initiate biliary differentiation. Moreover, when the intracellular domain of Notch, which is cleaved from the Notch receptor upon stimulation by Jagged-1 is overexpressed in liver, it can induce hepatoblasts to differentiate to biliary-like cells [27, 32].

There is also strong evidence for an involvement of other signaling pathways in cholangiocyte differentiation, but in contrast to Notch and TGFβ, these other pathways are not known to contribute to the periportal location of the differentiation process. Wnt signaling has attracted much attention and is now known to play key roles at all stages of liver development [33]. To learn more about Wnt/β-catenin signaling, please see Chap. 20. It is particularly difficult to unravel, given the high number of ligands and receptors expressed in liver, and the involvement of Wnt in a variety of cellular events such as proliferation, apoptosis, and differentiation. A key role for Wnt signaling in biliary differentiation was initially suggested in experiments in which treatment of liver explants with antisense to β-catenin led to absence of ck-19-positive cells and treatment with Wnt3a induced biliary differentiation [34, 35]. This interpretation was later supported by the analysis of mouse mutants in which the activity of the Wnt mediator β-catenin was either inhibited or stabilized: Inactivation of β-catenin by a Cre-loxP-mediated gene targeting approach resulted in multiple liver defects including duct paucity [36], and enhanced activity of β-catenin mediated by depletion of Adenomatosis Polyposis Coli was associated with increased biliary development [37]. The combination of these gain- and loss-of-function approaches suggests that Wnt signaling promotes cholangiocyte differentiation, but it remains unknown, which ligands and receptors participate in vivo in this process.

Finally, a role for the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) pathways in intrahepatic biliary development has recently been proposed. Using the chick liver as experimental model, it was shown that FGF-2 and FGF-7 synergize with BMP-4 and extracellular matrix components to promote differentiation of hepatoblasts to biliary cells [38]. Interestingly, in parallel experiments with mice, it was shown that the BMP mediator Smad5 is predominantly expressed in the periportal area [39], thereby suggesting one more mechanism to restrict biliary differentiation around the vicinity of the portal vein.

Transcriptional Network Initiating Intrahepatic Biliary Differentiation

Whereas cell–cell signaling mechanisms are essential to ensure that biliary cells are located near the portal vein, cell intrinsic cues must determine how the cholangiocytes acquire their specific properties. Transcription factors stand out in this process and it is not surprising that most of the available information derives from the phenotypic analysis of transcription factor-deficient mouse livers. Hepatocyte nuclear factor-6 (HNF-6), also called Onecut-1, was the first transcription factor shown to control biliary differentiation. It is expressed in hepatoblasts starting at the onset of liver development, and is maintained in hepatocytes and cholangiocytes, with highest levels in the cholangiocytes. In its absence in HNF-6 knockout mice, the expression of biliary markers is initiated prematurely, and periportal hepatoblasts display "hepatobiliary" features, i.e., they coexpress hepatocyte and biliary markers. Onecut-2 (OC-2), a paralog of HNF-6, is redundant to HNF-6, and double HNF-6/OC-2 knockouts display an even stronger phenotype in which not only the periportal hepatoblasts, but all the hepatic epithelial cells appear as hybrid hepatobiliary cells [20, 40]. This phenotype results from a severe perturbation of TGFβ signaling. Indeed, in the double HNF-6/OC-2 knockouts, the activity of the Activin/TGFβ pathway extends further in the parenchyma as compared with wild-type livers, leading to the interpretation that the hepatobiliary phenotype results from a superposition of a TGFβ-induced biliary program on the hepatocyte program. In other words, HNF-6 and OC-2 control biliary differentiation by adjusting TGFβ signaling to appropriate levels; this may occur via a control exerted on the expression of the TGFβ receptor type II (TβRII) and of the Activin/TGFβ inhibitor follistatin [20]. In parallel with its regulation of TGFβ signaling, HNF-6 was also shown to stimulate HNF-1β, another regulator of biliary development [40, 41]. Finally, the role of HNF-6 is conserved in other species. Knockdown of the functional ortholog of HNF-6 in zebrafish (ZfOnecut-3) resulted in fewer and poorly organized ducts and ectopic expression of biliary cytokeratin in the parenchyma [42].

Since HNF-6 and HNF-1β were the first factors identified as transcriptional regulators of biliary differentiation, subsequent studies of transcription factor-deficient mice usually investigated HNF-6 and HNF-1β expression, leading to the somewhat biased view that the cascade linking the two factors is central in the biliary transcriptional network. Taking this remark into consideration, it is still possible to draw a model of this network (Fig. 15.1).

The Notch intracellular domain is a transcriptional coregulator, which represses differentiation of isolated hepatoblasts towards hepatocytes, but promotes induction of the biliary markers CK19, CK7, HNF-6, and HNF-1β [43]. Interestingly, the intracellular domain of Notch, when associated with RBP-Jκ(καππα), binds to the Sox9 gene. These data further extend the notion that the initiation of biliary differentiation is marked by the expression of Sox9 and that Notch signaling contributes to this process. The zinc-finger transcription factor Sa114 has similar effects as Notch: When it is overexpressed in isolated hepatoblasts, it represses hepatocyte differentiation and enhances biliary differentiation [44]. This most likely occurs via the stimulation of Notch signaling, since both Jagged-1 and Notch2 are induced in the cultured cells. Other regulators of biliary marker expression

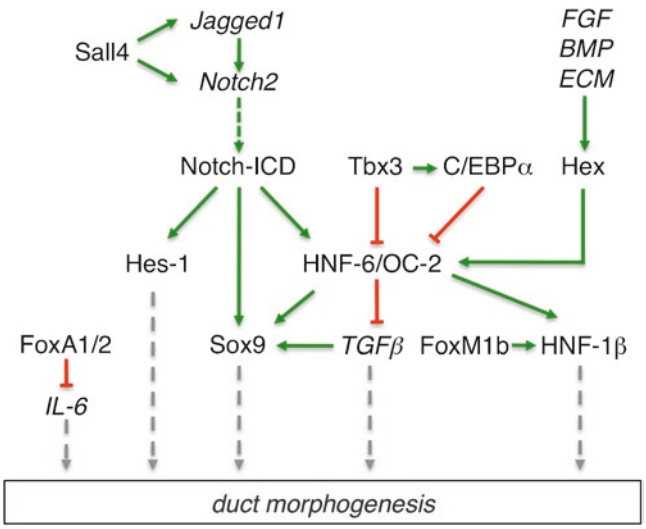

Fig. 15.1 Transcriptional network regulating biliary differentiation and bile duct morphogenesis. Transcriptional regulatory cascades are indicated, with *green arrows* referring to stimulatory interactions and *red bars* to inhibitions. Terms in *italics* are signaling factors or mediators

are the hematopoietically expressed homeobox factor (Hhex) and the forkhead box factor (Fox), FoxM1B. In Hhex knockouts, biliary differentiation is abnormal, and HNF-6 and HNF-1β expression is deficient [45], while in FoxM1B-deficient livers no cholangiocytes are detected [46].

Transcription factors that exert a negative regulation on biliary differentiation have also been identified. The T-box transcription factor 3 (Tbx3) represses HNF-6 and HNF-1β, and it stimulates hepatocyte differentiation. This regulation is somewhat peculiar. Indeed, Tbx3 knockout mice display a premature increase in the expression of HNF-6 and HNF-1β in the earliest hepatoblasts (E9.5) [47]. Therefore, Tbx3 may regulate the timing of differentiation. However, the mode of action of Tbx3 seems very dynamic. Indeed, at a slightly later stage (E12.5), cultured hepatoblasts in which Tbx3 is repressed continue to display increased expression of the biliary markers CK19 and CK7. However, unlike at E9.5, this effect is dependent on $p19^{Arf}$. In wild-type cells, at E12.5, Tbx3 normally represses $p19^{Arf}$ and in that way stimulates hepatocyte differentiation at the expense of biliary differentiation [48]. Interestingly, Tbx3 also initiates a forward negative loop by stimulating the expression of CCAAT/Enhancer Binding Protein alpha (C/EBPα), the latter being known as a repressor of HNF-6 and HNF-1β [49].

Importantly, this transcriptional network only provides the core of gene regulations, but it does not explain how specific biliary functions, such as polarity markers or ion transporters, are induced. This results from the limited number of differentiation genes that were identified as targets of the various transcription factors. The TGFβ receptor type II and the Activin/TGFβ inhibitor, follistatin, have been mentioned above as targets of HNF-6 and OC-2 [20]. Moreover, Sox9 and Hes-1 are targets of HNF-6 and of Notch signaling, respectively, and are transcriptional regulators of duct morphogenesis [14, 27, 50].

Intrahepatic Bile Duct Morphogenesis: A Multistep Process

In parallel to differentiation, cholangiocytes must organize to form ducts. This occurs according to a multistep process that is unique in many ways as illustrated in Fig. 15.2. During the initial morphogenic event, the differentiating cholangiocytes line up around the periportal mesenchyme, thereby forming a single-layered ring of cells called the "ductal plate." The cells that form the ductal plate are cholangiocyte precursors, which are partly polarized i.e., they have a basal pole that is separated from the mesenchyme by a basal lamina, but they have not yet acquired typical apical markers.

In the following stage, cells that display characteristics of hepatoblasts associate with specific areas of the ductal plate; they remain separated from the ductal plate by a lumen and so constitute asymmetrical ducts with the cholangiocyte precursors. These ducts are called primitive ductal structures (PDS), and are considered radially asymmetrical because they are lined on the portal side by cholangiocyte precursors and on the parenchymal side by hepatoblasts [14]. The PDS can be identified using a combination of markers: The cholangiocyte precursors on the portal side express Sox9, Jagged-1 and high levels of E-cadherin, whereas the hepatoblasts on the parenchymal side express HNF-4, TβRII, and lower levels of E-cadherin. Polarization of the cells progresses when the PDS are formed. As soon as a lumen is detectable, all the cells develop tight junctions and express osteopontin at the apical pole. Interestingly, the in vivo data are consistent with the idea that PDS develop by apposition of hepatoblasts to the ductal plate cholangiocytes. However, in an attempt to reconstitute tubulogenesis in vitro, it was shown that this process may be initiated by migration of cells of the single-layered ductal plate [51]. Cells grown in between two layers of extracellular matrix acquire ductal plate cell characteristics and form a monolayer; some cells then depolarize and move upwards to constitute a second layer followed by repolarization and delineation of a lumen. Whether this model faithfully recapitulates in vivo tubulogenesis still needs to be confirmed.

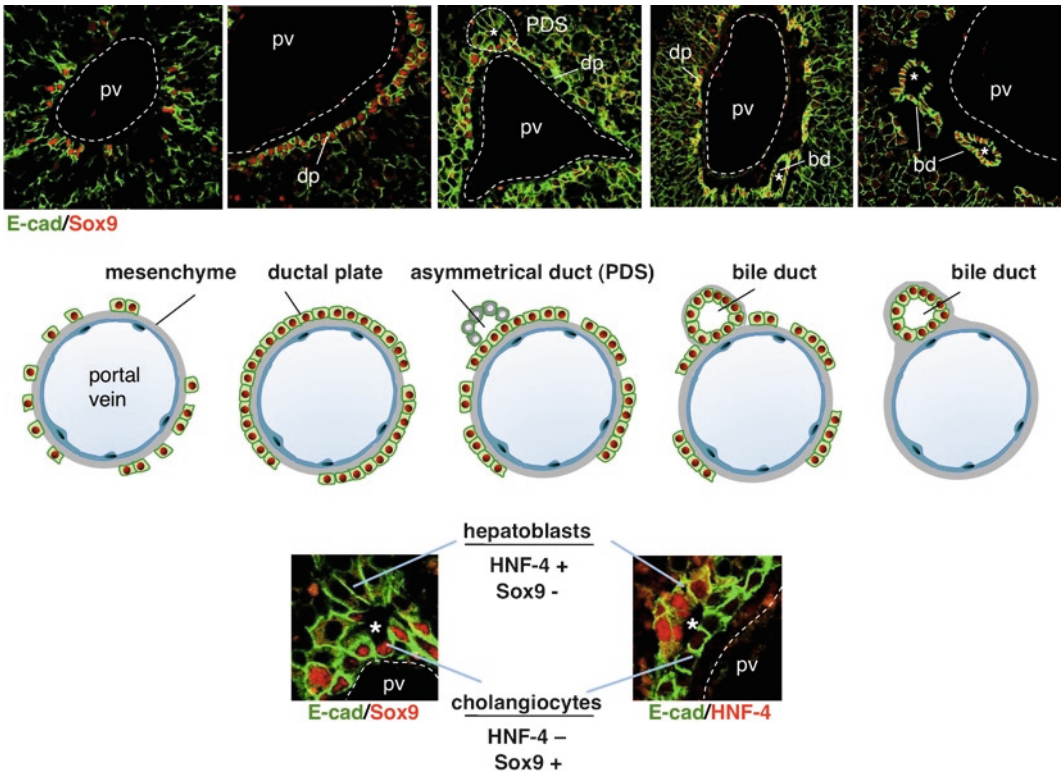

Fig. 15.2 Morphogenesis of the intrahepatic bile ducts. The *upper panels* illustrate the sequential stages of bile duct development on sections immunostained to detect E-cadherin (*green*) and Sox9 (*red*) which are strongly expressed in the cholangiocytes. The *middle panels* show a schematical representation of the corresponding *upper panels*. The *lower panels* illustrate the asymmetrical stage at which the primitive ductal structures (PDS) are lined on the parenchymal side by HNF-4-expressing hepatoblasts, and on the portal side by Sox9-expressing cholangiocytes. *bd* bile duct; *dp* ductal plate; *pv* portal vein

Beyond the stage of PDS, the forming tubules develop into mature and radially symmetrical ducts. The hepatoblasts on the parenchymal side repress the expression of the hepatoblast markers and acquire expression of cholangiocyte markers; all cells become fully polarized as indicated by the baso-lateral location of E-cadherin and the formation of a complete basal lamina surrounding the duct. Eventually the ducts are radially symmetrical as they are entirely lined by cholangiocytes.

During the formation of the PDS and their maturation in ducts, the ductal plate cells not involved in ductogenesis are eliminated, a process called "ductal plate remodeling." How this takes place is not well understood. It has been proposed that apoptosis contributes to the regression of the ductal plate in humans [52]. However, in mice, no obvious sign of apoptosis could be detected (unpublished). In addition, in the light of recent knowledge on the asymmetrical mode of biliary tubulogenesis, it is surprising that the apoptotic cells shown in human liver seem to correspond to the portal layer of PDS [52]; these cells are not those expected to be eliminated during ductal plate remodeling. Therefore, there is most likely a need to re-evaluate the fate of ductal plate cells in developing liver.

In the next step of duct morphogenesis, the periportal mesenchyme encircles the bile ducts. This step is often referred to as the "incorporation stage," but its mechanism is poorly understood. It could result from migration of the ducts in the portal mesenchyme, or of proliferation of mesenchymal cells around the ducts, or both. Duct migration would require that matrix proteinases are secreted by cholangiocytes and two studies indeed report that this is the case [53, 54].

Whereas the paragraphs above describe how a duct structure is formed, they do not explain how the ducts grow. In the prenatal period in mice, duct growth essentially results from differentiation of cholangiocytes and maturation of PDS. Duct development progresses from the hilum to the periphery of the lobes and this explains why in developing liver all stages of duct maturation can be found within a liver lobe. At the end of gestation, the number of proliferating cholangiocytes increases, indicating that further duct extension in length and diameter also depends on proliferation.

When duct development is terminated, the cholangiocytes become mitotically quiescent.

Cell-Cell Signaling and Intrahepatic Bile Duct Development

The two signaling pathways that have been best characterized at the cholangiocyte differentiation stage are also involved in tubulogenesis. Indeed, beyond the stage of ductal plate formation, Notch and TGFβ signaling remain important for duct morphogenesis.

Jagged-1 expression in developing ducts is first restricted to the cells located on the portal side of the PDS. However, when these structures mature to form radially symmetrical ducts, Jagged-1 expression becomes detectable in all cholangiocytes [27]. From a functional point of view, elegant studies in which Notch signaling was inhibited by temporal-specific Cre-mediated ablation of RBP-Jκ(καππα) indicated that this signaling pathway is required both for differentiation and tubulogenesis [27]. These data extended earlier findings in which the Notch-induced gene Hes-1 was shown to be required for duct formation. In Hes-1 knockout mice, the ductal plate develops normally, but no PDS can be detected [50]. In addition, mice deficient in Jagged-1 and Notch2 showed abnormal bile duct formation [26, 30, 31].

TGFβ signaling promotes differentiation of hepatoblasts to cholangiocytes and was therefore a good candidate to regulate the maturation of PDS into symmetrical ducts. The analysis of TGFβ signaling in the PDS revealed unexpected features, whereas the cholangiocytes of the single-layered ductal plate express TβRII, this receptor is repressed in cholangiocytes on the portal side of the PDS. The hepatoblasts on the parenchymal side of the PDS maintain TβRII expression until they have matured to cholangiocytes [14]. Interestingly, in mice with inactivation of Sox9, duct maturation is delayed and this is associated with prolonged expression of TβRII in PDS [14]. Therefore, based on the expression of TβRII, it is suggested that TGFβ signaling plays a dynamic role during duct maturation.

Other signaling pathways were considered when investigating bile duct morphogenesis, but these studies were performed before the notion of transient asymmetry became known. Much effort has been devoted to understand the role of extracellular matrix components, which may control duct development by interacting with integrins expressed by the cholangiocytes. The latter express a specific and complex set of laminin receptors, namely integrins α(αλπα)6β1,α 2β1,α 3β1, and α6β4 [18]. The demonstration that growth of hepatoblasts in a laminin gel can induce differentiation and polarization of the cells as well as lumen formation further supports the role of laminin [55]. Tenascin, another extracellular matrix constituent, is detected in the vicinity of ducts, only at the stage when they become surrounded by mesenchyme. This suggests that tenascin may play a role at this specific stage of biliary development [56].

In the phase of duct extension, it is likely that cell–cell signaling regulates cholangiocyte proliferation. Extracellular factors involved in cholangiocyte proliferation were in most cases identified in experimental models in which proliferation was induced by cholestasis, leaving open the question of their involvement in normal cholangiocyte proliferation [57]. However, cholangiocytes proliferate in response to Insulin-like growth factor-1, growth hormone, estrogen, histamine, and Interleukin-6 (IL-6), suggesting that these factors participate in normal duct extension [58–60]. At the intracellular level, the cAMP-, phosphatidylinositol-3-kinase- and Ca^{2+}-regulated pathways control proliferation (reviewed in [2, 57]).

Gene Regulation and Intrahepatic Bile Duct Development

The phenotypic analysis of mice knockout for the transcription factors HNF-6 and HNF-1β not only revealed differentiation anomalies, they also suggested that these factors regulate duct morphogenesis. Mice with a liver deficient in either of the two factors show abnormal cyst-like biliary structures and persistence of biliary cells, which are not involved in tubulogenesis. The latter phenomenon is very similar to human DPM [40, 41]. Gene targets of HNF-6 and HNF-1β have not yet been identified, with the exception that the HNF-1β gene contains HNF-6 binding sites and is stimulated by HNF-6 in vivo and in transfection experiments. Sox9 is required for proper timing of duct maturation and is also downstream of HNF-6, but it is unknown if this regulation is direct or indirect [14]. In other organs, HNF-6 and HNF-1β were found to regulate genes that are involved in polycystic diseases. Indeed, in the pancreas, HNF-6 stimulates *Cys1* and *Pkhd1,* and in kidney, HNF-1β controls the expression of *Ift88/Tg737/Polaris, Pkd2,* and *Pkhd1* [61, 62]. Although it is not yet known if HNF-6 and HNF-1β target the same genes in cholangiocytes, it is tempting to speculate that they do so.

Since HNF-6 and HNF-1β were the first transcription factors shown to regulate bile duct morphogenesis, most subsequent studies included the analysis of their expression. This showed that mice deficient in C/EBPα, which have ductal plate-like malformations, overexpress HNF-6 and HNF-1β in the abormal biliary cells [49]. Interestingly, in Hhex knockout livers, HNF-6 expression in hepatoblasts is reduced

only when *Hhex* has been deleted at the earliest stage of liver development. However, when the *Hhex* gene is inactivated at later stages, HNF-6 expression seems normal despite that biliary cells develop cystic structures; the latter are lined by a mixed cell population comprising hepatobiliary cells of which many fail to express HNF-1β [45]. These data can be integrated into the model describing transcriptional regulation of bile duct development (Fig. 15.1). However, since differentiation and morphogenesis are tightly linked, it is often unclear if a specific transcription factor is a regulator of differentiation or tubulogenesis, or both. This is further illustrated by the functional analysis of the transcription factor Sa114, which when overexpressed in hepatoblasts grown on Matrigel, stimulates differentiation and induces formation of bile duct like structures [44].

As indicated above, bile duct development is terminated when cholangiocytes stop proliferating. This proliferation arrest is critically dependent on the presence of the transcription factors FoxA1 and FoxA2 [60]. By using a conditional gene deletion approach, it was shown that in the absence of these factors, the cholangiocytes continue to proliferate and generate hyperplastic bile ducts. To induce proliferation arrest, FoxA1 and FoxA2 normally recruit the glucocorticoid receptor to the IL-6 gene promoter, and this results in repression of IL-6 production. In contrast, in the absence of FoxA1 and FoxA2, the hormone receptor cannot bind to the IL-6 gene and is replaced by nuclear factor καππα-light-chain-enhancer of activated B cells (NF-κ(καππα)B), a positive regulator of IL-6. In that case, overexpression of IL-6 stimulates proliferation of cholangiocytes, leading to biliary hyperplasia.

Finally, whereas most efforts have been devoted to the study of transcriptional regulation of duct morphogenesis, recent work addressed the importance of posttranscriptional regulation of duct development by microRNAs. Liver-specific ablation of Dicer, which results in the inhibition of synthesis of all miRNAs, did not reveal an involvement of miRNAs in biliary development. However, in these experiments microRNA depletion only occurred after birth, thereby precluding any analysis of duct development [63, 64]. In contrast, using a knockdown approach in zebrafish, it was found that miR-30a and miR-30c, two microRNAs specifically expressed in developing bile ducts, are necessary for normal biliary development. Although the exact mode of action of these two microRNAs is not known, it is supected that they regulate duct development by targeting the mRNAs coding for the Epidermal Growth Factor receptor and Activin A, which control cholangiocyte proliferation and differentiation, respectively [65]. A further function of miRNAs in duct development has been found when studying the miR23b miRNA cluster. Inhibition of miR-23b miRNAs during differentiation of a cultured liver stem-cell line, promoted expression of biliary genes and of the TGFβ mediators Smads, whereas ectopic expression of miR-23b during biliary differentiation of the stem cells had the opposite effect. These data indicate that tight regulation of miR-23b is required for development of the ducts [66]. Finally, a role for microRNAs in cholangiocyte proliferation has also been uncovered: miR-15a represses the cdc25 mRNA, and this results in repression of proliferation [67].

Development of the Extrahepatic Biliary Tract

Much less is known about the development of the extrahepatic biliary tract, as compared with the intrahepatic ducts. The extrahepatic biliary tract develops from a diverticulum located at the ventral part of the liver. This part has often been referred to as pars cystica, to distinguish it from the liver bud, which is then referred to as pars hepatica. The extrahepatic biliary tract grows in length and develops into a gallbladder, cystic duct, and common bile duct. Their lumen is lined by cholangiocytes derived from the endoderm, whereas the outer layers derive from the mesenchyme. The hepatic ducts develop from the liver bud (pars hepatica) and anastome with the rest of the extrahepatic biliary tract (reviewed in [9]).

The extrahepatic biliary tract develops in the neighborhood of the ventral pancreas. Importantly, the relationship between the two organs extends beyond their anatomical location. Indeed, genes involved in pancreas development also impact on extrahepatic bile duct development. This is the case for Hes-1, which controls endocrine development in the pancreas and prevents extrahepatic cholangiocytes from differentiating into pancreatic tissue [68, 69]. Similarly, the pancreatic and duodenal homeobox factor-1 (Pdx-1) is required for development of the pancreatic bud, as well as for differentiation of mucin-producing cells and peribiliary glands in the extrahepatic ducts [70]. Also, Hhex is required for ventral pancreas organogenesis and epithelial morphogenesis of the liver bud, and in its absence the extrahepatic ducts are replaced by duodenal-like tissue [45].

During development of the extrahepatic ducts, the mesenchyme condenses around the epithelium, suggesting that this process is regulated by molecular interactions between the epithelium and the mesenchyme. This hypothesis is substantiated by observations revealing that the absence of the epithelial factors HNF-6 or HNF-1β and the haploinsufficiency of the mesenchymal transcription factor FoxF1 are associated with gallbladder and common bile duct dysgenesis [40, 41, 71]. Moreover, during gallbladder and cystic duct elongation, the orphan leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) is expressed in the epithelium; at later stages it is found in the mesenchyme. In hypomorphic *Lgr4* mutant mice, the gallbladder and cystic ducts are absent, again pointing to possible mesenchyme–epithelium interactions [72].

Lessons from Developmental Diseases of the Biliary Tract

The understanding of biliary development not only relies on basic science, but also on a thorough analysis of the mechanisms that are dysfunctional in human diseases. Providing a complete overview of the hereditary and developmental diseases of the biliary tract goes beyond the scope of this chapter, and we refer to reviews that address this issue [73–76]. However, some important conclusions were drawn about normal development based on the analysis of human disease.

Bile Duct Paucity

Bile duct paucity, also called "ductopenia," may be a manifestation of abnormal biliary development. The Alagille syndrome, which results from defective Notch signaling, is a good illustration of ductopenia. However, whereas studies in mice concluded that Notch signaling is required for biliary differentiation and duct formation, the analysis of patient biopsies revealed in some cases that the bile duct/portal tract ratio decreases with age [77]. In line with this, a patient showed normal ducts near the liver hilum, but ductopenia was observed at the periphery of the lobes [78]. Since there was no sign of bile duct destruction, these observations suggest that in humans, the Notch pathway may be involved in elongation and branching of the ducts, two processes which are still poorly characterized at the molecular level.

Deficient HNF-1β/TCF2 function was recently identified in humans as another rare cause of ductopenia [79]. The patient showed bile duct paucity, which is somewhat in contrast to mouse *Hnf1b* knockout livers in which bile duct paucity occurs in association with bile duct dysplasia, a feature not found in the patient [41]. Therefore, the exact role of HNF-1β in biliary development deserves more investigation.

Ductopenia is also observed in patients with "Arthrogryposis-Renal dysfunction and Cholestasis syndrome." This syndrome is associated with mutations in the *VPS33B* gene, a regulator of vesicular membrane fusion which interacts with SNARE proteins [80]. Therefore, intracellular trafficking is essential for normal biliary development. This was further investigated at the genetic level in zebrafish, in which knockdown of *vps33b* leads to duct paucity, and in which the expression of VPS33B was shown to be stimulated by the HNF6/HNF1β cascade [81]. The data on VPS33b in humans prompted the search for other vps genes linked with biliary development. In zebrafish again, it was shown that *vps18* is required for normal duct development, since zebrafish deficient in this gene develop cholestasis as a result from bile duct paucity and bile canalicular defects [82].

Ciliopathies

In a number of diseases, bile ducts are present but develop abnormally. This is the case in autosomal dominant polycystic kidney disease (ADPKD), in which hepatic biliary cysts may develop in parallel with renal cysts. Biliary cysts are characterized by cholangiocyte hyperproliferation, and this results from increased sensitivity to estrogens and Insulin-like growth factor-1 [83]. This in turn induces overproduction of vascular endothelial growth factor, which promotes proliferation of the cyst cholangiocytes [84]. The study of ADPKD not only uncovered a role of proliferation regulators, but it also highlighted the role of primary cilia. Indeed, *PKD1* and *PKD2,* the two genes whose mutations are associated with the disease, code for Polycystin-1 and -2, which together form a calcium channel located at the membrane of primary cilia. This and other observations brought these organelles to light in the context of polycystic diseases. Primary cilia are situated at the apical pole of the cells and play a role in osmo-, mechano-, and chemosensing [85]. In ADPKD, the cholangiocyte cilia are shorter or absent [83], and dysfunction of Polycystin-1 and -2 is associated with perturbed intracellular levels of Ca^{2+} and cAMP, two regulators of proliferation [86]. Therefore, dysfunctional cilia are associated with biliary dysgenesis and cilia must be considered to fully understand the mechanisms of biliary development.

In patients affected with autosomal recessive polycystic disease (ARPKD), the liver may show multifocal dilations of the bile ducts. The disease is associated with mutations in *PKHD1,* a gene which codes for a transmembrane protein known as polyductin or fibrocystin [87, 88]. The function of the latter is unknown, but it is located at the cilia, basal body, and apical membrane of the cholangiocytes. Development of hepatic cysts in ARPKD is, like ADPKD, dependent in part on hyperproliferation, which results from increased cAMP signaling and reduced intracellular calcium levels [89], as well as from decreased levels of miR-15a [67]. Moreover, animal models of ARPKD include the cpk and Oak Ridge polycystic kidney disease mouse models, which have mutations in the *Cys1* and *Ift88/Tg737/polaris* genes that code for proteins associated with Polycystin-1 and -2 at the primary cilia [90–92]. Therefore, the study of ARPKD opened new venues in the search for regulators of biliary development.

Importantly, not all polycystic diseases result from defects that primarily affect the cilia. Indeed, Autosomal Dominant Polycystic Liver Disease, a rare disease affecting the liver and not the kidneys, is associated with mutations in Hepatocystin and Sec63p, two proteins which are involved in glycosylation and transport of glycoproteins [93–96].

Ductal Plate Malformations

Such lesions are found in ARPKD, Jeune syndrome, congenital hepatic fibrosis, the embryonic form of biliary atresia, Caroli syndrome, as well as in Meckel–Gruber syndrome and related diseases (Joubert syndrome and nephronophthisis). DPM are defined as abnormal remodeling of the ductal plate leading to persistence of embryonic biliary structures [97]. In most cases, the genes associated with the syndrome have been identified [75, 76, 98], but often the exact function of the corresponding proteins remains elusive. Given the concept that DPM results from persistence of embryonic structures, it is likely that these genes are involved in regression of the ductal plate. Along these lines, Meckel syndrome-affected patients show DPM associated with decreased apoptosis of the ductal plate cells [99]. The Jeune syndrome-associated gene codes for IFT80, a protein regulating transport of proteins in the cilium; ARPKD was mentioned above to result from mutations in the cilium-associated protein polyductin/fibrocystin. This indicates that the phenotypic spectrum of ciliopathies extends from cyst formation to DPM and that ductal plate regression in normal conditions is controlled by cilium function.

Diseases of the Extrahepatic biliary Tract

The most common hepatobiliary disorder in children is biliary atresia, characterized by a progressive and inflammatory process that leads to sclerosing obliteration of the extrahepatic biliary tree. The fetal or syndromic form of the disease shows several organ malformations in addition to the biliary defect. These include laterality defects, such as situs inversus, suggesting that cilium-associated proteins that regulate left–right asymmetry participate in the disease process. These proteins include Inversin, Cryptic, and Zic3. *Inv* knockout mice show situs inversus and hepatobiliary defects [100], whereas mutations in *CRYPTIC* and *ZIC3* were found in patients with biliary atresia [101, 102]. In addition, when the transcriptome of livers from patients with the fetal form of biliary atresia was compared with that from patients suffering from the nonsyndromic form of biliary atresia, it was found that the laterality genes *SPROUTY4, LEFTYA*, and *ZIC3* were differentially expressed in the two forms of the disease [103]. Also, when comparing normal liver with nonsyndromic biliary atresia liver, it was shown that genes regulating morphogenesis were abnormally expressed in the disease [104]. Whether these gene expression anomalies contribute to the pathogenesis of biliary atresia, remains to be determined.

Finally, the study of rare familial cases of choledocal cysts is expected to provide insight into the mechanisms of extrahepatic biliary development. In this case, clinical work may also benefit from the recent identification of the neurofibromatosis type 2 (*NF2)* gene as a regulator of common bile duct development. Indeed, a mutant zebrafish screen uncovered that *Nf2* mutants develop gallbladder and choledocal cysts, in association with cystic intrahepatic ducts [82].

Conclusions

In recent years, rapid progress has characterized the field of biliary development. An increasing number of regulators have been identified, leading to a better understanding of human disease. In the opposite direction, knowledge gained from the analysis of patients has considerably fed the research on normal biliary development. Such a fertile dialog between basic research and clinical science – "from bench to bedside" and from "bedside to bench" – is obviously the best guarantee for future progress.

Acknowledgments Work by the author is supported by the Interuniversity Attraction Poles Program (Belgian Science Policy), the Fund for Scientific Medical Research (Belgium), the D.G. Higher Education and Scientific Research of the French Community of Belgium, and the Alphonse and Jean Forton Fund.

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Chapter 16
Hepatic Progenitors in Development and Transplantation

David A. Shafritz, Michael Oertel, and Mariana D. Dabeva

High Regenerative Potential of the Liver

The rationale for studies to repopulate the liver with transplanted cells is essentially based on three observations: (1) The well-known finding that the liver can fully regenerate after acute hepatotoxic injury or surgical reduction in liver mass, (2) the regenerated liver functions normally, without long-term impairment, and (3) a unique portal (venous to venous) circulation exists in the liver that provides ready access of transplanted cells to the parenchyma through the hepatic sinusoids.

In the normal adult liver, hepatocytes are in a quiescent state, turning over very slowly (only 2–3 times/year). However, in 1931, Higgins and Anderson reported that after removal of the large median and left lateral lobes of the rat liver by a simple surgical procedure (two-thirds partial hepatectomy), the remaining lobes increase rapidly and replace the lost hepatic tissue [1]. After this procedure, hepatocytes rapidly enter the cell cycle, undergo mitosis and massively proliferate (peaking between 24 and 36 h), during which 70–90% of the remaining host hepatocytes engage in DNA synthesis [2]. From these and other studies, it has been concluded that the proliferative activity of residual adult hepatocytes in the normal liver is sufficient to regenerate the parenchymal mass following two-thirds partial hepatectomy (PH) and that participation of stem cells is not required [3]. A comprehensive description of cellular and molecular events occurring during liver regeneration is presented in Chap. 18.

Role of YAP in Molecular Regulation of Liver Mass

The liver size (mass) is proportional to total body weight, ranging from 3 to 5% in different mammalian species. Since the adult liver is essentially quiescent, it is quite surprising how rapidly the liver regenerates after two thirds PH and that the liver mass returns to normal, despite the fact that the lobular architecture is permanently modified. The remaining lobes increase in size and cell number, and restore a normal liver-to-body weight ratio. This process is referred to as "compensatory hyperplasia," as there is no actual regeneration of the removed lobes. In humans, when an undersized liver is transplanted, it grows to the expected full size for the host and when an oversized liver is transplanted, it subsequently reduces in size to the appropriate mass compared to total body weight. However, until very recently, nothing was known regarding how this process is regulated.

In most interesting studies, Pan et al. [4] have shown that mammalian genes, homologous to Drosophila genes that regulate wing mass during development (members of the Drosophila Hippo kinase signaling cascade), control hepatocyte proliferation. When YAP, the mammalian counterpart to Drosophila Yorki (the last gene in the Drosophila Hippo signaling pathway), is overexpressed in a transgenic mouse model, hepatocyte proliferation becomes unchecked and there is massive liver hyperplasia, as well as hepatic carcinogenesis. When YAP hyperexpression is turned off, liver size returns to normal [4]. Similarly, knockout of the mst1 and mst2 genes that are upstream of Yap in the mammalian hippo kinase signaling cascade also leads to liver hyperplasia and tumorigenesis [171].

Yap is a transcriptional activator of many target genes and is considered to be a "tumor suppressor gene," operating through a phosphorylation/dephosphorylation mechanism. However, when this gene is hyperexpressed, it leads to cellular hyperplasia and oncogenesis. Which downstream targets of Yap either suppress or induce oncogenesis and other questions concerning how the mammalian hippo kinase signaling cascade regulates liver mass still remain to be addressed. In addition, whether Yap is a normal physiologic regulator of liver size and whether its expression or phosphorylation changes during liver regeneration also remains to be determined. Once these issues are better understood, it may be possible to modify the mammalian hippo kinase signaling cascade or use Yap to engineer hepatocytes with augmented proliferative potential that can effectively repopulate the liver.

D.A. Shafritz (✉)
Department of Medicine, Cell Biology and Pathology, Marion Bessin Liver Research Center, Albert Einstein College of Medicine of Yeshiva University, New York, NY, USA
e-mail: david.shafritz@einstein.yu.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_16, © Springer Science+Business Media, LLC 2011

Hepatocyte Transplantation into the Regenerating Liver

Since many genetic-based liver disorders are caused by simple dysfunction of hepatocytes without underlying liver injury, it should be possible to treat these disorders by transplantation of normal (wt) hepatocytes, This is especially true under circumstances in which replacement of a small percentage of cells would be therapeutically effective. Representative examples of such disorders include hemophilia, hyperbilirubinemia, ornithine transcarbamylase deficiency, hypercholesterolemia and phenylketonuria. In some of these disorders, cell transplantation could, in principle, also be performed under circumstances in which the patient's own cells are genetically manipulated ex vivo and then transplanted back into the liver (autologous cell transplantation). This would circumvent the need for immunosuppression and indeed this has been done, but with only temporary success [5]. In other genetic-based disorders in which there is active and continuous liver injury, such as Wilson's disease, α1-antitrypsin deficiency (P_1ZZ phenotype), and inherited hemachromatosis, these disorders could also, in principle, be alleviated by transplantation of wt hepatocytes, but this would require substantially greater levels of hepatocyte replacement. However, a major current difficulty is, only a small percentage of the total hepatocellular mass (~1–2% maximally) can be replaced by hepatocyte transplantation without causing portal hypertension and hepatic infarction. Therefore, to obtain effective cell therapy, in most instances it will be necessary to expand the cells in the host after they have been transplanted.

Attempts to increase the number of transplanted hepatocytes in the repopulated liver simply by stimulating liver regeneration (for example, through the use of PH or carbon tetrachloride (CCl_4)-induced hepatic necrosis) have generally been unsuccessful. This is not surprising, since, on an average, hepatocytes need to undergo only one or two rounds of cell division to replace all of the liver mass removed by two thirds PH [2, 6]. Both endogenous and experimentally transplanted hepatocytes should contribute equally to this limited proliferative response. Therefore, one would not expect the percentage of transplanted hepatocytes to increase significantly simply by inducing liver regeneration. Although performing repeated partial hepatectomies or cell transplantations increases the level of liver repopulation by transplanted hepatocytes, the results are quite modest [7, 8]. However, high levels of liver repopulation can be achieved with adult hepatocytes under circumstances in which there is both massive and sustained liver injury and preferential selection of transplanted cells, or under circumstances in which host hepatocytes have been rendered incapable of cellular proliferation (see below).

Special Animal Models to Repopulate the Liver by Transplanted Hepatocytes

For many years, it was thought that mature hepatocytes could undergo only 2–3 divisions after which they become terminally differentiated and are incapable of further proliferation. However, during the last decade, it has been shown in several rodent models that under specialized circumstances, hepatocytes exhibit high proliferative capability and can extensively repopulate the liver. In the first such model, Sangren et al. [9] developed a transgenic mouse in which a protease, urokinase plasminogen activator (uPA), is expressed exclusively in hepatocytes under control of the albumin promoter. This protease was supposed to be expressed exclusively on membrane-bound polyribosome of the endoplasmic reticulum and then secreted into the serum. However, small amounts of uPA remained in the liver tissue causing extensive liver injury. This led to sub-fulminant hepatic failure and death of the mice at 4–6 weeks of age. However, some mice survived and in these mice, there were nodules of normal liver tissue of varying sizes scattered throughout the hepatic parenchyma (Fig. 16.1a). This occurred by deletion of the uPA transgene from individual hepatocytes, which then expanded clonally into large clusters and replaced damaged tissue. Subsequently, Rhim et al. [10] transplanted normal hepatocytes, marked with a β-galactosidase transgene, into uPA mice and observed extensive liver repopulation (Fig. 16.1b). They estimated that, on average, each transplanted hepatocyte that had engrafted into the uPA host liver underwent ~12–14 cell divisions [10].

Grompe et al. [11] developed a second mouse model to repopulate the liver by mature hepatocytes through targeted disruption of fumarylacetoacetate hydrolase (Fah), the last gene in tyrosine catabolism. Deletion of Fah leads to accumulation of upstream intermediates in tyrosine catabolism, some of which (primarily fumarylacetoacetate) are toxic and cause extensive and continuous liver injury. The Fah null mouse represents an animal model for the human metabolic disorder Hereditary tyrosinemia, Type 1 (HT1), which causes extensive liver injury, hepatocellular carcinoma, and death at an early age [12]. Administration of 2 (2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC), a pharmacologic inhibitor of tyrosine catabolism upstream of homogentisic acid, prevents accumulation of fumarylacetoacetate and is partially successful in treating patients with HT1 [12], although it does not prevent hepatocarcinogenesis [13].

In Fah null mice, Grompe et al. demonstrated that liver repopulation by transplanted wt hepatocytes can be totally regulated by NTBC administration. They showed specifically that NTBC suppresses repopulation by transplanted wt hepatocytes in Fah null mice, because, under these conditions,

there is no selective advantage for wt hepatocytes to survive over host Fah null hepatocytes. When wt hepatocytes are transplanted into Fah null mice maintained on NTBC, only scattered small clusters of transplanted hepatocytes are detected (Fig. 16.1c). However, if NTBC treatment is discontinued at the time of cell transplantation, liver injury resumes and transplanted cells proliferate extensively, forming large clusters within 3 weeks and replacing most of the liver mass within 6 weeks (Fig. 16.1d). Therefore, cyclic administration and withdrawal of NTBC can be used

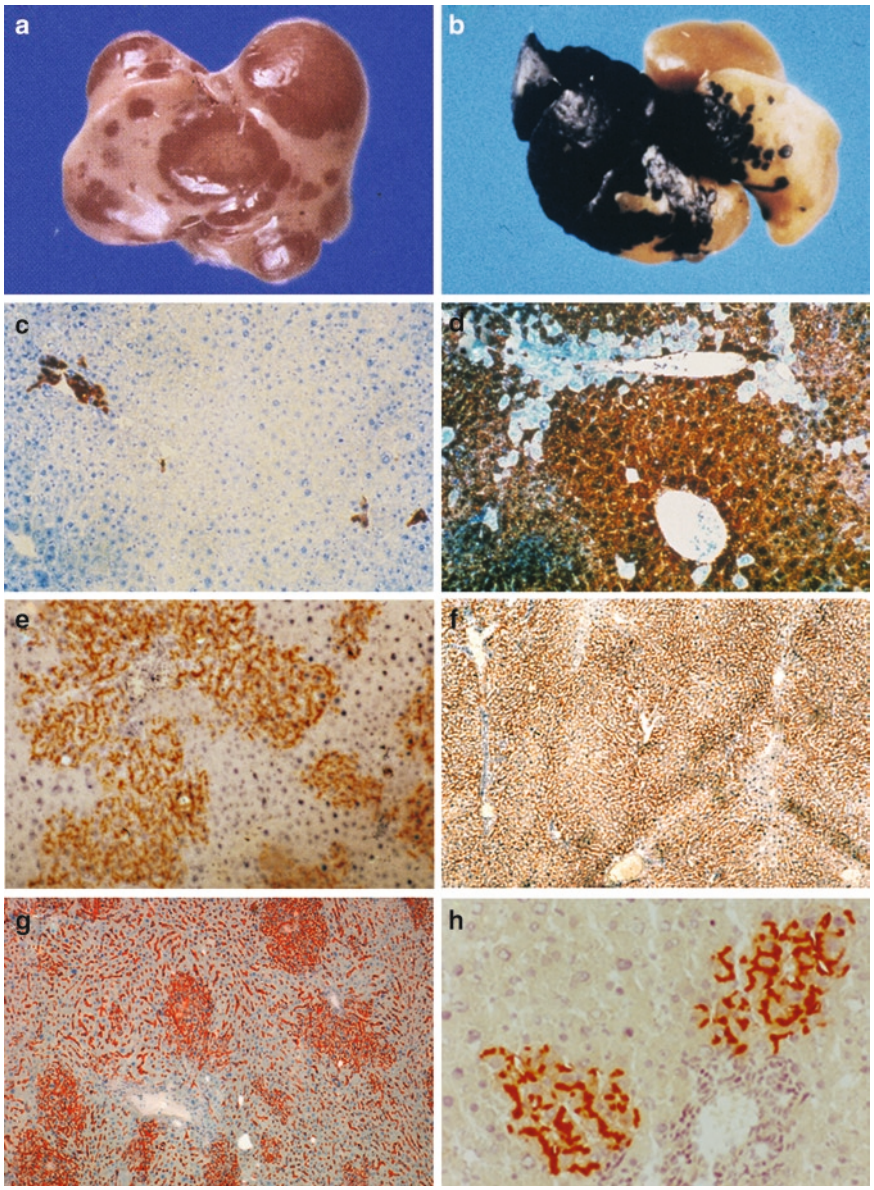

Fig. 16.1 Major models for liver repopulation by transplanted hepatocytes. (**a**) Spontaneous liver repopulation in Alb-uPA transgenic mouse by revertant hepatocytes that have deleted the uPA transgene. From Sangren et al. [9], used with permission. (**b**) Repopulation of uPA transgenic mouse liver by transplanted β-galactosidase expressing normal hepatocytes. From Rhim et al. [10], used with permission. (**c**) Scattered Fah positive hepatocytes in Fah null mouse transplanted with wt hepatocytes but maintained on NTBC. From Overturf et al. [11], used with permission. (**d**) Massive repopulation of Fah null mouse with transplanted wt (Fah positive) hepatocytes at 6 weeks after withdrawing NTBC administration. From Overturf et al. [11], used with permission. (**e**) Integration of transplanted wt ($DPPIV^+$) hepatocytes that have expanded massively into the hepatic parenchyma of a retrorsine/PH treated $DPPIV^-$ mutant rat. From Laconi et al. [15], used with permission. (**f**) Near total (99%) repopulation of the liver by transplanted wt ($DPPIV^+$) hepatocytes at 9 months after their transplantation into $DPPIV^-$ mutant rat treated with retorsine/PH. From Laconi et al. [15], used with permission. (**g**) Repopulation of $DPPIV^-$ mutant rat liver by wt ($DPPIV^+$) hepatocytes after treatment of the host with x-irradiation/PH. Courtesy Chandan Guha, used with permission. (**h**) Repopulation of $DPPIV^-$ mutant rat liver by wt ($DPPIV^+$) hepatocytes after treatment of the host with x-irradiation/ischemic liver injury. From Malhi et al. [21], used with permission

as a tool to control liver failure in Fah null mice and this allows transplanted normal hepatocytes (expressing the Fah gene) to expand when NTBC administration is discontinued. Fah null mice with livers repopulated by wt hepatocytes remain healthy, have normal liver function tests and show a relatively normal liver structure for many months after hepatocyte transplantation [11]. These studies were the first to show that liver repopulation can effectively cure a metabolic disease, namely, the mouse equivalent to HT1.

In Fah null mice, not only do transplanted wt hepatocytes replace Fah null hepatocytes, but the transplanted cells can also be serially transplanted through seven consecutive Fah null mice, while retaining full ability to proliferate and replace host hepatocytes [14]. In these studies, it was calculated that each serially transplanted hepatocyte underwent an average of at least 69 cell divisions. Thus, murine hepatocytes exhibit essentially infinite capacity to proliferate and restore liver function under circumstances in which there is (1) both massive and continuous liver injury and (2) the transplanted hepatocytes have a significant selective advantage for survival compared to host hepatocytes. (Both of these conditions have been produced experimentally in uPA transgenic and Fah null mice.)

A third method to obtain a high level of liver repopulation by transplanted hepatocytes is to impair proliferation of endogenous hepatocytes, i.e., render them incapable of cell division, and then transplant normal hepatocytes in conjunction with a liver proliferative stimulus. This was first achieved by treating rats with retrorsine, a plant alkaloid that is taken up and metabolized by hepatocytes to produce an active intermediate that crosslinks cellular DNA and disrupts hepatocyte division [15]. When retrorsine or a closely related compound, monocrotaline, is administered to rats or mice [15–18], there is a long-lived inhibition of hepatocyte proliferation. However, essential metabolic functions are maintained in these DNA damaged hepatocytes and the animals survive. After the effects of acute chemical injury have subsided (2–4 weeks), the animals are subjected to two-thirds PH or CCl_4 administration in conjunction with transplantation of hepatocytes from normal animals. This leads to a brisk regenerative response by transplanted hepatocytes and there is extensive replacement of DNA-damaged host hepatocytes within several months [15–18]. What was most surprising in the retrorsine/PH model is that the transplanted hepatocytes do not develop into hyperplastic nodules, i.e., the liver remodels and the transplanted hepatocytes become fully integrated into the hepatic plates (Fig. 16.1e). Transplanted hepatocytes form hybrid canaliculi with neighboring host hepatocytes; the liver structure becomes and remains essentially normal for many months after cell transplantation and more than 99% of host hepatocytes can be replaced (Fig. 16.1f) [15]. Using the retrorsine/PH model, we have transplanted wt allogenic hepatocytes into albumin deficient Sprague Dawley rats, i.e., the Nagase analbuminemic rat, under an immunosuppressive protocol. Under these conditions, there was extensive replacement of albumin-deficient host hepatocytes by wt albumin expressing hepatocytes, which led to a 7,000-fold increase in albumin production and restoration of serum albumin levels to the normal range [19].

Another method to achieve effective liver repopulation by transplanted hepatocytes is to induce DNA damage by selective liver irradiation in conjunction with hepatocyte transplantation and either two-thirds PH, CCl_4 administration or ischemic liver injury [20, 21]. In the retrorsine/PH model, thyroid hormone (an hepatocyte mitogen in rats) can partially replace PH as an inducer of liver repopulation [22]. Most recently, in x-irradiated mice, administration of HGF has been used to replace PH as a liver regenerative stimulus [23]. With retrorsine or monocrotaline treatment of the host liver, transplanted hepatocytes have a proliferative advantage over host hepatocytes, as the latter have been rendered incapable of cell division. After retrorsine-induced DNA damage, host hepatocytes also exhibit an increased level of apoptosis [22], which also contributes to liver replacement by transplanted cells.

Other methods to achieve liver repopulation are to transplant donor hepatocytes that have augmented proliferative potential or a selective survival advantage into a host with a normal liver and stimulate cycles of regeneration by repeated liver injury. The latter has been achieved by transplanting Bcl-2 transgenic mouse hepatocytes that are resistant to apoptosis into wt mice in which apoptosis has been induced by repeated injections of anti-FAS Ab [24]. $p27^{KIP1}$ is a cyclin kinase inhibitor that regulates the cell cycle, and deletion of this gene provides a proliferative advantage to p27 null hepatocytes over normal host hepatocytes when transplanted in conjunction with repeated liver injury induced by CCl_4 injection [25]. In both of these models, there was a modest to moderate level of liver repopulation (2–16% range). Hopefully, in the future, pharmacologic methods will be developed to achieve these same effects, which might then be adapted for clinical applications.

Progenitor ("Oval") Cells for Liver Repopulation

The term "oval cells" was introduced by Farber [26] to describe non-parenchymal cells in the periportal region that were present after treating rats with carcinogenic agents, such as ethionine, α-acetaminobenzene (2-AAF), and 3-methyl-4-diethylaminobenzene. Other methods to induce proliferation of "oval cells" are to treat rats with D-galactosamine [27, 28], a choline deficient/ethionine substituted

diet [29, 30], or allyl alcohol [31], or to treat mice with dipin [32], or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) [33]. In each of these models, cells are induced that have a small oval-shaped, pale-stained blue nucleus and very scant, lightly basophilic cytoplasm. Farber did not believe that "oval cells" are hepatocyte progenitors [34], but Thorgeirsson and coworkers [35] demonstrated that "oval cells" induced to proliferate in the periportal region after treatment of rats with 2-AAF, followed by two-thirds PH, subsequently differentiate into distinct clusters of basophilic hepatocytes. This was demonstrated by pulse labeling of the liver with ^{3}H-thymidine and following the progression of labeled cells from the periportal region into clusters of hepatocytes in the mid-parenchyma [35, 36]. Other indirect evidence suggesting that "oval cells" are hepatic progenitors came from reports that "oval cells" express c-kit [37], CD34 [38], flt3 receptor [39], and LIF [40], all known to be markers for hematopoietic stem cells or their immediate derivatives. Sca-1, another cell surface protein expressed by hematopoietic stem cells in the mouse, is also expressed in fetal liver epithelial cells [17] and in "oval cells" of the adult mouse liver [41, 42]. Most recently, using lineage tracing studies in a double transgenic mouse expressing a β-galactosidase reporter gene under control of the Fox11 promoter, Greenbaum and coworkers [43] showed production of both hepatocytes and bile duct epithelial cells from progenitor cells that were induced to proliferate by bile duct ligation or feeding mice a DDC-containing diet. These findings strengthen conclusions from earlier studies in rats using 2-AAF/PH treatment [35, 36].

"Oval cells" are induced massively when liver injury is superimposed on circumstances in which hepatocyte proliferation is impaired. These cells exhibit many features of progenitor cells, dividing rapidly and appearing to differentiate into both hepatocytes and bile duct epithelial cells. Thorgeirsson et al. [44] performed extensive immunohistochemical and ultrastructural studies in which they demonstrated that "oval cells," induced to proliferate by 2-AAF/PH, are derived from undifferentiated cells in the Canals of Hering. Subsequently, these cells pass through discontinuities in the laminar basement membrane of the ductal limiting plate and join together with stellate cells as they enter the hepatic parenchyma, proliferate, and differentiate into hepatocytes.

Attempts to establish specific markers for "oval cells" to distinguish them from mature hepatocytes and bile duct epithelial cells and to determine their lineage origin (mesoderm or endoderm) have led to conflicting findings. All investigators agree that "oval cells" express common liver epithelial progenitor cell markers, such as α(alpha)-fetoprotein (AFP) and albumin (Alb) for hepatocyte progenitors and CK-19 (and OV6 in the rat) for bile duct progenitor cells. However, the term "oval cells" is used to identify a highly heterogenous population of cells. Multiple different cell types are induced in livers undergoing "oval cell" activation, and it is not clear whether "oval cells" from different animal species or from different hepatic injuries are in fact comparable. "Oval cells" were initially thought to express hematopoietic stem cell markers, c-kit, CD34, and Thy 1 [37, 38, 45, 46], but several recent studies have shown that both fetal liver progenitor cells and "oval cells" are negative for c-kit, CD34, and Thy1 [47–50]. It has also been demonstrated recently that Thy1 is expressed in stellate cells that proliferate together with "oval cells" in various activation models [51]. These issues may be clarified through the use of antibodies to detect "oval cells" in rats [52, 53] and mice [33, 54, 55], once the specificities of these antibodies have been fully established.

Transplantation of "Oval Cells"

Since hepatocytes do not effectively repopulate the normal adult liver, an obvious alternative would be to transplant progenitor ("oval") cells that should have a higher proliferative potential than adult hepatocytes. Twenty years ago, Faris and Hixson [56] reported that "oval cells," isolated from the liver of rats fed a choline-deficient (CD) diet, treated with 2-AAF and transplanted into the liver of secondary hosts, produced "colonies" or clusters of cells with an hepatocytic phenotype in recipients that had also been subjected to the CD diet. However, the level of liver repopulation by transplanted CD/2-AAF "oval cells" was not determined. "Oval cells" isolated from the liver of rats treated with D-galactosamine also proliferate and differentiate into hepatocytes after transplantation into rats undergoing two-thirds PH [57]. However, in these studies, which were conducted in a nonselective tissue environment, liver repopulation by transplanted D-galactosamine-induced "oval cells" was low (not quantified). Duct-like epithelial cells, isolated from the atrophic pancreas of rats treated with a copper chelating agent, also proliferate modestly after transplantation into normal rat liver in conjunction with two thirds PH [57]. This probably occurs by reprogramming of epithelial progenitor cells in the Cu-deficient pancreatic ducts to the hepatocytic lineage, as evidenced by their expression of AFP and Alb. This appears to represent a reversible switch between the hepatic and pancreatic lineages, similar to a process recently described by Chan and coworkers called "transdetermination," in which hepatocytic progenitor cells switch their lineage program to pancreatic islet cell-like gene expression after in vivo transduction of mice with an adenovirus vector expressing neurogenin 3 [58]. This is distinct from "transdifferentiation" in which a fully differentiated cell type switches its gene expression program to another fully differentiated cell type, a process that will be discussed later in this chapter in

reference to studies reporting bone marrow cells differentiating directly into hepatocytes. After transplantation of "lineage-switched" pancreatic progenitor cells into the hepatic microenvironment, these cells fully differentiate into hepatocytes [57]. Isolated pancreatic cells from normal mice also repopulate the liver of Fah null mice [59]. "Oval cells" isolated from the liver of DDC-fed mice also repopulate the liver of Fah null mice, although perhaps with a reduced efficiency compared with mature hepatocytes [60]. Similarly, "oval cells" from GFP transgenic mice maintained on a DDC diet also repopulate the liver of wt mice treated with monocrotaline in conjunction with PH [61]. Other recent studies have shown effective repopulation of the liver by purified "oval cells" in both retrorsine treated rats [62] and Fah null mice [60], but not in animals with a normal liver.

Most recently, "oval cells" have been isolated from normal mouse and dog liver [42, 63, 64]. These cells exhibit properties characteristic of hepatic progenitor cells in culture, but in vivo repopulation data are very limited. Numerous studies have reported the isolation and in vitro passage of "oval cells" and "oval cell" lines from mice and rats, as well as from humans. These cells are clonal, bipotent and exhibit other stem and progenitor cell properties in vitro and in vivo [47, 63, 65–73]. However, liver repopulation by "oval cell" lines has generally been very low, even under conditions in which the host liver is highly compromised (highly selective conditions).

Properties of Stem and Progenitor Cells Relevant to Liver Reconstitution/ Repopulation

Based on studies conducted in vivo in the bone marrow, skin and intestinal epithelium, stem cells are generally considered to exhibit four major properties:

- Self-renewal or self-maintenance (generally slowly cycling)
- Multipotency (producing progeny in at least two lineages)
- Functional, long-term tissue reconstitution;
- Serial transplantability

Two methods to identify self-maintaining stem cells in the liver are:

- To show that specific populations of cells are undergoing asymmetric division [74, 75], during which one of the progeny remains undifferentiated, whereas the other differentiates into an hepatocyte or cholangiocyte
- To identify cells that retain a nuclear marker of DNA synthesis long after the tissue has turned over or undergone regeneration, i.e., the cells have presumably undergone proliferation and have then become quiescent ("label retaining cells")

Both of these methods have been established in rapidly turning over tissues, but, in principle, they could be used to study specific populations of cells in a slowly turning over tissue, such as the liver, following an acute liver injury. However, this would require a large or sustained liver regenerative stimulus. Although one study on asymmetric cell division has been conducted in vitro with hepatic-derived cell lines [76], none have been reported in vivo.

Regarding "label retaining cells," most studies have been conducted in the skin and intestinal epithelium. In one of these studies, using the nuclear marker histone 2B-GFP, "bulge cells" in the hair follicles retained the label [77]. "Bulge cells" have been shown to generate complete new hair follicles [78], but studies in hair follicles are complicated, as other populations of stem cells have also been identified [79]. The validity of the label-retaining method to identify stem cells has also recently been challenged [80].

In the liver, recent studies have reported four distinct populations of "label-retaining cells," i.e., Canal of Hering cells, intralobular bile duct cells, periductular "null" or undifferentiated cells, and peribiliary hepatocytes [81]. These cells were identified by pulse labeling with BrdU during acute liver injury with acetaminophen, followed 2 weeks later by a second liver regenerative stimulus using acetaminophen to "wash out" the label. Animals were then examined for cells retaining BrdU in the liver at 4 and 8 weeks after the second acetaminophen treatment. However, further studies will be necessary to establish the specific importance of these various populations of "label-retaining cells" in liver biology.

Most recently, lineage tracing experiments with double transgenic mice (Fox11-cre x rosa 26-loxP-β-gal) have demonstrated that β-galactosidase marked hepatic progenitor cells induced by DDC, are permanently labeled after they have been induced and can differentiate into both hepatocytes and mature bile duct epithelial cells [43]. This represents a significant step forward; however, the contribution of progenitor cells vs. adult hepatocytes to new hepatic mass generated following acute liver injury, as well as during normal liver cell turnover, still remains to be determined.

In adult tissues, stem cells are maintained at low levels and then proliferate very slowly. Progenitor cells, the progeny of stem cells, proliferate rapidly and differentiate into somatic populations; however, they do not maintain themselves. Like stem cells, progenitor cells may have multi-lineage potential, but they may also be unipotent. Regardless of whether they are multipotent or unipotent, progenitor cells are capable of only short-term tissue reconstitution. In reality, there may be a continuum of progenitor cells along the

hepatic lineage that progressively lose their stem cell properties as they differentiate toward more mature phenotypes.

The proliferative capability and differentiation properties of a specific cell may also vary depending on the tissue context in which that particular cell is located. Therefore, the designation of a particular cell as a stem or progenitor cell may be somewhat arbitrary. As new liver progenitor cell marker genes are established, these and other related issues can be addressed. In other tissues that are rapidly turning over, progenitor cells have also been termed "transit amplifying cells" [82]. "Oval cells," that have been activated to proliferate in various rodent model systems, exhibit many features of "transit amplifying cells" and thus may represent the liver counterpart to progenitor cells identified in other organs, such as the skin and intestinal epithelium.

Hepatic Stem Cells in the Developing Liver

Cellular, molecular, and morphologic studies have traced the proliferation and differentiation of stem cells during liver development. In the mouse, stem cells begin to proliferate from the ventral wall of the endoderm when it becomes positioned next to the developing heart, which occurs on embryonic day (ED) 8.0 [83–85]. Specification toward hepatic epithelial lineages occurs at ED8.5 and requires fibroblast growth factor (FGF) signaling from the cardiogenic mesoderm [86], as well as bone morphogenic protein (BMP) signaling from the septum transversum mesenchyme [87]. These cells begin to express GATA4 and liver-enriched, nuclear transcription factor HNF4α on ED 9.0–9.5, as well as liver-specific genes, AFP followed by Alb [84, 85, 88]. The hepatic-specified cells are now referred to as hepatoblasts, which proliferate massively and invade the septum transversum mesenchyme that contains stellate cells and sinusoidal endothelial cells. These latter cells secrete a variety of cytokines and growth factors that are known to be involved in liver development, such as EGF, FGF, HGF, TGFβ, BMPs, TNFα, and IL-6 [84–87, 89]. A visible liver structure is formed at ED11, at which time the hepatoblasts continue to expand rapidly and begin to express numerous liver-specific genes [90–92]. Some cells express genes that are specific for both the hepatocytic (AFP or Alb) and cholangiocytic (cytokeratin-19) lineages, and these cells are considered bipotential. Just prior to ED16, hepatoblasts diverge along these two distinct lineages, hepatocytes and cholangiocytes [88, 93], with Notch signaling promoting cholangiocytic differentiation and HGF and oncostatin M expression promoting hepatocytic differentiation [94]. After ED16, the percentage of bipotent cells falls dramatically, and most of the cells are unipotent and irreversibly committed to either the hepatocytic or cholangiocytic lineage [95–97]. As organogenesis proceeds, intrahepatic bile ducts are formed in the vicinity of large portal vein branches, beginning on ~ED17 [98]. A more comprehensive discussion of liver development can be found in Chap. 13.

Thus, the fetal liver contains cells that are in different stages of hepatic epithelial lineage progression. These cells have been isolated, cultured and transplanted into various animal model systems and fetal liver epithelial cells exhibit highly superior properties compared to either mature hepatocytes or adult liver progenitor cells when they are transplanted into the normal adult liver under nonselective conditions [57, 96, 99]. In contrast, under selective conditions, all three cell types (fetal liver cells, progenitor cells from the adult liver, and mature hepatocytes) efficiently repopulate the massively injured liver [10, 11, 15–21, 60–62, 96, 100]. However, the level of repopulation varies, depending on the animal species and the specific injury model used.

Liver Repopulation Using Fetal Liver Stem/Progenitor Cells

As indicated previously, the ultimate test for a putative stem cell is to demonstrate its ability to functionally repopulate a tissue or organ, long-term. Sandhu et al. [96] reported 5–10% repopulation of DPPIV$^-$mutant F344 rat liver by transplanting wt ED14 fetal liver epithelial cells in conjunction with two thirds PH. The transplanted cells were integrated into the host parenchyma, forming hybrid canaliculi with host hepatocytes, and the bulk of the repopulating clusters contained both hepatocytes and mature bile duct cells [96]. Liver repopulation by transplanted ED14 fetal liver cells increased slowly and progressively, and remained stable over 6 months. These results were obtained in a normal (nonselective) tissue environment, requiring only a two thirds PH to initiate the repopulation process. These findings are comparable to those in the hematopoietic system, where extensive ablation is required for bone marrow replacement by transplanted hematopoietic stem cells. Thus, transplanted rat ED14 fetal liver epithelial cells exhibit three major properties of stem cells: (1) extensive proliferation, (2) bipotency, and (3) long-term repopulation in vivo.

In ED14 rat fetal liver, there are three distinct populations of epithelial cells, those positive for AFP and Alb but negative for CK-19; those positive for AFP, Alb and CK-19; and those positive for CK-19, but negative for AFP and Alb [95, 96]. The number of AFP$^+$/Alb$^+$/CK-19$^+$ cells decreased

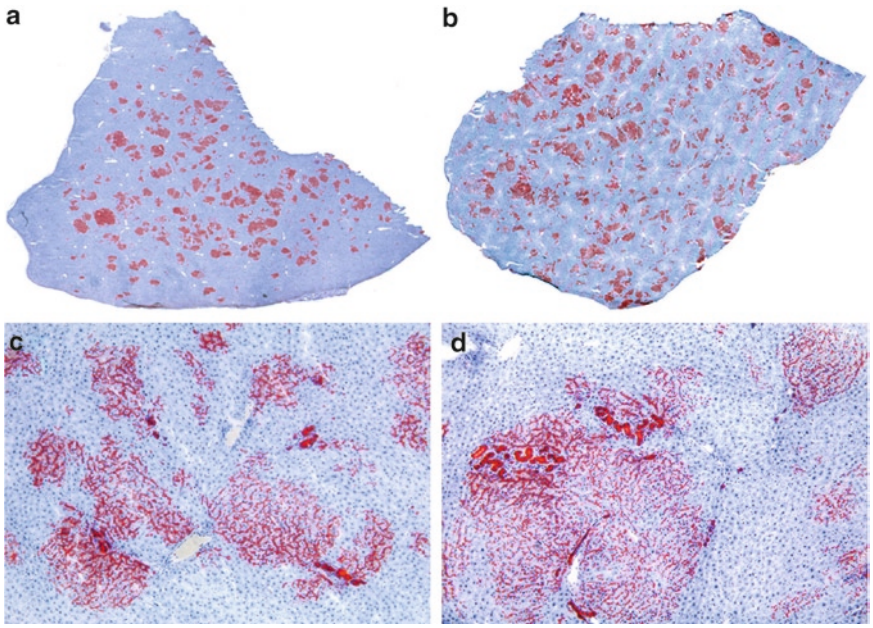

Fig. 16.2 High repopulation of the normal (non-retrorsine treated) rat liver by transplanted ED14 fetal liver stem/progenitor cells. Major lobes from two rat livers repopulated with fetal liver stem/progenitor cells scanned with a high resolution scanner (**a**, **b**) and at original magnification, 100× (**c**, **d**). Note: The majority of repopulating clusters derived from transplanted cells contain both hepatocytes (incorporated into the liver parenchyma) and cholangiocytes (incorporated into bile ducts)

dramatically at ED16, after which liver repopulation potential of rat fetal liver cells also decreases dramatically [96]. The level of liver repopulation by ED14 fetal liver cells under nonselective conditions (i.e., in a normal liver) can also be increased to 20–25% simply by increasing the number of ED14 fetal liver cells transplanted (Fig. 16.2) [99]. Repopulation continues to increase for up to 1 year, reaching an average of ~30% for the total liver. Repopulation remains stable for the life of the animal, which is consistent with the slow turnover of parenchymal cells in this organ [Oertel et al., unpublished data]. In this normal rat model, there is a several thousand fold amplification of transplanted fetal liver epithelial cells in the repopulated normal host liver [99]. Both hepatic parenchymal cords and mature bile ducts are formed by transplanted fetal liver cells, as well as whole new liver lobules, and the progeny of the transplanted cells express normal levels of hepatocytic and cholangiocytic genes in the respective cell types. As serial transplantation (an indicator of self-renewal) has not yet been demonstrated with fetal liver epithelial cells, they are referred to as fetal liver stem/progenitor cells [99, 101].

The mechanism for liver repopulation by rat fetal liver stem/progenitor cells has been shown to be cell competition between the transplanted cells and host hepatocytes [99], a process originally described in Drosophila during wing development [102, 103]. Rat fetal liver stem/progenitor cells have been cryopreserved with full ability to repopulate the normal adult liver after thawing [104] and they have been enriched to 95% purity by selection with immunomagnetic beads [105].

Liver Repopulation by Extrahepatic and Embryonic Stem Cells

Various studies have reported that cells released from the BM into the circulation migrate to the liver and differentiate into hepatocytes. However, the extent to which this occurs and the mechanism(s) involved remain controversial (for reviews, see [106–109]). Originally, Petersen and coworkers reported that BM stem cells from DPPIV$^+$ F344 rats transplanted into sublethally irradiated DPPIV$^-$ F344 rats, repopulate the BM and then migrate to the liver and "transdifferentiate" into hepatocytes through the liver "oval cell" progenitor pathway [110]. Subsequently, Theise et al. [111, 112] and Alison et al. [113] reported that mouse and human BM cells "transdifferentiated" in hepatocytes, and Lagasse et al. [114] that liver repopulation by BM cells or hematopoietic cells was much higher (30–50%) in Fah$^{-/-}$ mice. However, studies by Wang et al. [60] showed that BM cells did not enter the "oval cell" pool in mice treated with DDC. Menthena et al. [115] also showed that DPPIV$^+$ BM

cells transplanted into DPPIV$^-$ rats contributed <1% to the "oval cell" pool in three different models of "oval cell" activation: (1) 2-AAF/PH, (2) retrorsine/PH or (3) D-gal induced liver injury. These [60, 115] and other studies in both mice and rats [107–109] have demonstrated that "transdifferentiation" of hematopoietic stem cells into "oval cells" is at best a rare event, which probably does not have physiologic significance.

In Fah$^{-/-}$ mice and other model systems, it has been shown that cell fusion and reprogramming, rather than transdifferentiation, is the mechanism by which hematopoietic cells acquire a hepatocytic phenotype. Initial studies in cell culture showed that BM and neuronal cells can fuse with ES cells in culture [116, 117]. Wang et al. [118] and Vassilopoulos et al. [119] subsequently showed that hematopoietic stem cells fuse with hepatocytes in Fah null mice to produce cells that express the deficient enzyme, which then expand massively to restore liver mass and function [118]. Fusion also occurs between hematopoietic cells and neurons or muscle cells [120, 121], and it has been shown that myelomonocytic cells can fuse with hepatocytes [122, 123] or muscle cells [124] to produce somatic hybrids expressing genes from both parental cell types.

Other studies have reported that fusion is not required for BM-derived cells to differentiate into hepatocytes [125–127]. Unfractionated or CD34$^+$ enriched cells from human cord blood [128–131], multipotent adult progenitor cells (MAPC) [132, 133], or mesenchymal stem cells [134–136] have been transplanted into the liver of immunodeficient mice. These transplanted cells express a differentiated hepatocytic phenotype [128–139], but liver repopulation was once again very low. Several studies have reported that mesenchymal stem cells isolated from adipose tissue can also engraft in the liver parenchyma and contribute to liver regeneration [140, 141]. One of these studies [141] reported large repopulation clusters with hepatocyte-differentiated mesenchymal stem cells, but this required retrorsine pretreatment of the recipients and the overall level of liver repopulation was not reported. These studies are promising, as mesenchymal stem cells from adipose tissue are readily available through liposuction and could be used clinically if more robust liver repopulation could be achieved.

Many studies have demonstrated that ES cells in culture can be induced along the endodermal and hepatocytic lineages by addition of specific cytokines and growth factors [142–151]. The first step typically involves the generation of embryoid bodies, followed by the induction of definitive endoderm using activin A. The endodermal population is then further specified towards the hepatic lineage using BMP-4 and basic FGF. Cells produced in this fashion express typical hepatocytic markers, such as AFP and Alb. They can then be transplanted into the liver with differentiation into both mature hepatocytes [148–151] and bile duct epithelial cells [149], but the level of liver repopulation obtained with hepatocyte-differentiated ES cells is very low, although somewhat higher when the cells are transplanted into MUP-uPA/SCID mice [151]. To date, all ES differentiation protocols generate "hepatocyte-like" cells [148–151], but not fully mature hepatocytes. More mature "hepatocyte-like" cells have been selected using surface markers, such as the asialoglycoprotein receptor, and selected cells have shown higher levels of differentiated function after their transplantation [151]. Such methods may ultimately be successful in the future to isolate lineage-specified ES cells that will be therapeutically effective.

Induced Pluripotent Stem Cells

Because of their extensive proliferative capacity, pluripotent stem cells are an attractive potential source of transplantable hepatocytes [152]. Not only can these cells divide extensively, but they also retain the ability to differentiate into many different mature cell types [152]. Pluripotent cells can now be derived by direct genetic reprogramming of somatic cell types, such as dermal fibroblasts [153–156] and most recently, iPS cells derived from human skin fibroblasts have been differentiated along the hepatocytic lineage (B,C). In one of these studies, hepatocytic differentiated human iPS cells have been injected into the liver parenchyma of newborn mice with maintenance of hepatocytic morphology and human albumin expression [172].

Human "Oval Cells" and Stem Cells

A human counterpart to "oval cell" activation has been described in liver tissue obtained from patients with extensive chronic liver injury or submassive hepatic necrosis i.e., the so-called "ductular reaction" (for detailed description, see [157]). "Ductular reactions" are comprised of cells in ductular arrays that have the same morphologic appearance and immunohistochemical markers as found in rodent "oval cells." These cells are present primarily in the portal tracts, and express both hepatocytic and bile ductular markers, as well as certain neuroendocrine genes [157–160]. Using double and triple label immunohistochemistry, Zhou et al. [161] have shown that "ductular reactions" are bipolar structures with cells at one pole exhibiting hepatocytic morphology and gene expression (HepPar1 or HepPar1/NCAM), and cells at the other pole exhibiting biliary morphology and gene expression (CK-19 or CK-19/NCAM). Undifferentiated epithelial cells are found in the center of these bipolar structures and express only NCAM. Cells with similar morphologic and immunohistochemical properties have also been

identified in the human fetal liver beginning in the 4th week of gestation [162].

Several investigators have isolated, cultured, and/or passaged human fetal liver epithelial cells with bipotent properties, and several of these studies have demonstrated their differentiation into hepatocytes after transplantation into SCID or nude mice [71–73, 163]. Schmelzer et al. [163] have identified two populations of hepatic progenitor cells from human fetal, neonatal, and pediatric liver that exhibit stem cell properties. One population is thought to represent a hepatic stem cell (AFP^-/Alb^+) and the other a slightly more differentiated hepatoblast (AFP^+/Alb^+). More recently, Schmelzer et al. [164] reported data suggesting that these cells may reside in the Canals of Hering. One unexpected difference from their rodent counterpart is that human hepatic-specified stem cells are Alb positive but AFP negative. This is opposite to what one might expect based on expression of AFP before Alb during liver development [88]. Nonetheless, these studies suggest that a human liver somatic stem cell might exist and hopefully future studies will demonstrate in vivo self-renewal and long-term repopulation of the liver by these cells, proving that they are indeed stem cells.

Xenorepopulation Models

Two models capable of supporting extensive repopulation of the mouse liver with human hepatocytes have been reported. In 2001, Dandri et al. first showed that immune-deficient uPA transgenic mice could be engrafted with human hepatocytes and used as a model for hepatitis B [165]. Subsequently, this model has been further developed to permit extensive repopulation, reaching levels as high as 90% human cells [166, 167]. Fah knockout mice can also be repopulated extensively with human cells from a variety of sources when they are crossed onto severely immune deficient backgrounds [168]. This model has the advantage that the level of hepatic injury can be titrated by NTBC. Both systems are likely to be important in identifying stem cell derived "hepatocytes" that can be used in cell therapy. Table 16.1 summarizes the results of all cell transplantation/liver repopulation studies reviewed above, and indicates the model systems and specific physiologic or pathophysiologic circumstances, i.e., selective or nonselective conditions, under which liver repopulation has been achieved.

Future Horizons

Although substantial progress has been made during the past 10–15 years concerning the possibility of liver repopulation by transplanted cells, much still needs to be learned. Factors governing engraftment of transplanted cells into the liver and their homing to the correct niche, factors regulating proliferation and differentiation of transplanted cells into specific phenotypes required for organ function and specific host conditions under which effective liver replacement can be achieved, all need to be determined. The best starting point for therapeutic liver repopulation will probably be a genetic disorder with ongoing liver injury, which will hopefully induce or augment proliferation of transplanted cells in the host liver. One example of such a condition is Wilson's disease, in which transplanted cells might also have a modest selective advantage [169], since they will not store high levels of copper. Another example is α_1-antitrypsin deficiency, in which a mutated form of α_1-antitrypsin (PiZZ phenotype) remains within the hepatocyte and causes liver injury [170].

To date, very few patients have been transplanted with fetal liver cells. Studies in rats have shown that fetal liver cells, i.e., fetal hepatoblast, have the capacity to proliferate in the host, replace hepatic mass with functional hepatocytes and maintain differentiated hepatic function, long-term [96, 99]. This requires a liver proliferative stimulus or liver injury at the time the cells are transplanted, but no selective advantage other than the inherent ability of fetal hepatoblasts to replace host hepatocytes by cell competition, a process during which the transplanted fetal liver stem/progenitor cells, which have a higher proliferation potential than normal mature hepatocytes, induce apoptosis in the latter and replace them in the liver tissue [99]. Cells with similar properties to fetal liver progenitor cells have been identified in the normal adult liver and these cells can be activated to proliferate under conditions in which there is an impairment in the ability of mature hepatocytes to undergo cell division These are the so called "oval cells," but their ability to effectively repopulate the adult liver occurs only under highly selective conditions.

Stem cells from pediatric or adult cadaveric liver or from other sources, such as bone marrow, cord blood or ES cells, as well as cell lines from various sources, are possibilities for liver repopulation, as well as cultured fetal liver cells or adult hepatocytes modified to favor engraftment and proliferation in the host. However, this will require substantial additional research. Unfortunately, existing cell lines show only limited repopulation potential in the normal liver at the current state-of-the-art. To further advance the field of liver cell therapy, it will be necessary to find conditions under which cells and cell lines derived from ES cells, iPS cells, fetal liver or adult liver, can be expanded in culture and successfully repopulate the liver under conditions that will be clinically acceptable. Although we are not yet there, restoration of liver function by therapeutic cell transplantation holds great promise for the future.

Table 16.1 Liver repopulation by transplanted cells of hepatic and nonhepatic origin

Type of cell	Model systems used	Comments	Selected publications
Cells of hepatic origin			
Adult hepatocytes[a]			
Mouse, rat or human	uPA transgenic mouseFah null mouse Retrorsine (or monocrotaline) treated rat or mouse plus PH or CCl_4 administration x-Irradiation of rat or mouse liver plus pH, HGF or ischemic liver injury Bcl-2 transgenic mouse hepatocytes into anti FAS Ab treated mouse p27 null mouse hepatocytes into wt mouse plus CCl_4 at 4–8 weekly intervals	Effective repopulation (range, 30–90%), requires highly selective conditions, including: Massive and continuous liver injury or sustained disruption of host hepatocyte proliferation Selective advantage for transplanted cells to survive compared to host hepatocytes With Bcl-2 transgenic hepatocytes in normal mice treated with weekly injections of anti FAS Ab for 8–12 weeks, repopulation was 3–16% With p27 null mouse hepatoytes in normal mice with 4–8 weekly injections of CCl_4, repopulation was 2–6%	Rhim et al. [10]Overturf et al. [11] Laconi et al. [15] Mignon et al. 1998; [24] Guha et al. [20] Malhi et al. [21] Dandri et al. [165] Yuan et al. [25] Tateno et al. [166] Witek et al. [18] Azuma et al. [168] Haridass et al. [100]
Oval/progenitor cells[b]			
Mouse and rat (2-AAF or D-gal activated liver)	Fah null mouseRetrorsine/PH treated rat	Effective repopulation requires highly selective conditions (range, 20–80%)	Wang et al. [60]Song et al. [61] Yovchev et al. [62]
Fetal hepatoblasts			
ED14 rat	$DPPIV^-$ rat/PH	Effective repopulation occurs under nonselective conditions (5–25%)	Sandhu et al. [96]Oertel et al. [99]
ED11.5–13.5 mouse	$DPPIV^{-/-}$ mouse/$CCl_4$$DPPIV^{-/-}$ mouse retrorsine/CCl_4 Alb-uPA/$Rag2^{-/-}/\gamma_c^{-/-}$ mouse	Repopulation <1% (nonselective conditions)Repopulation 10–80% (highly selective conditions) Repopulation 2–20% (highly selective conditions)	Nierhoff et al. [17]Haridass et al. [100]
"Stem" cells			
Human fetal, neonatal or adult liver	NOD/SCID mouse/±CCl_4	Short-term repopulation (2–8 days), similar results ± CCl_4 (% repopulation not determined)	Schmelzer et al. [164]
Cultured hepatic epithelial cells and cell lines			
Mouse, rat and human	$DPPIV^-$ ratsMouse/CCl_4 SCID mouse/CCl_4 uPA/SCID mouse $Rag2^{-/-}$ mouse/ retrorsine/CCl_4 $Rag2^{-/-}/\gamma_c^{-/-}$/50% PH Fah null mouse	Repopulation minimal under nonselective conditions (<0.1% or not determined)Under highly selective conditions, repopulation still very low (≤2%)	Coleman et al. [66]Yasui et al. [67] Suzuki et al. [68] Strick-Marchand et al. [69] Fougère-Deschatrette et al. [70] Suzuki et al. 2008 [63] Malhi et al. [71] Mahieu-Caputo et al. [72] Dan et al. [73]
Cells of nonhepatic origin			
Bone marrow or cord blood (hematopoietic) stem cells[c]			

(continued)

Table 16.1 (continued)

Type of cell	Model systems used	Comments	Selected publications
Mouse and human	Rat/2-AAF/CCl_4SCID mouse NOD/SCID mouse Fah null mouse Patients with BM or liver transplants	Human "hepatocyte-like" cells observed in all cases, but repopulation generally low, except for one human liver biopsy from patient with chronic HCV infection (40%) and in FAH null mice under highly selective conditions (30–50%)	Petersen et al. [110]Theise et al. [111] Theise et al. [112] Alison et al. [113] Lagasse et al. [114] Danet et al. [128] Kakinuma et al. [130] Kollet et al. [131] Newsome et al. [125]
Mesenchymal stem cells			
Cultured mouse and human	NOD/SCID mouse*Pfp*/$Rag2^{-/-}$/CCl_4 mouse uPA/$Rag2^{-/-}$ mouse	Repopulation generally very low or not studied (nonselective conditions)	Jiang et al. [133]Aurich et al. [139] Brulport et al. [135] Kuo et al. [136]
Adipose stem cells			
Cultured rat or human	Balb c nude mouseDPPIV$^-$ rat/retrorsine	Scattered cells observed expressing human alb or α_1-AT, levels of repopulation not studied	Banas et al. [140]Sgodda et al. [141]
Embryonic stem (ES) cells			
Mouse and human	DPPIV$^{-/-}$/$Rag2^{-/-}$ mouse/Retrorsine/CCl_4 uPA/SCID mouse NOD/SCID mouse	Scattered cells or cell clusters with "hepatocyte-like" phenotype; % repopulation low or not reported, except for [149] (1.94%)	Gouon-Evans et al. [148]Heo et al. [149] Duan et al. [150] Basma et al. [151] Si-Tayeb et al. [172] Sullivan et al. [173]
Induced pleuripotent stem (iPS) cells			
Mouse and human	Differentiation of human iPS cells along hepatocytic lineage in cell culture and expression of human albumin after transplantation directly into newborn mouse liver parenchyma		

[a]In a normal rat or mouse host liver, repopulation by transplanted hepatocytes was minimal (usually less than 1%), even after repeated liver injuries or multiple cell infusions (Rajvanshi et al. [7, 8])

[b]In a normal host liver (rat), repopulation by transplanted oval/progenitor cells is <1% (Dabeva et al. [57])

[c]In Fah null mice, fusion between transplanted hematopoietic cells and hepatocytes has been reported (Wang et al. [118]; Vassilopoulos et al. [119]; Camargo et al. [122]; Willenbring et al. [123])

Acknowledgements The authors would like to thank Anna Caponigro and Emily Bobe for assistance in typing this manuscript.

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Chapter 17
Adult Liver Stem Cells

D. Hunter Best and William B. Coleman

Introduction

The search for stem-like progenitor cells of the adult liver has been ongoing for many decades. However, as recently as 20 years ago the existence of liver cells with stem-like potential was critically questioned and not generally accepted [1, 2]. In contrast, it has long been known that tissues with high cellular turnover (like skin, intestine, and bone marrow) contain stem cells that function to maintain tissue homeostasis through continuous renewal of the cell lineage [3–8]. Evidence for stem-like liver progenitor cells first emerged from studies of liver injury, regeneration, and carcinogenesis in rodent models. Some evidence for stem-like progenitor cells of the adult human liver has appeared in the literature during the last 20 years. Since that time, investigations into the roles that these stem-like progenitor cells play in response to hepatic injury and carcinogenesis have escalated. In addition, there is tremendous interest in pursuing the potential application of stem-like progenitor cells for treatment of liver disease through gene therapy and cell transplantation approaches [9–13].

New insights into cell lineage generation and new understanding of the relationships of cells composing a lineage in adult organisms have modified the traditional thinking that stem-like progenitor cells are necessarily undifferentiated cells with a limited degree of potency [14]. However, modification of the paradigm has not resulted in a universally accepted definition of what a stem-like progenitor cell represents [15]. Rather, there has been a reevaluation of the contributions of various cell types, both differentiated and undifferentiated, to normal lineage renewal, response to injury, and tissue regeneration. In the liver, these changing concepts have given rise to a recognition that there are multiple liver epithelial cell types that have the potential to originate new cell populations [16, 17]. In addition, a number of intriguing new observations have been made that suggest the stem cells may yield progeny that evince a high degree of phenotypic plasticity [18, 19]. Several reports have demonstrated that adult stem-like progenitor cells from one tissue can be induced to differentiate into parenchymal cells of other tissues when transplanted into appropriate sites, including the derivation in the liver of hepatocytes by transplantation of stem-like progenitor cells from extrahepatic sources [20, 21]. These new results suggest that stem-like progenitor cells of adult tissues may not be intrinsically restricted in their differentiation commitment, and/or that they are capable of responding to different tissue microenvironments with an alternative cellular differentiation that is appropriate for the new site [22].

In this chapter, we review the evidence for stem-like progenitor cells of the adult liver, including (1) the types of cells in the liver that exhibit stem-like potential, (2) sources of liver stem-like progenitor cells from extrahepatic tissues, (3) evidence for human liver stem-like progenitor cells, (4) isolation, culture, and characterization of liver stem-like progenitor cells from rats, and (5) evidence for the differentiation potential of cultured rat liver stem-like progenitor cells. Given the scope of this undertaking and the large number of published studies on these topics, we have not attempted to comprehensively review the literature. Therefore, in the latter sections of this chapter, we will focus our review on studies of the well characterized WB-F344 rat liver epithelial stem-like progenitor cell line [23]. Furthermore, where possible, the reader is directed to the excellent reviews on these subjects that are available in the literature. There are additional chapters in this textbook on cell therapies, liver development, and cancer stem cells for readers interested in these topics.

History of Liver Stem-Like Progenitor Cell Biology

The idea that the liver of adult rodents contain stem-like progenitor cells that can give rise to the epithelial cell types of the liver (hepatocytes and biliary epithelial cells) developed

W.B. Coleman (✉)
Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, USA
e-mail: wbcolemn@med.unc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_17, © Springer Science+Business Media, LLC 2011

from evidence accumulated from over a hundred years of investigation [17, 24]. Early in the last century, potential lineage relationships among biliary epithelial cells, transitional cells, and hepatocytes were recognized in several studies of liver regeneration [25–28]. Studies on hepatocarcinogenesis in experimental animals produced additional evidence that hepatocytes could be generated through the activation, proliferation, and differentiation of transitional cells possessing features of both ductular cells and hepatocytes [29, 30]. Subsequently, Wilson and Leduc [31] suggested that cells contained in the cholangioles or terminal bile ductules constitute a compartment of stem-like progenitor cells that can proliferate and generate hepatocytes in some forms of liver injury. Since then, the concept of the liver stem-like progenitor cell has generated considerable controversy, argument, and discussion [1, 2, 16, 32–36].

The debate surrounding the liver stem-like progenitor cell is fueled by the fact that stem cells and their phenotypic characteristics are largely intuitive concepts. As it is true for other tissues, putative stem-like progenitor cells of the liver have not been identified microscopically in situ and have not been prospectively isolated from the normal liver in pure form. In addition, hepatocytes and biliary epithelial cells of the normal liver demonstrate very little cellular turnover and do not operate as a typical stem cell-fed lineage system [37, 38] like those of other self-renewing tissues, such as intestine [5, 39–41], skin [42–44], and bone marrow [45, 46]. These observations and the fact that the adult liver retains the capacity for complete and rapid renewal of cell numbers in response to cell loss through activation and proliferation of fully differentiated cells (both hepatocytes and biliary epithelial cells) seem to argue against the need for a stem-like progenitor cell in the adult liver tissue. However, these observations do not eliminate the possibility that cells possessing a broader differentiation potential are present in the adult liver as a "reserve" [32] or "facultative" [47, 48] stem-like progenitor cell compartment, or that cells with stem-like properties may serve a physiological function other than (or in addition to) lineage renewal. It has been suggested that participation of stem-like progenitor cells in liver growth processes may depend upon several factors, including the presence or absence of liver injury, the type and extent of injury, and the capacity of hepatocytes to respond to growth stimuli [10], consistent with the concept of the "facultative" liver stem cell [47, 48]. Despite the controversy surrounding the concept of the liver stem-like progenitor cell, considerable evidence has accumulated that supports the notion that the adult rodent liver contains cells with stem-like properties that can serve as progenitor cells for both hepatocytes and biliary epithelial cells under certain pathophysiological circumstances [17, 49]. Three major sources of evidence support the existence of liver stem cells: (1) the founding of lineages of hepatocytes and biliary epithelial cells from hepatoblasts during embryonic development of the liver and the expression of differentiated cell-specific traits in cultured hepatoblasts; (2) the reestablishment of epithelial lineages following the proliferation of simple epithelial cells (oval cells) in livers subjected to carcinogenic or noncarcinogenic liver injury; (3) the isolation and propagation from the livers of adult rodents of simple epithelial cells that demonstrate the ability to differentiate into hepatocytes and/or biliary epithelial cells when transplanted into appropriate sites in vivo, or when cultured under specific conditions ex vivo. These sources of evidence have been comprehensively discussed in several reviews [17, 32, 49–57].

Essential Properties of Stem-Like Progenitor Cells

The major properties thought to characterize stem-like progenitor cells have been inferred from investigations of classic stem cell-fed lineage renewal systems, including bone marrow, intestinal epithelium, and skin. Essential properties expected of stem-like progenitor cells include the capacity to (1) proliferate repeatedly, (2) renew the stem cell population, and (3) generate sufficient differentiated progeny to maintain or regenerate the functional capacity of a tissue [39, 43]. Classic stem cells are thought to exhibit undifferentiated cellular phenotypes, to express variable differentiation potentials, and to be able to proliferate continuously (actual stem cells), or to be proliferationally quiescent until needed (potential or facultative stem-like progenitor cells) [39, 43]. While classic stem cell-fed lineage systems have been used to infer the properties of stem-like progenitor cells, evidence now suggests the existence of stem-like cells in many tissues that do not contain active stem cell-fed lineages [58], such as the central nervous system [59–63] and liver [17, 24, 49]. These newly discovered stem-like cells appear to have properties that differ from those proposed for classic stem cells.

Stem-Like Progenitor Cells of the Adult Liver

Unlike rapidly renewing epithelial tissues (such as the intestinal mucosa or skin), in which an active stem cell lineage system continually initiates replacement of differentiated cells that are shed [64, 65], the liver is normally a quiescent organ with minimal/slow rates of cell turnover in the adult [66]. Nonetheless, the liver possesses an extraordinary capacity for the regeneration of tissue mass following loss of normal hepatocyte numbers due to partial tissue loss (surgical resection) or hepatotoxic injury (necrosis). A number of different cell types can be activated to repair or regenerate the liver depending

upon the nature and extent of injury and/or tissue deficit [17, 49]. In an otherwise healthy liver, the replacement of hepatocytes (and tissue mass) lost to surgical resection or toxic injury is achieved through the proliferation of differentiated, normally quiescent hepatocytes contained in the residual (viable) tissue (Fig. 17.1). For additional information on the subject of liver regeneration, please refer to Chap. 18. However, certain forms of liver injury impair the capacity of the remaining differentiated hepatocytes to proliferate in response to liver tissue deficit. When this occurs, a reserve or facultative stem-like progenitor cell compartment is activated to proliferate and replace the lost hepatocytes. Recent evidence suggests that there are at least two distinct cell populations that can be activated to generate new hepatocytes and/or cholangiocytes. Rodent livers contain a population of normally quiescent (facultative), undifferentiated stem-like progenitor cells that reside in or around the biliary ductules of the portal tracts, which can be activated under certain pathological conditions to reestablish a proliferating-differentiating lineage (the oval cell reaction), capable of generating hepatocytes and some other cell types [67, 68]. In addition, the adult liver contains a population of incompletely differentiated small hepatocyte-like progenitor cells (SHPC) that can be activated to replace lost hepatocytes in some forms of tissue injury [69]. These observations combine to suggest that there are at least three distinct populations of cells with stem-like potential in the adult rat liver (Fig. 17.1). It is conceivable that all these stem-like progenitor cells of the adult liver are derived from the same primordial stem cell population of the developing liver. In the following sections, the evidence for the existence of each of these stem-like progenitor cell populations in liver will be reviewed.

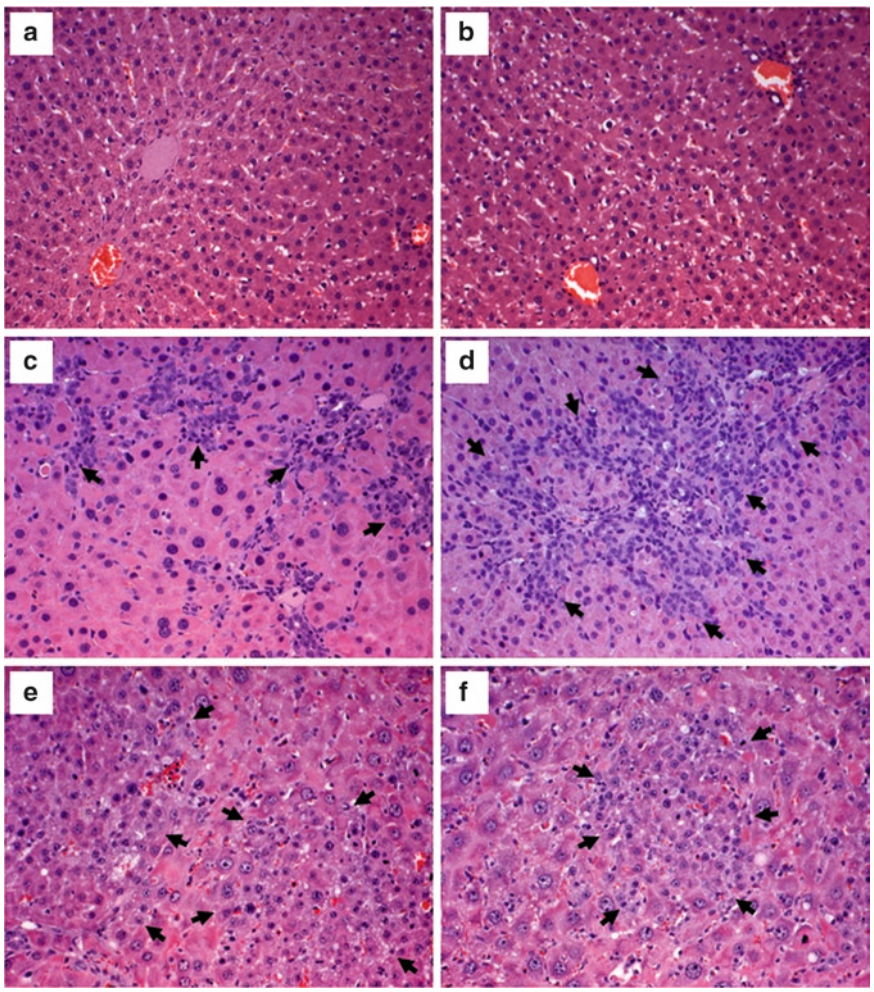

Fig. 17.1 Stem-like progenitor cell responses to signals for liver regeneration. liver regeneration after surgical partial hepatectomy involves proliferation of differentiated hepatocytes (**a**, **b**). However, when differentiated hepatocytes cannot proliferate in response to the regenerative stimulus, other liver progenitor cell populations are activated, such as oval cells in the 2-AAF model of liver injury (**c**, **d**), or small hepatocyte progenitor cells (SHPC) after retrorsine-mediated liver injury (**e**, **f**). *Arrows* denote oval cells (**c**, **d**) and SHPC (**e**, **f**). Original magnification: ×10

Unipotential Liver Stem-Like Progenitor Cells: Differentiated Hepatocytes and Biliary Epithelial Cells

The liver possesses an enormous capacity to replace cells that are lost to surgical resection or necrosis [10, 70–74]. Activation of undifferentiated stem-like progenitor cells does not occur after cell loss when mature hepatocytes and biliary epithelial cells are capable of proliferating to restore the normal liver mass and structure [75, 76]. In rats subjected to surgical partial hepatectomy, the residual (viable) hepatocytes undergo a rapid burst of proliferation that ultimately restores the normal hepatocyte number [66, 77–79]. Likewise, biliary epithelial cells proliferate after partial hepatectomy to form expansions of the intrahepatic duct system [66, 80, 81]. Irrespective of their location within the parenchyma (periportal to pericentral), virtually all hepatocytes proliferate and divide at least once during restoration of the hepatocyte number [82, 83]. The ability of quiescent hepatocytes to reenter the cell cycle and proliferate in response to liver deficit has fascinated investigators throughout history. More recently, the extensive growth potential and enormous proliferative (replicative) capacity of the mature hepatocyte has become evident. In rats, hepatocytes proliferate and divide at least 8–12 times during the prolonged process of liver growth following five consecutive partial hepatectomies [84]. In transgenic mice that express the urokinase gene in the liver under the direction of the albumin promoter-enhancer, the majority of hepatocytes succumb to the toxic transgene product [85]. In this model, the toxic transgene becomes inactivated in random hepatocytes enabling them to proliferate, undergoing 10–12 cycles of cell division to yield discrete nodular aggregates (clones) that repopulate the liver parenchyma [86]. In a similar experimental system, transplanted normal hepatocytes repopulate the livers of transgenic mice that lack fumarylacetoacetate hydrolase ($FAH^{-/-}$) enzyme activity [87] due to the targeted disruption of exon 5 of the *Fah* gene [88]. In this model, the transplanted FAH-expressing hepatocytes exhibit a selective growth advantage over host FAH-deficient hepatocytes, allowing the former to repopulate the livers of mutant mice. In these studies, it was estimated that transplanted hepatocytes proliferated through at least 15 cell divisions during repopulation of mutant livers [87]. In other studies, wild-type male hepatocytes were serially transplanted at limiting dilution through the livers of female $FAH^{-/-}$ mice [89]. Complete repopulation of the diseased liver was accomplished in each round. The complete replacement of host liver by the progeny of transplanted hepatocytes through seven rounds of transplantation suggests that the transplanted hepatocytes were capable of at least 100 population doublings [89]. In addition, these investigators found that both diploid and polyploid hepatocytes were capable of liver repopulation in the $FAH^{-/-}$ mouse model system [90]. More recently, immunodeficient $FAH^{-/-}$ mice were pretreated by infection with an adenovirus expressing urokinase followed by transplantation of human hepatocytes [91]. In these animals, the engrafted human hepatocytes replaced up to 90% of the liver parenchyma, and could be serially transplanted through several animals, suggesting that human hepatocytes have an extensive replicative capacity similar to that of rodent hepatocytes [91]. Together, these studies demonstrate the incredible capacity for cell proliferation by differentiated hepatocytes (including human hepatocytes), consistent with the suggestion that these cells represent a unipotential stem-like progenitor cell population of the adult liver.

Unipotential Liver Stem-Like Progenitor Cells: Small Hepatocyte Progenitor Cells

In several experimental models, hepatocytes are rendered incapable of proliferation through treatment with mito-inhibitory compounds, facilitating the outgrowth of stem-like progenitor cells in response to liver deficit. We have described the cellular responses and time course for liver regeneration after surgical partial hepatectomy (PH) in rats with retrorsine-induced hepatocellular injury [69]. Similar to other models of chemical liver injury [17, 24], systemic exposure to retrorsine, a member of the pyrrolizidine alkaloid (PA) family, results in a severe inhibition of the replicative capacity of fully-differentiated hepatocytes [69, 92–94]. When confronted with a strong proliferative stimulus such as PH [69, 92, 93] or hepatocellular necrosis [95], retrorsine-injured hepatocytes synthesize DNA, but are unable to complete mitosis, and arrest as non-proliferative giant cells (megalocytes). In this model, neither retrorsine-injured, fully-differentiated hepatocytes nor oval cells proliferate sufficiently to contribute significantly to the restoration of liver mass after PH. Instead, the entire liver mass is reconstituted after PH through a novel cellular response that is mediated by the emergence and rapid expansion of a population of small hepatocyte-like progenitor cells (SHPCs), which share some phenotypic traits with fetal hepatoblasts, oval cells, and fully-differentiated hepatocytes, but are morphologically and phenotypically distinct from each [69]. SHPCs emerge in all regions of the liver lobule (periportal, pericentral, and midlobular) after PH and are derivative of the modest oval cell outgrowth in periportal regions, suggesting that SHPCs represent a novel cell population [69].

Small hepatocyte progenitor cells morphologically, most closely resemble differentiated (but small) hepatocytes at early time-points after PH, perhaps suggesting that SHPCs are a subset of retrorsine-resistant hepatocytes and not a novel progenitor cell population. However, the phenotype of SHPCs

indicate that they are in fact distinct from fully-differentiated hepatocytes, since a subset of SHPCs express the oval cell/bile duct/fetal liver markers OC.2 and OC.5 through 5 days post-PH [69]. Co-expression of hepatocyte markers and oval cell markers by early-appearing SHPCs suggest that these cells are not fully-differentiated, and that they display a phenotype similar to that expected for a cell type transitional between the bipotential hepatoblast (E14) and a fetal hepatocyte (E18–E20). Retrorsine-exposed rats are able to regenerate their liver mass completely after PH as is evidenced by liver weights and liver/body weight ratios [69]. At 30 days post-PH, liver weights and liver/body weight ratios do not differ significantly after either retrorsine/PH, or control/PH [69]. By this time, the progeny of SHPCs occupy virtually the entire parenchyma in retrorsine/PH rats (~90% by area). However, comparison of the time-course for liver regeneration in control and retrorsine-exposed rats after PH shows that liver regeneration through activation and expansion of SHPCs is a much more protracted process. Complete regeneration of the liver mass in retrorsine-exposed animals requires nearly 30 days, compared to about 10 days in control rats [69].

Using a combined approach involving gene expression analysis of tissues isolated using laser capture microdissection and in situ immunohistochemistry, the expression patterns of select mRNAs and proteins were examined in the earliest (least-differentiated) SHPCs that emerge after PH in retrorsine-exposed rat livers [96]. The results show that early-appearing SHPCs (at 3–7 days post-PH) express mRNA and/or protein for all of the major liver-enriched transcription factors (HNF1α, HNF1β, HNF3α, HNF3β, HNF3γ, HNF4, HNF6, C/EBPα, C/EBPβ, and C/EBPγ), WT1, α-fetoprotein, and P-glycoprotein [96]. Compared to surrounding hepatocytes, early-appearing SHPCs lack (or have significantly reduced) expression of mRNA for hepatocyte differentiation markers tyrosine aminotransferase and α1-antitrypsin [96]. Likewise, SHPCs that emerge and proliferate during the early phases of liver regeneration lack or have reduced expression of several hepatic CYP proteins that are known to be induced in rat livers after retrorsine exposure (CYP2E1, CYP1A2, and CYP3A1). However, by 30 days post-PH, expression patterns of all markers expressed by SHPCs mirrors those expected for fully-differentiated hepatocytes. Both α-fetoprotein and WT1 protein are uniquely expressed by SHPCs during the early phase of liver regeneration, suggesting that these markers may be used to identify the earliest progenitors of these cells [96]. These results suggest that SHPCs represent a unique parenchymal (less-differentiated than mature hepatocytes) progenitor cell population of adult rodent liver [69, 96]. The progenitor cell of origin of the SHPCs is a hotly debated topic with some investigators suggesting that these cells represent an intermediary between oval cells and mature hepatocytes [10, 97], others suggesting that these cells are derived from mature (fully-differentiated) hepatocytes [98–100], and still others suggesting that these cells represent an independent progenitor cell population [69, 101, 102]. As a result, several studies have been performed by our group and others to elucidate the origins of the SHPCs [100, 103, 104].

To address the possible progenitor relationship between oval cells and SHPCs, retrorsine-exposed Fischer 344 rats were treated with the mitoinhibitory agent 2-acetamidofluorene (2-AAF), 7 days prior to PH [103]. In marked contrast to animals treated only with retrorsine, the livers harvested from 2-AAF/retrorsine-treated rats at 3 and 7 days post-PH showed extensive proliferation of oval cells, but no proliferation of SHPCs [103]. Clusters of "small hepatocytes" are first observed 10 days post-PH, continue to proliferate through 14 days post-PH, and restore liver weight to control levels by 21 days post-PH [103]. Labeling of proliferating oval cells with bromodeoxyuridine (BrdU) at 6 days post-PH demonstrated that the "small hepatocytes" observed at 10 days post-PH in this model are progeny of oval cells and not an independent cell population [103]. Further evidence that these "small hepatocytes" are not the same population as SHPCs was obtained by treating retrorsine-exposed rats with 2-AAF 7 days post-PH. In these animals, the administration of 2-AAF resulted in a blockade of SHPC expansion suggesting that these cells are susceptible to 2-AAF poisoning [103]. As the "small hepatocytes" observed in 2-AAF/retrorsine-exposed animals proliferate in the presence of 2-AAF, this is strong evidence that, although histologically similar, these cells are not SHPCs. The suggestion that oval cells and SHPCs do not share the same cellular origin has been confirmed recently in studies by Pichard et al. [100]. Additional evidence that oval cells are not the source of SHPCs emerged from experimental models that employ the bile duct toxin diaminodiphenylmethane (DAPM) [104]. In this study, retrorsine-exposed Fischer 344 rats were treated with DAPM 24 hours prior to PH. In this model, DAPM treatment results in significant damage to the bile ducts, but had no effect on liver regeneration. Oval cells were never observed in DAPM/retrorsine treated animals but SHPC clusters were observed beginning at 1–3 days post-PH. These clusters continued to proliferate and expand through the subsequent interval in a manner identical to that observed in animals treated only with retrorsine until liver weight was restored by the end of the 30 day experimental period [69, 104]. These results provide direct evidence that oval cell proliferation is not necessary for SHPC-mediated liver regeneration and provides the strongest evidence yet that these cells are not derived from oval cells. Instead, the findings of this study suggest that the cell of origin of the SHPC is likely to be located in the liver parenchyma. However, the exact cell type giving rise to these cells remains unknown. Our group has posited that SHPCs may arise from a population of cells that are histologically

similar to mature hepatocytes but are phenotypically distinct [101, 102]. This is based on the original characterization of the SHPCs where it was demonstrated that SHPCs exhibit characteristics of fetal hepatoblasts, oval cells, as well as mature hepatocytes [69, 96]. These characteristics suggest that a mature (fully-differentiated) hepatocyte is unlikely to be the source of a less differentiated cell population. Regardless, studies have been published suggesting that mature hepatocytes are the source of SHPCs. In one recent study, Ferry and colleagues used a method of genetically labeling hepatocytes in a modified version of the retrorsine model of liver injury to investigate the possibility that mature hepatocytes are the source of SHPCs [98]. In this study, the authors utilize a retroviral vector containing the β-galactosidase gene to label mature hepatocytes in retrorsine-treated Sprague Dawley rats. The animals were then subjected to PH, biopsied 28 days post-PH, and euthanized 56 days post-PH [98]. The authors observed the presence of SHPCs in the livers of these animals at both the time of biopsy and sacrifice. β-galactosidase staining was observed in some of the cell clusters present in the livers of these animals suggesting that some retrovirally labeled cells were the source of the SHPCs [98]. Based on these findings, the authors concluded that mature hepatocytes are the source of SHPCs. However, these findings do not address the phenotypic differences between those observed in SHPCs and that of mature (fully-differentiated) hepatocytes. In addition, the findings of this study do not rule out the possibility that the cell of origin of the SHPC is a less differentiated cell type located in the liver parenchyma. As such, additional studies are required to definitively establish the source of these interesting cells.

Multipotential Liver Stem-Like Progenitor Cells: Oval Cells

Oval cells, which proliferate in several hepatocarcinogenesis models and in some forms of noncarcinogenic liver damage, may be related to liver stem-like progenitor cells. A number of different experimental models elicit the proliferation of oval cells [17, 50, 76, 105]. All of these models are characterized by concurrent stimulation of liver growth and inhibition of normal mechanisms for liver tissue restoration (i.e. blockade of the proliferation of hepatocytes). The stimulus for liver growth can be satisfied through several different methods, including surgical resection, nutritional stress, or chemically-induced necrosis. Blockade of hepatocyte proliferation is frequently achieved by the use of chemicals (such as 2-acetylaminofluorene) that impede or prevent mitotic division of mature hepatocytes [67, 68]. The cellular response common to each of these models involves the proliferation of small cells with scant cytoplasm and ovoid nuclei that are morphologically described as oval cells [106]. While most of the models of oval cell proliferation involve rats, similar models have been developed using carcinogen-treated mice and transgenic mice that express viral oncogenes or other transgenes [107–112].

The timing of cellular events differs, sometimes dramatically, among the various models of oval cell proliferation [17]. However, the majority of oval cell proliferation models share a common sequence of events: (1) Proliferation of oval cells in or around the portal spaces, (2) invasion of the lobular parenchyma by the proliferating oval cells, (3) appearance of transitional cell types and immature hepatocytes, and (4) maturation of hepatocytes and restoration of normal liver structure. Oval cells are initially seen in the portal zones of the liver lobule in the regions of terminal bile ductules or cholangioles [32, 113]. Proliferating oval cells are recognized to represent a collection of phenotypically distinct cells that compose a heterogeneous cell population or "compartment" [37, 114, 115]. Morphologically, the typical oval cell possesses cellular characteristics similar to those cells of terminal bile ductules [116–118]. However, the oval cell compartment also contains transitional cells that display morphologic features intermediate between oval cells and hepatocytes [118, 119].

Proliferating oval cells form irregular duct-like structures that are connected to pre-existing bile ducts [118, 120, 121]. As they proliferate, oval cells migrate from the portal regions into the lobular parenchyma, sometimes occupying a large percentage of the liver mass. Groups of small basophilic hepatocytes appear among oval cells, and these immature hepatocytes proliferate and differentiate as the oval cells gradually disappear and the normal liver structure is restored [118, 122–125]. The possibility that oval cells might possess stem-like properties and give rise to hepatocytes and/or biliary epithelial cells has been recognized for some time [29, 31, 106]. Several studies have attempted to document the fate of oval cells that proliferated in various hepatocarcinogenesis models and after noncarcinogenic liver injury, producing evidence that oval cells are precursors of hepatocytes [122–126]. Using the modified Solt-Farber model of oval cell proliferation, Evarts et al. [122, 124] demonstrated unequivocally that oval cells radiolabeled with ^{3}H-thymidine could directly give rise to tagged basophilic hepatocytes. More recently, Alison and colleagues examined the proliferation and fate of oval cells in rats using the modified Solt-Farber model with various doses of 2-acetylaminofluorene [127]. Proliferation of oval cells also has been described in the chronic injury produced in mouse liver by transgenic expression of both hepatitis B virus [111] and SV40 T antigen [109]. Bennoun and colleagues [109] demonstrated a transition between proliferating oval cells in SV40 T antigen transgenic mice and newly formed hepatocytes. Likewise in mice treated with diethylnitrosamine, oval cells proliferate

and subsequently differentiate into hepatocytes [107]. Employing the D-glactosamine model of oval cell proliferation in rats, Lemire et al. [125] and Dabeva and Shafritz [123] also demonstrated the transfer of the radiolabel from oval cells to small hepatocytes. In both of these studies, transition from oval cells to hepatocytes was accompanied by a shift from the biliary epithelial/oval cell phenotype (expression of α-fetoprotein, γ-glutamyltranspeptidase, and biliary epithelial-type cytokeratins) to a cellular phenotype characteristic of hepatocyte differentiation (expression of albumin, glucose-6-phosphatase, and other hepatocyte markers; reduction of α-fetoprotein expression) [123, 125].

Liver Stem-Like Progenitor Cells from Extrahepatic Tissues

In addition to the progress characterizing stem-cell responses in liver and the various populations of liver cells with stem-like potential, advances have also been made in the identification of multipotent adult stem cells with broad differentiation potential that includes liver. A number of studies have identified extrahepatic sources of stem-like cells that can colonize the liver and/or give rise to hepatocytes in vivo or in culture. Most recently, several investigators have reported that progenitor cells contained in bone marrow or peripheral blood can give rise to cells of the liver [21]. In somewhat older studies, the ability of pancreatic cells to give rise to hepatocytes has been described. In addition to these two extrahepatic sources of stem-like progenitor cells for liver epithelial cells, several other sources have also been suggested, including neural stem cells. Evidence that extrahepatic stem cells can give rise to liver is summarized in the sections that follow.

Liver from Progenitor Cells of the Bone Marrow

Bone marrow contains several different cell types with stem-like potential, including hematopoietic, stromal, and mesenchymal stem cells. In addition to these progenitor cell types, it has been suggested that bone marrow contains a multipotent adult stem-like progenitor cell that expresses a broader tissue differentiation potential. However, whether this multipotent progenitor cell compartment of the bone marrow represents a single cell type with broad differentiation capacity, or whether it represents an admixture of several tissue stem cell types has not been resolved [128]. In fact, the multipotent stem-like progenitor cell of bone marrow may be related (or identical) to one of these cell types (hematopoietic or mesenchymal stem cells) of the bone marrow. Bone marrow transplants generate cell lineages of the blood, and have now been suggested to give rise to a number of other cell types, including cardiac muscle [129], skeletal muscle [130, 131], neurons [132–134], and lung epithelium [135]. In the liver, bone marrow-derived stem-like progenitors have been suggested to give rise to oval cells [136], hepatocytes [137–141], and biliary epithelial cells [135, 138]. Putative bone marrow progenitor cell-derived hepatocytes have been described in rats [136], mice [137, 140, 141], and humans [138, 139]. A recent review of much of the literature related to the potential derivation of hepatocytes from bone marrow progenitor cells concluded that hematopoietic cells do not transdifferentiate into hepatocytes and that these cells do not significantly contribute to formation of new hepatocytes under normal physiological conditions, or in pathology [142]. Despite this conclusion, some of the evidence that suggests that bone marrow-derived stem-like progenitor cells can give rise to hepatocytes in experimental models and humans will be reviewed.

Transdifferentiation of bone marrow progenitor cells into hepatocytes has yielded variable replacement of liver, possibly related to the animal model employed. Transplantation of unfractionated bone marrow from male donors into lethally irradiated syngeneic female mice (B6D2F1) resulted in efficient reconstitution of the host hematopoietic system and generation of donor-derived hepatocytes in the host livers [137]. The bone marrow-derived hepatocytes were identified within the hepatic plates of recipient livers using Y-chromosome in situ hybridization [137]. Quantitative analysis suggested that 1–2% of hepatocytes were bone marrow-derived [137]. These results suggested that bone marrow-derived stem cells could engraft and give rise to hepatocytes, albeit at low frequency, in normal liver. Grompe and colleagues employed the murine model of hereditary tyrosinemia type I [87, 88] to investigate the potential for bone marrow-derived stem cells to repopulate diseased liver [140, 141]. Transplantation of unfractionated bone marrow into $FAH^{-/-}$ mice resulted in replacement of 30–50% of the liver mass after a period of selection [140]. Furthermore, when highly purified KTLS ($c\text{-}kit^{high}Thy^{low}Lin^{-}Sca\text{-}1^{+}$) hematopoietic stem cells from male ROSA26/BA mice were transplanted into lethally irradiated female $FAH^{-/-}$ mice, liver engraftment and hepatocytic differentiation of transplanted cells was observed [140]. The hepatocyte phenotype of engrafted cells was confirmed by expression of albumin and bile canalicular dipeptidylpeptidase IV [140]. In addition, the engrafted cells expressed the FAH protein and were positive for β-galactosidase and the Y-chromosome [140]. It was also suggested that the $c\text{-}kit^{-}$ and Lin^{+} fractions of the bone marrow do not contain significant numbers of progenitor cells that can give rise to hepatocytes [140]. While substantial liver repopulation by bone marrow-derived hepatocytes was observed when selective conditions were employed [140, 141], negligible hepatocyte replacement

was observed in the absence of selective pressure [141]. In a similar study, unfractionated bone marrow from transgenic mice expressing Bcl2 under the control of the liver pyruvate kinase gene promoter was transplanted into normal mice, some of which were subjected to lethal irradiation [143]. In this study, the frequency of hepatocyte differentiation from transplanted bone marrow progenitor cells was rare. However, when selection pressure was applied through the administration of anti-Fas antibodies, the small numbers of bone marrow-derived hepatocytes present in the livers of recipient mice expanded 6-fold to 20-fold, ultimately occupying approximately 1% of the liver mass [143]. These studies show that positive selection pressure can result in a higher degree of replacement of liver by bone marrow-derived hepatocytes. Other investigators have failed to detect donor-derived hepatocytes in undamaged livers after bone marrow transplant, despite reconstitution of the hematopoietic system and replacement of the liver endothelium [144, 145]. These observations suggest that generation of hepatocytes from bone marrow stem cells is uncommon in the absence of strong selective pressure, such as that in the $FAH^{-/-}$ mouse model.

A few studies have examined the differentiation potential of bone marrow-derived progenitor cells in vitro. Verfaillie and colleagues have isolated and established cultured, multipotent, adult progenitor cells from bone marrow of humans, mice, and rats [146, 147]. These multipotent adult progenitor cells co-purify with the mesenchymal stem cell fraction of the bone marrow [146, 147]. When these cells are propagated on Matrigel in the presence of FGF-4 and HGF, hepatocyte-like cells expressing albumin, CK18, and HNF3β resulted after 14 days of culture [148]. Furthermore, these multipotent adult progenitor cell-derived hepatocytes expressed several functional characteristics of mature hepatocytes, including secretion of urea and albumin, expression of phenobarbital-inducible cytochrome P450, the capacity to store glycogen, and the ability to take-up LDL [148], suggesting that the bone marrow of adult mammals contains progenitor cells with the potential to give rise to hepatocytes, and that these cells can be propagated in culture without loss of potency [148].

Liver from Stem-Like Progenitor Cells of the Pancreas

Several experimental models have been developed in which large eosinophilic cells that morphologically and phenotypically resemble hepatocytes are induced in the pancreas of rats and hamsters following severe pancreatic injury. Rats maintained on a copper deficient diet for 8–10 weeks show widespread injury to exocrine elements of the pancreas [149, 150]. When the copper-deficient diet is replaced with a normal diet, hepatocyte-like cells develop during the regeneration of the pancreatic tissue [149, 150]. In this model, cells that resemble hepatic oval cells are thought to represent the progenitor cells for pancreatic hepatocytes [150]. These observations coupled with other studies, led to the suggestion that liver and pancreas may share a common stem cell [151]. To examine the possibility that pancreatic oval cells could serve as liver progenitor cells, proliferating pancreatic oval cells were isolated and introduced into the livers of dipeptidylpeptidase IV-deficient rats via transplantation into the spleen [152]. Following transplantation, hepatocyte-like cells that express dipeptidylpeptidase IV activity were observed in the livers of recipient dipeptidylpeptidase IV-deficient animals, suggesting that oval cells proliferating in response to pancreatic injury caused by the copper-deficient diet can serve as hepatocyte progenitor cells [152]. In a similar study, suspensions of pancreatic cells from normal adult mice were transplanted into $FAH^{-/-}$ mice to examine the possibility that the normal pancreas contains a population of hepatocyte progenitor cells [153]. When selection pressure was applied, extensive liver repopulation (>50% replacement of liver) was observed in a subset of recipient mice, and another subset showed nodules of donor-derived hepatocytes [153].

Chen et al. [154] have examined the fate of normal rat pancreatic ductal epithelial cells following implantation into the abdominal subcutaneous tissue or intraperitoneal cavity of adult syngeneic rats. In these studies RP-2 pancreatic duct epithelial cells [154] were embedded in a gel composed of extracellular matrix (collagen I and Matrigel) prior to implantation [154]. Eight weeks following subcutaneous implantation, nests of eosinophilic epithelioid cells and rare duct structures were observed in the recovered extracellular matrix gel [154]. Six weeks following intraperitoneal implantation, trabeculae and clusters of large polygonal epithelioid cells with granular eosinophilic cytoplasms, resembling mature hepatocytes of the adult liver, were observed [154]. These hepatocyte-like cells expressed high levels of tyrosine aminotransferase, albumin and transferrin, and stained positively with HES6 monoclonal antibodies [154].

These studies demonstrate that normal pancreatic duct epithelial cells can differentiate into functional hepatocytes following implantation into an appropriate host microenvironment. They provide additional support for the suggestion that liver and pancreas share a similar stem cell whose differentiation options are determined by the tissue microenvironment.

Liver from Neural Stem Cell Cultures

Cultured neural stem cells isolated from mice [155], have been shown to give rise to neurons and glia following transplantation into brain tissue of host animals [156]. Such neural

stem cells have been suggested to have a differentiative plasticity when transplanted into various tissue microenvironments other than the brain. For instance, neural stem cells derived from adult donor tissue differentiate into hematopoietic lineages when engrafted into the bone marrow [157]. When introduced into developing mouse blastocysts, neural stem cells contribute to cells of various germ layers and tissues of chimeric embryos, including the liver [158]. Additional studies will be required to demonstrate whether neural stem cells can give rise to differentiated hepatocytes in the adult liver.

Human Liver Stem-Like Progenitor Cells

Evidence for Liver Stem-Like Progenitor Cells in Humans

In recent years numerous investigators have attempted to identify and isolate human liver stem-like progenitor cells and/or have made observations in pathological human livers that suggest the existence of these cells. In many instances, investigators have attempted to determine if activation and proliferation of oval cells occurs in humans in a fashion similar to the oval cell reaction observed in rodents [159]. Using morphologic criteria and/or immunohistochemical staining, several reports suggest the presence of cells resembling oval cells in several different human liver diseases (reviewed in [17, 49]). In some studies, cells exhibiting a phenotype that is consistent with oval cells were observed in normal human liver [160, 161].

Liver from Bone Marrow Stem-Like Progenitor Cells in Humans

In rodents, bone marrow has been suggested to contain stem cells with hepatocytic differentiation potential. Likewise, several studies have suggested that human bone marrow contains stem-like progenitor cells that can differentiate into hepatocyte progeny under various conditions [139]. The first evidence that bone marrow-derived progenitor cells give rise to hepatocytes in humans emerged from a study of archival autopsy or biopsy liver specimens from gender-mismatched transplant patients [138]. Liver tissue from two female patients who received therapeutic bone marrow transplants from male donors, and four male patients who received orthotopic liver transplants from female donors were studied [138]. Variable numbers of Y-chromosome-positive hepatocytes and biliary epithelial cells were detected using in situ hybridization [138]. In the latter study, the interval from transplant to liver sampling was 1 month to 2 years, and the numbers of Y-chromosome-positive hepatocytes observed varied from 1 to 8% among the patients [138]. However, when the investigators adjusted the data to account for their assessment of the insensitivity of Y-chromosome in situ hybridization, the corrected results suggested that 5–40% of hepatocytes observed were derived from circulating progenitor cells, probably of bone marrow origin [138]. In a similar study, the livers of nine female patients who received bone marrow transplant from male donors and the livers of eleven male patients who received orthotopic liver transplants from female donors were evaluated for Y-chromosome-positive hepatocytes [139]. Among these two groups of patients, 0.5–2% of hepatocytes were found to be derived from extrahepatic progenitor cells, and clusters of Y-chromosome-positive hepatocytes were observed in several instances, suggesting that hepatocyte progeny had clonally expanded after colonization of the liver by circulating progenitor cells [139]. In a third study, patients with hematologic or breast malignancies received high-dose chemotherapy and transplants of allogeneic peripheral blood stem cells from donors that were pretreated with granulocyte colony-stimulating factor [162]. Y-chromosome-positive hepatocytes were detected as early as 13 days post-transplant, and 4–7% of hepatocytes examined were found to be donor-derived [162]. In all of these studies, small numbers of hepatocytes that were derived from bone marrow (or other circulating progenitor cells) were detected, and the interval from transplant to sampling of the liver was short (less than a year in most cases). It has been suggested that the engraftment of bone marrow-derived stem cells in the liver may represent an early feature in liver transplants, but that hepatocytes derived from bone marrow progenitor cells may not persist as a long-term feature of grafted livers [163]. In a study of gender-mismatched liver transplant patients with long interval between liver transplant and biopsy (1.2–12 years), no host-derived hepatocytes were detected when the Y-chromosome was used in in situ hybridization experiments [163].

Isolation and Culture of Adult Liver Stem-Like Progenitor Cells

Several types of epithelial cells can be isolated from rodent livers and established in primary or propagable cultures [47, 48]. Differentiated hepatocytes and bile duct cells can be maintained in primary culture for short periods of time, but generally these cell types exhibit limited propagablity and lifespan in culture [164, 165]. In contrast, simple (undifferentiated) liver epithelial cells can be readily established and propagated in culture [166]. These simple liver epithelial cell

types possess some stem-like properties, suggesting that they may represent the cultured counterpart of epithelial stem cells in the adult liver [167].

Early Studies of Propagable Liver Epithelial Cells

Early long-term rat liver epithelial cell cultures were established from cell outgrowths in liver tissue explant cultures [168]. Development of enzymatic techniques for the preparation of viable single-cell suspensions of liver cells made possible the selective culture of several liver epithelial cell types [169, 170]. Brief digestion of liver tissue with collagenase produces liver cell suspensions enriched for hepatocytes [169, 171], whereas enrichment of nonparenchymal epithelial cell types can be accomplished by the selective removal of hepatocytes from collagenase-dispersed liver using various strong proteases, such as Pronase or trypsin [172]. The nonparenchymal cells that remain following protease treatment of liver cell suspensions include macrophages (Kupffer cells), endothelial cells, bile ductular cells, Ito cells, and various hematopoietic cells [172, 173]. Also present in dispersed liver cell suspensions are simple epithelial cells [166, 174].

Rat Liver Epithelial Stem-Like Progenitor Cells: Oval Cell Lines

Oval cells have been isolated from diseased liver and established in culture by several laboratories. Morphologically, cultured oval cells are cuboidal and grow in a monolayer [175, 176]. Some established lines of oval cells are stably diploid or pseudodiploid, are nontumorigenic, and do not proliferate in soft agar [175–177]. Ultrastructurally, cultured oval cells exhibit catalase-positive peroxisomes that proliferate in response to treatment with clofibrate [176]. Cultured oval cells generally express glucose-6-phosphatase activity and lactate dehydrogenase isozymes 2–5, and they are variably positive for albumin and α-fetoprotein [176–179]. A few oval cell lines have been characterized for cytokeratin expression and shown to express CK8 and CK18, and to variably express or not express CK7 and CK19 [176, 177]. As with some other liver epithelial cell lines, cultured oval cells tend to be antigenically simple. Cultured LE/6 oval cells do not express antigens for monoclonal antibodies OC.1, OC.2, BD.1, H.1, or H.2 (reviewed in [24]). The presence of peroxisomes and glucose-6-phosphatase activity in cultured oval cells suggests that these cells are part of the hepatocyte lineage.

Rat Liver Epithelial Stem-Like Progenitor Cells: The WB-F344 Cell Line

Several lines of rat liver epithelial cells have been established from the livers of normal adult rats [24]. The WB-F344 rat liver epithelial cell line represents one such propagable rat liver epithelial cell line that was clonally derived from a single epithelial cell [23]. WB-F344 cells are small (9–15 μm diameter) polygonal cells that grow in a monolayer and are phenotypically similar to other established rat liver epithelial cell lines [24]. Ultrastructurally, WB-F344 cells exhibit a relatively simple cytoplasm with few organelles. Adjacent cells in confluent monolayers are joined by numerous desmosomes [23] and nexus junctions containing connexins 26 and 43, and are dye coupled. Cells are polarized, surfaces directed to the growth medium interface contain microvillus-like projections, and a basement membrane-like material containing fibronectin is deposited at the substrate interface [23]. WB-F344 cells possess a stable diploid or quasidiploid karyotype [23]. They do not proliferate in soft agar culture and are nontumorigenic following transplantation into neonatal syngeneic rats [23]. WB-F344 cells share some phenotypic traits with both hepatocytes and biliary epithelial cells, but their overall phenotype differs distinctively from either differentiated cell type [23]. Most notably, WB-F344 cells are null for the major antigens that typify and distinguish hepatocytes or biliary epithelial cells.

Stem-Like Progenitor Cells from the Human Liver

Very few studies have appeared that report the isolation and culture of human liver cells. Strom and colleagues have isolated a cell population from human liver and established it in propagable culture [180]. The resulting cell line, AKN-1, has been characterized in culture, and shows many characteristics of biliary epithelial cells [180]. It is tempting to speculate that AKN-1 cells represent a cultured counterpart to putative undifferentiated human liver stem cells. However, these cells contain chromosomal abnormalities, display an aneuploid DNA content, and are tumorigenic following transplantation into nude mice [180], indicating that the AKN-1 cell line may not represent propagable normal human liver stem cells. In a similar study, cells expressing c-kit and CD34 have been isolated and cultured from diseased human liver [161]. These cells were localized to portal tracts in close proximity to bile ducts in cirrhotic livers [161]. In cell culture, these cells expressed markers (such as CK19) that suggested differentiation towards the biliary epithelial cell lineage [161]. Of great significance, cells positive for c-kit

and CD34 were also isolated from normal human liver, albeit in smaller numbers, and these cells also acquire biliary epithelial differentiation in vitro [161].

Evidence for the Multipotential Differentiation of Adult Liver Stem-Like Progenitor Cells In Vivo

The ultimate proof that a rat liver epithelial cell line represents cultured stem-like cells requires the demonstration that these cells can give rise to hepatocytes and/or biliary epithelial cells following transplantation into appropriate sites in host animals. To investigate the differentiation potential of cultured rat liver stem-like progenitor cells, WB-F344 cells have been transplanted into livers or extrahepatic sites of syngeneic animals to examine their fate in vivo. Several weeks following the transplantation of WB-F344 cells into the interscapular fat pads of syngeneic Fischer 344 rats, small clusters of cells morphologically resembling hepatocytes were identified [167]. However, whether these cells possessed functional attributes of hepatocytes was not determined. Nonetheless, this observation suggested that transplanted WB-F344 cells could acquire characteristics of hepatocytes in vivo. In order to demonstrate a precursor-product relationship between transplanted cells and differentiated cell types in the liver, methods had to be established that would allow the definitive identification of the progeny of the transplanted cells among host cells in the adult liver. We have utilized three different strategies to examine the fate of WB-F344 rat liver epithelial cells following transplantation into adult rat livers: (1) Introduction of a genetic tag/marker enzyme into WB-F344 cells [167, 181], (2) transplantation of normal WB-F344 cells into the livers of rats that are deficient for dipeptidylpeptidase IV enzyme activity [182], and (3) transplantation of normal WB-F344 cells into the livers of Nagase analbuminemic rats [183]. In all of these model systems, transplanted WB-F344 cells (or BAG2-WB cells) integrated into hepatic plates, and morphologically and functionally differentiated into hepatocytes. The results from these studies combine to show that WB-F344 cells are multipotent and differentially responsive to the tissue microenvironment of the transplantation site.

Hepatocytic Differentiation by Transplanted WB-F344 Rat Liver Stem Cells

To facilitate transplantation studies, WB-F344 rat liver epithelial cells were genetically modified by infection with the CRE BAG2 retrovirus which encodes the *Escherichia coli* β-galactosidase gene and the Tn5 neomycin resistance gene [184]. The resulting cells, termed BAG2-WB, were transplanted into the livers of adult Fischer 344 rats and the livers of these rats were examined for the presence of β-galactosidase-positive cells at various times following transplantation. In these studies, β-galactosidase-positive hepatocyte-like cells were detected in the hepatic plates of recipient rats among the host hepatocytes phenotypically similar to other established rat liver epithelial cell lines [181]. The size and morphologic appearance of these cells is indistinguishable from that of the host hepatocytes [167, 181]. β-galactosidase-positive cells were observed at all time points examined, up to greater than one year following transplantation. Subsequent studies demonstrated that the β-galactosidase-positive hepatocyte-like cells express functional differentiation typical of hepatocytes, including expression of albumin, transferrin, α1-antitrypsin, and tyrosine aminotransferase [167]. Several methods were utilized to demonstrate further that the β-galactosidase-positive cells observed in these livers were derived from the transplanted BAG2-WB cells. In some studies, cells were labeled with the lipophilic fluorescent membrane dye PKH26-GL [181]. Examination of liver sections demonstrated the presence of fluorescent cells in the hepatic plates of the host livers, consistent with the observations made using the β-galactosidase marker enzyme [181]. In addition, the neomycin resistance gene of the CRE BAG2 retroviral construct could be detected by PCR in genomic DNA prepared from livers of rats that received BAG2-WB cell transplants [181]. Together, these studies demonstrate that transplanted WB-F344 rat liver epithelial cells incorporate into the hepatic plates of the host liver, morphologically and functionally differentiate into hepatocytes, and remain a stable component of the hepatic parenchyma over long periods of time.

In other transplantation studies [182], normal WB-F344 cells were transplanted into the livers of German Fischer 344 rats, which are deficient for dipeptidylpeptidase IV enzyme activity [185, 186] to examine the fate of these cells in a transplantation model that does not depend upon the use of exogenous marker enzymes. Dipeptidylpeptidase IV is a bile canalicular enzyme that is expressed by mature hepatocytes in normal rats [187, 188]. The WB-F344 cell line was isolated from an American strain adult Fischer 344 rat [23] that expresses normal levels of dipeptidylpeptidase IV activity in hepatocytes. Following transplantation into the dipeptidylpeptidase IV-deficient rats, WB-F344 cells incorporate into hepatic plates and morphologically differentiate into hepatocyte-like cells that express dipeptidylpeptidase IV enzyme activity [182]. These dipeptidylpeptidase IV-positive hepatocytes are easily distinguished from the host hepatocytes using a histochemical staining reaction for dipeptidylpeptidase IV activity. The dipeptidylpeptidase IV-positive hepatocytes in hepatic plates were comparable to adjacent

host hepatocytes in size and morphology. Close physical contact between the differentiated progeny of the transplanted cells and host hepatocytes was verified through co-localization of dipeptidylpeptidase IV staining and ATPase staining of hybrid bile canaliculi. In addition, the localization of dipeptidylpeptidase IV staining to bile canaliculi shows that the surface membranes of differentiating WB-F344 cells acquired the polarization characteristic of fully differentiated hepatocytes [182]. These results provide additional evidence that WB-F344 cells morphologically and functionally differentiate into hepatocytes following their transplantation into the liver microenvironment of adult rats.

In a third transplantation model, wild-type WB-F344 cells were transplanted into Nagase analbuminemic rats [189, 190] to examine the efficacy of liver stem-like cell transplant for phenotypic correction of a genetic liver defect [184]. In previous studies, transplanted WB-F344 cells (or BAG2-WB cells) gave rise to differentiated hepatocyte progeny that expressed albumin [167, 182]. Therefore, in this transplantation model, albumin serves dual roles: (1) As a marker for detection of the progeny of transplanted WB-F344 cells, and (2) as a metabolic marker for monitoring phenotypic correction of analbuminemia. Albumin-positive hepatocytes were detected in the hepatic plates among albumin-negative host hepatocytes in all rats receiving cell transplants. In some cases, individual albumin-positive hepatocytes were observed, whereas in other instances, clusters of albumin-positive hepatocytes were detected. The WB-F344 cells were transplanted into Nagase rats that were treated with cyclosporin to minimize rejection of the transplanted cells due to strain-specific differences between the Fischer 344 rat cells and the Sprague Dawley rat hosts [183]. However, once engrafted into the livers of these rats, albumin-positive WB-F344 hepatocyte progeny could be detected for up to four weeks following the cessation of cyclosporin treatment [183].

Multipotential Differentiation of Adult Liver Stem Cells in Culture

Various approaches have been used to examine the differentiation potential of cultured rat liver stem cells. These include modification of culture mediums to contain differentiation-promoting agents, and the use of combinations of extracellular matrix materials as culture substrates. These studies have yielded evidence that cultured stem-like epithelial cells can be induced to express characteristics of differentiated liver cell types in culture. In the following sections we will review the results of our studies with WB-F344 rat liver epithelial cells, as well as some studies from the literature on the differentiation of oval cells in culture.

Hepatocytic Differentiation of Oval Cells In Vitro

Oval cell cultures established from rats treated with 3′-methyl-4-dimethylaminoazobenzene exhibit a typical epithelial morphology in culture, express various cytokeratins, vimentin, γ-glutamyltranspeptidase, and BDS7 antigen [191]. The phenotype of these cells can be modified by culturing them on fibronectin-coated dishes in medium containing various differentiation-promoting agents. Inclusion of sodium butyrate in the growth medium inhibits cellular proliferation and produces dramatic morphological alterations in the cultured oval cells [191]. In the presence of sodium butyrate alone or in combination with dexamethasone, cultured oval cells synthesize albumin and express tyrosine aminotransferase activity [191]. Steinberg and co-workers have also examined the effects of differentiation-promoting chemicals on the phenotypic characteristics of cultured oval cell lines [177]. Oval cell lines (OC/CDE) were established from cells isolated from rats maintained on a choline-deficient diet supplemented with ethionine [177]. Exposure of OC/CDE cell lines to either sodium butyrate or dimethylsulfoxide resulted in cessation of cell proliferation, increase in cell size, expression of albumin (in 35–40% of cells), and enhancement of glucose-6-phosphatase, γ-glutamyltranspeptidase, and alkaline phosphatase activities [177]. Tyrosine aminotransferase activity was not detected in OC/CDE cell cultures treated with either sodium butyrate or dimethylsulfoxide [177]. These studies combine to demonstrate that cultured oval cells can be induced to express some characteristics of differentiated hepatocytes.

Fausto and colleagues [192] developed a three dimensional cell culture system that supports the hepatocytic differentiation of oval cell lines. In this system, LE/2 and LE/6 oval cells were cultured within a collagen I gel matrix, supported by a fibroblast feeder layer. After several weeks in culture, these oval cells acquired a phenotype characterized by typical hepatocyte morphology and ultrastructure, expression of an hepatocytic cytokeratin pattern ($CK8^+$, $CK18^+$, $CK19^-$), and production of albumin [192]. In the absence of a fibroblast feeder layer, oval cells cultured in this model system with defined growth factors HGF and/or KGF) produced ductal structures, suggesting differentiation towards the biliary epithelial cell lineage [192]. This study shows that cultured oval cells are bipotent and that their differentiation fate is influenced by soluble factors (growth factors and others) produced by stromal cells.

Hepatocytic Differentiation by RLE-13 Rat Liver Epithelial Cells in Culture

A number of different simple epithelial cell lines have been established in culture from normal rat livers (reviewed in [17]), including RLE-13, which was established from a normal adult Fischer 344 rat [193]. Thorgeirsson and colleagues have induced hepatocytic differentiation of RLE-13 cells by treatment with 5-aza deoxycytidine, followed by culture in defined growth medium containing FGF1/2, oncostatin M, HGF, and dexamethasone [194]. Culture of the RLE-13 cells under these conditions resulted in significant enlargement of cell size, increased organelle complexity, and decreased proliferation. Concurrent with the morphological alterations, differentiating RLE-13 cells expressed hepatocyte-specific markers, including tyrosine aminotransferase, and liver-enriched transcription factors, including HNF4 [194]. These results combine to suggest that RLE-13 cells acquire an hepatocytic phenotype when maintained in culture under defined conditions.

Hepatocytic Differentiation of WB-F344 Rat Liver Stem-Like Progenitor Cells in Culture

Exposure to sodium butyrate in culture inhibits proliferation, alters normal cellular morphology (increased cell size and decreased nuclear/cytoplasmic ratio), and dramatically increases cellular protein synthesis in WB-F344 rat liver epithelial cells [195]. Ultrastructurally, sodium butyrate-treated WB-F344 cells demonstrate a complex cytoplasm with extensive rough endoplasmic reticulum, numerous mitochondria, and large numbers of primary and secondary lysosomes. Sodium butyrate-treated WB-F344 cells also express dexamethasone-inducible tyrosine aminotransferase enzyme activity [195], which is a marker of hepatocyte differentiation. The dexamethasone-inducible tyrosine aminotransferase activity developing in these sodium butyrate-treated WB-F344 cells responds to the modulating effects of insulin and L-tyrosine in a manner that closely resembles that of cultured hepatocytes and hepatoma cell lines [196, 197]. These studies demonstrate that WB-F344 cells can be induced to express traits of differentiated hepatocytes in vitro.

Using a similar cell culture system, Couchie and colleagues have evaluated the ability of WB-F344 cells to differentiate into the biliary epithelial cell lineage [198]. WB-F344 cells cultured on dishes that were coated with laminin-rich Matrigel in the presence of growth medium containing sodium butyrate demonstrated a reduction in proliferation and formed cord-like structures between islets of cells [198]. WB-F344 cells in the cord-like structures were elongated, and expressed several biliary markers, including BDS7, CK19, and γ-glutamyl transpeptidase [198]. These results suggest that WB-F344 cells adopt a biliary epithelial cell phenotype in response to specific factors/signals in cell culture. More recently, Yao and colleagues [199] demonstrated that stable transfection of WB-F344 cells with RhoA expression vectors drives biliary differentiation in vitro.

Stem-Like Progenitor Cell Responses in Liver Injury and Repair

The mammalian liver possesses tremendous flexibility in its capacity to respond to injury and loss of cell numbers (function). At least three different cell populations have been implicated in liver regeneration: (1) differentiated hepatocytes (in otherwise normal liver), (2) SHPC (observed in retrorsine-exposed rats), and (3) oval cells (observed in numerous models of liver injury). Given multiple sources of regenerative cells and the observed differences in timing of activation of these cell populations in response to liver deficit, it is intriguing to speculate that there is a hierarchy of potential cellular responses and that the nature of the regenerative cell population is determined by (a) the presence or absence of liver injury, (b) the type and extent of injury, and (c) the capacity of each cell population to respond. In this model, activation and proliferation of mature hepatocytes represents the primary (preferential) cellular response, and the activation, emergence, and proliferation of reserve stem-like progenitor cell populations (SHPC or oval cells) represent secondary cellular responses, occurring only when the primary response is blocked or impaired. Several studies from the literature suggest that IL6 may be a common signaling molecule that functions to regulate the liver's response to injury [200–203]. There may be redundancy in the cellular reactions to liver injury, but a common regulatory pathway that directs the process of regeneration. Thus, following a signal for liver regeneration, all possible progenitor cell populations may be concurrently primed for activation (IL6-mediated), but that the secondary reserve progenitor cell responses do not manifest unless the primary response (hepatocyte-mediated) fails. Further studies are required to address these possibilities.

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Chapter 18
Liver Regeneration

George K. Michalopoulos

General Considerations

Loss of tissue in large proportions compromises the functional capability of an organ or the entire organism. Small wounds in skin, muscle and soft tissues are usually associated with local inflammatory response and restoration of the local structural integrity, mediated by processes collectively termed as "wound healing". This typically occurs in skin and mesenchymally derived tissues such as bone, muscle and heart, and the central nervous system. However, in cases of major loss of tissue, especially in tissues derived from endoderm, the affected organs have the capacity to expand in size and attempt to restore the totality of the original function by increasing the size of the residual organ to eventually approximate the initial size as it was before the tissue loss. Kidney, an organ with epithelial components but derived from mesenchyme, has a substantial capability to slowly increase the residual size of the organ to restore most of function as it was prior to tissue loss. Thyroid, pancreas, intestine, and lungs also display significant capacity to restore part of the original function and mass.

Liver has received special attention as a regenerative champion, because it outstrips other organs in the speed, magnitude, and the capacity for multiple repeats of its regenerative response [1–5]. Loss of tissue in a clinical setting occurs as a result of a diffuse acute injury, mediated by toxic chemicals, viruses, metabolic abnormalities, or acute vascular disorders. Such episodes lead to a massive loss of hepatocytes and compromise the capacity of the organ to deliver functions vital for body homeostasis. Such episodes are associated with parallel development of both an *inflammatory* and a *regenerative* response. Inflammation involves mobilization of extrahepatic and intrahepatic macrophages (Kupffer cells), and polymorphonuclear leucocytes, mobilized to remove necrotic or apoptotic cells, tissue debris, etc. Other humoral and cellular components of the immune response (e.g. lymphocytes of different types) are mobilized to combat specific offending agents, especially viruses. Chronic liver damage is often mediated by exposure to the same offending agents causing the acute offense. Even though the tissue responses in the acute and chronic setting are probably mediated by the same signaling mechanisms, the net results are often different, with chronic offenses resulting in distortion of the hepatic tissue architecture.

It has been the challenge of experimental tissue biology to study the mechanisms associated with initiation and termination of the regenerative response and to establish the scientific basis to explain how a prolonged response to offending agents, mobilizing regenerative events on a chronic basis, leads to permanent tissue damage. In order to dissociate the otherwise confabulated occurrences of inflammatory and regenerative signals associated with diffuse loss of hepatocytes, the model of liver regeneration after *two-third partial hepatectomy (PHx)* was established by Higgins and Anderson in 1931 [6]. Taking advantage of the existence of multiple lobes in rodent liver, a simple operation was designed that allowed rapid surgical removal of two-third of the liver, leaving structurally intact two residual liver lobes, which then grow to the size of the original liver mass. The anatomical architecture of the original liver (four or five lobes based on anatomic conventions) is not re-established. The regenerated liver returns to two lobes, but the final mass after regeneration is that of the total of the original five lobes. The advantages of this approach for study of liver regeneration are several:

- The process of removal of two-third of the liver is of very short duration, since the removal of the large lobes is done after the lobes have been externalized. Thus, the sequence of signaling events following surgery can be very precisely timed.
- There is no histologic damage to the residual lobes. Thus, there is no inflammatory response to remove dead hepatocytes and no cell migration into the liver that is required to assist with removal of the tissue debris. The signals observed after PHx are purely those associated with the regenerative response.

Despite the obvious merits of PHx as a tool to study liver regeneration, it does not bear resemblance to most of the

G.K. Michalopoulos (✉)
Department of Pathology, University of Pittsburgh,
Pittsburgh, PA, USA
e-mail: michalopoulosgk@upmc,edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_18, © Springer Science+Business Media, LLC 2011

clinical situations causing liver damage, since those typically involve diffuse hepatocyte necrosis and apoptosis. Partial hepatectomies are performed in humans as well for specific circumstances, such as following trauma or for the removal of solitary metastatic lesions. However, in most situations associated with massive loss of hepatocytes, exposure to the offending agent, associated inflammation, and regenerative signals proceed in tandem. Thus, it should be always understood that full explanation of restoration of tissue architecture in diseased liver should at some level integrate the regenerative and the inflammatory signals. The latter may affect (positively or negatively) the effects of the former. However, the lessons learned from PHx and other experiments models have defined the repertoire of signals controlling hepatocyte proliferation, and the proliferative behavior of hepatocytes in an inflammatory setting is still governed by the same signals.

How Much Liver Does the Body Need? Is There a "Hepatostat"?

Before discussing the current understanding on signaling pathways associated with liver regeneration, it is important to emphasize that the overall parameters determining the normal liver to body weight ratio are not fully understood. Early studies have shown that transplantation of livers of small animals into larger ones caused an enlargement of the liver size until a new size proportionate to the larger body of the host had been attained [7]. This has been shown for dogs [8] as well as humans [9]. Unfortunately, there were no detailed studies of cellular kinetics etc. in these early studies to help us understand the process. The same phenomena were observed when a baboon liver was transplanted to an immunosuppressed human. The baboon liver grew to a size of a normal liver within 7 days [7]. It is intuitively postulated that a sensor system ("hepatostat", for the purposes of this chapter) operates between the liver and peripheral tissues to facilitate this process. A primary candidate agent is the overall flow of the portal blood. A small liver would face an increased portal vein blood flow immediately after transplantation. Expansion of the liver mass would result in a portal flow to liver size ratio comparable to the expected for the body size. Thus, the portal vein flow would be the body weight "surrogate" in the "hepatostat" signaling, for appropriate adjustment of liver size. This hypothesis is bolstered by experimental and clinical findings following diversion of portal vein flow from the liver directly into the inferior vena cava. This procedure is called portacaval shunt. It results in substantial decrease in liver weight to less than half the original size, with decrease in size of individual hepatocytes, measurable hepatocyte apoptosis, and a mild inflammatory response [10, 11]. Restoration of the flow by reversal of the shunt is associated with rapid liver enlargement mediated by multiple mitoses and increase in the size of the individual hepatocytes. The same effects can be seen by injecting insulin alone [10, 11]. Insulin is present at high concentration in the portal blood, secreted by the beta cells of the pancreatic islets, and appears to be involved in mediating these changes. On the one hand, the role of other potential portal blood components such as epidermal growth factor (EGF) may have such an effect, though they were not found to be as effective as insulin when directly infused in dogs after portacaval shunt [12]. Also, in support of the role of portal blood flow as a component to the stimuli leading to adjustment of liver size is the fact that after PHx, the entire flow of the portal vein is proceeding through the liver remnant (one-third of the original size), thus mimicking the situation in which a liver from a smaller animal is transplanted into an animal of a much larger size. However, it is not clear whether the relative increase in blood flow per se or the enhanced availability (per hepatocyte) of specific portal blood components is responsible for the observed effects. Partial diversion of the portal vein flow after PHx is associated with a decreased regenerative response and decreased activation of hepatocyte growth factor (HGF), a very important hepatocyte mitogen (see below) [13].

The "hepatostat" may be adjusted to reflect specific physiologic needs. Pregnancy is associated with liver enlargement [14]. Estrogens are known to enhance the effects of EGF and HGF on hepatocytes, in parallel with a decrease of the mito-inhibitory effects of TGFβ(beta) (see below) [15]. Administration of estrogens to normal rats is associated with increase in liver body weight and increase in size of hepatocytes [16, 17]. On the other hand, the effect of pregnancy on liver size is abrogated in mice that are genetically deficient in FXR, a transcription factor which binds and mediates effects of bile acids [14]. These studies suggest that in addition to portal flow, other superimposed physiologic signals may adjust the hepatostat, and that hormones and hepatocyte products such as bile acids may play a role in this process. Since bile acids are produced exclusively by the liver, they are candidate products for acting as "sensors" for the hepatostat, but their role in this regard has not been examined. Recent discoveries have implicated the Hippo kinase homologues Mst1 and Mst2 and the protein known as YAP for their involvement in regulation of specifically the size of liver [18–20]. This is presented in more detail in the section "Termination of Liver Regeneration," below. These findings bring YAP in the forefront of regulation of liver size. Studies related to ILK [21] and GPC3 (see below) suggest that this YAP protein related system is in part under control of ECM signaling, but the pathways connecting these signaling systems remain to be established, hopefully in the near future.

Liver Regeneration After PHx

An outline of the signals described in this section is shown in Fig. 18.1.

Histologic Changes and Cellular Proliferation Kinetics

Earlier studies of nuclear labeling following injection of tritiated thymidine or bromodeoxyuridine (BRDU) have demonstrated that regeneration after PHx involves practically all hepatocytes in the liver for at least one round of DNA synthesis. It should be clearly emphasized that all cell types and pre-existing cell populations in the liver enter into cell proliferation and carry out the regenerative process. Liver regeneration after PHx under normal circumstances is not mediated by a growth of a selective "stem cell" population. Hepatocytes are the first liver cells responding to the regenerative signals by entering into DNA synthesis and cell proliferation [22]. Short labeling pulses demonstrate a peak of hepatocyte DNA synthesis at 24 h after PHx for the rat (approximately 36 h for the mouse) [22]. Most, but not all, hepatocytes are involved in this first proliferative event. A smaller wave of hepatocyte proliferation follows the first one, with a peak between 48 (rat) and 72 h (mouse) [22]. These observations have been registered in animals with very precisely defined conditions of light/dark cycles and feeding, as it has been shown that these events influence the timing of regeneration [23]. In most recent studies, there is no strict adherence to these issues and there is considerable variation in the observed results between different groups. (Adherence to some form of standardized regimen is important within each laboratory to obtain more reproducible results). Hepatocyte proliferation proceeds as a "wave" from periportal to pericentral areas of the hepatic lobule [24]. TGFβ(beta) immunoreactivity on hepatocytes also proceeds as a wave in the same direction, with a band of hepatocytes in mitosis following the TGFβ(beta) +/−interface [25]. Expression of metalloproteinases on hepatocytes follows the same pattern [26]. Proliferation of stellate cells and biliary epithelial cells occurs very shortly after proliferation of hepatocytes. Proliferation of endothelial cells proceeds as a much broader process from day 2 to 6 after PHx [22]. There is a small wave of apoptosis at the end of regeneration, perhaps reflecting a corrective mechanism to avoid hepatocyte surplus [27]. The early proliferation of hepatocytes prior to the initiation of proliferation of endothelial cells creates small nests of poorly vascularized hepatocytes [28]. Endothelial cells, stimulated by angiogenic factors produced by these hepatocytes (Angiopoietins 1 and 2, VEGF, FGF1, FGF2, and TGFβ(beta), see below) enter into proliferation and invade the nests of the

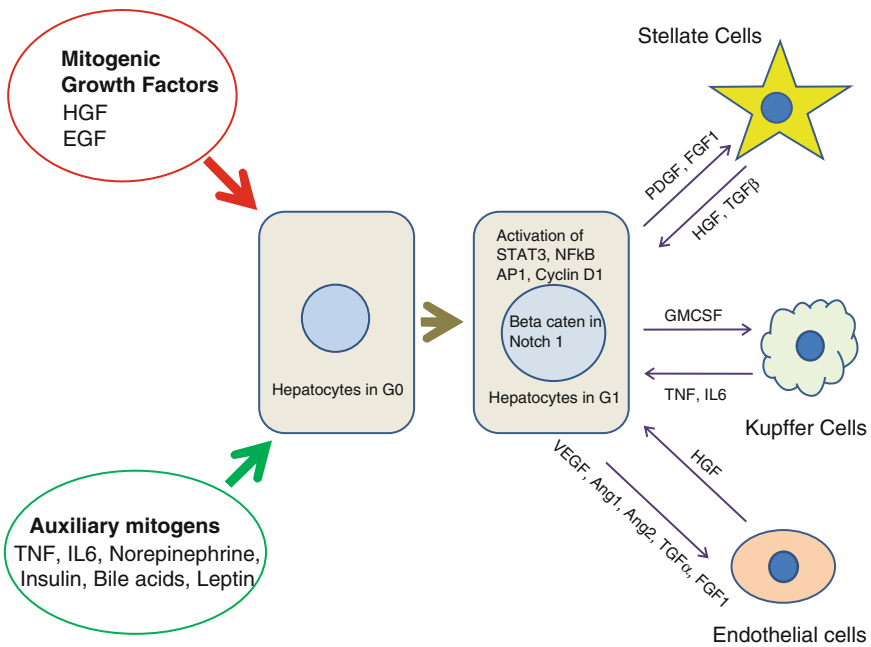

Fig. 18.1 Combined signals initiated by the mitogenic growth factors HGF and EGF and by the auxiliary mitogens impinge upon hepatocytes in G0 phase of the cycle and induce entry into the G1 phase. This is associated with multiple signaling events, including activation of STAT3, NFKB, formation of AP1, activation of cyclin D1, and migration of beta-catenin and Notch1 intracellular domain (NICD) to hepatocyte nuclei. the activated new gene expression patterns facilitate the trajectory of hepatocytes from G1 to S to G2 and mitosis. During this stage and thereafter, hepatocytes receive and transmit mitogenic signals from other hepatic cell types, such as stellate cells, endothelial cells, and kupffer cells (macrophages)

poorly vascularized hepatocytes, separating them into the typical hepatocyte plates, and eventually re-establishing sinusoidal architecture [29].

Extracellular Matrix

Most epithelial cell types in multicellular organisms are surrounded by extracellular matrix (ECM). This is composed of multiple proteins, with various degrees of glycosylation. Such proteins include various forms of collagen, fibronectin, tenascin, laminin, and many others. Each of these proteins exists in multiple isoforms. Several proteins with heavy degrees of glycosylation are also present as glycosaminoglycans (GAG) in the "pericellular" space and are often anchored with GPI linkages to the plasma membrane. Such proteins include glypicans, syndecans, perlecan, brevican, decorin, etc. Several heparin-binding growth factors including HGF, HB-EGF, FGF1, etc., are present in this complex milieu, contained on low affinity binding sites on GAG. There are complex relationships between growth factors and their receptors with the complex milieu of ECM [30, 31]. Many growth factors are available to the epithelial cells from the ECM reservoir consisting of low affinity binding sites. ECM components often bind to the growth factor receptors serving as co-receptors or antagonists. Decorin, capable of binding TGFβ(beta)1, EGFR, and MET, is a good example of this complexity of interactions between ECM components and plasma membrane receptors and ligands [32–35]. ECM components directly interact with receptors of their own, belonging to the integrin family of proteins [36]. Integrins also have extensive signaling cross-talk with growth factor receptors [37]. Members of the discoidin family of proteins are functioning as receptors for specific collagen types in addition to integrins [38]. Multiple types of proteinases (metalloproteinases (MMP), members of the ADAMS, etc.) and inhibitors to the proteinases are also present [39]. The complexity of the system and the interactions between all its parts allow for multiple degrees of freedom in terms of stimuli and observed responses.

Epithelial cells proceed to remodel the ECM through complex pathways prior to cell proliferation. There are two pathways that control this process. The first pathway is initiated by urokinase plasminogen activator (uPA), which catalyzes activation of plasminogen to plasmin. The latter is involved in activation of multiple MMP [40]. uPA activity rises within less than 5 min after PHx and it is the first demonstrable biochemical event following surgery [41]. This is also associated with increase of uPA receptor on hepatocyte plasma membrane [41]. There is subsequent activation of plasminogen to plasmin and activation of MMP9 [26]. There is intense expression of MMP9 in regenerating hepatocytes and it proceeds as a wave from periportal to pericentral areas following the wave of the regenerative response. The second pathway for ECM remodeling is activated by participation of MT MMP ("membrane type metalloproteinase", bound to the plasma membrane) and TIMP2. Acting in partnership, these two proteins activate MMP2. This pathway also operates during liver regeneration starting at 12 h after PHx. These events lead to decrease of several ECM associated proteins within a few hours after PHx [42] and with release of large amounts of hyaluronic acid in the peripheral circulation [43]. The degradation of ECM is reversed towards the later stages of LR. Increased synthesis of different collagen types and pericellular ECM proteins has been noted in the rat starting at 72 h after PHx [44, 45]. Glypican 3, a protein which is intensely over-expressed in human hepatocellular carcinomas, but which appears to have growth inhibitory effects on hepatocytes also increases towards the end of LR and seems to play a growth regulatory role in association with a tetraspanin protein known as CD81 [46]. The remodeling as well as the subsequent re-synthesis of ECM is associated with growth enhancing (early stage) or growth inhibitory signals (later stages) of LR.

Signals Associated with Hepatocyte Mitogenesis

There are complex mitogenic signals mobilized very early after PHx which drive and control the progress of hepatocytes during the cell cycle. Several recent publications have provided detail review of the literature associated with this topic [1–5, 43, 47]. Earlier work involving transplantation of hepatic fragments [48], implantation of isolated hepatocytes [49] and animals connected through parabiotic circulation [50, 51] had demonstrated that after PHx there is a set of signals appearing in the peripheral blood which drives hepatocytes into proliferation, even in ectopic sites. Subsequent work utilizing hepatocyte cultures resulted in the isolation and characterization of hepatocyte growth factor [52–54] (HGF, see below). Because the isolation assay was directed towards detecting substances mitogenic for hepatocytes in culture, other substances which are not directly mitogenic, but which have a regulatory signaling role and also become elevated after PHx, were overlooked. The list includes TNFα(alpha), norepinephrine, IL6, leptin, serotonin, bile acids, components of complement, etc. These non-mitogenic participants should be viewed as auxiliary mitogens. The role of these two groups and their members will be discussed below.

Mitogenic Growth Factors

Stimulation of DNA synthesis in hepatocyte cultures maintained in chemically defined serum free media is the basis for defining this group. Additionally, its members stimulate

hepatocyte DNA synthesis and liver enlargement when injected in sufficient amounts in normal rodents. Two such growth factors fulfill that role, HGF [55, 56] and the ligands of EGFR [57, 58]. Some members of the FGF family have weak mitogenic effects on hepatocytes in culture, but have not been shown to stimulate hepatocyte DNA synthesis and liver enlargement when injected into normal rats and mice [59]. Receptors for other growth factors are expressed in hepatocytes, and they become activated in culture, without stimulating hepatocyte DNA synthesis [60].

HGF and Its Receptor (cMET)

There are several detailed reviews of the functions associated with HGF and its receptor [61, 62]. Additional details are also found in Chap. 20. HGF is synthesized by mesenchymal cell in many organs, especially in lungs and liver [63]. It receptor (cMET) is expressed mostly in epithelial cells. In the central nervous system, both glial cells and neurons, variably in specific locations, express either HGF or its receptor [64]; recent evidence has shown that HGF may be involved in the early migration of cells supporting the layering and design of the cerebral cortex [65]. In liver, HGF is synthesized by predominantly by stellate cells [66] and (after PHx) also by endothelial cells [67]. In liver, the receptor for HGF is expressed in hepatocytes, biliary cells, and endothelial cells. Stellate cells appear to synthesize HGF on a continual basis, as a single chain polypeptide of 100 kDa with heavy degree of glycosylation. The single-chain HGF is biologically inactive and bound to GAG in the pericellular matrix, especially in periportal areas of the hepatic lobule [68]. Large quantities of HGF can be extracted from hepatic ECM [63]. The single chain inactive HGF can be activated to the active form by uPA [69]. The immediate enhancement of uPA activity after PHx is associated with release of HGF from GAG (due to ECM remodeling and degradation) and activation of single chain HGF to active two chain HGF, which is in part utilized locally and in part released to the peripheral blood [52]. The precise contributions of the local or peripherally released HGF are not clear, but the HGF receptor shows increased tyrosine phosphorylation within 30–60 min after PHx [70]. Following a measurable decline of HGF concentration in hepatic ECM in the first 3 h after PHx, there is enhanced synthesis of new HGF from both stellate and endothelial cells, starting at 3 h and reaching a peak at 24 h [71]. It should be noted that there is increased synthesis of new HGF not only in the liver but also in lungs [72]. The precise role of the newly synthesized HGF is not clear. "Knockdown" of HGF mRNA using ShRNA against HGF resulted in elimination of synthesis of new HGF, but only a small decline in hepatocyte DNA synthesis [73]. On other hand, ShRNA against cMET had strong suppressive effects of the hepatocyte cell cycle with complete elimination of the events of the first 24 h after PHx and absence of mitoses [73]. However, there was also an increase in several pro-apoptotic genes, without measurable increase in hepatocyte apoptosis [73]. The effects on cell cycle were eventually compensated with a rebound at 48 h after PHx, after the cDNA expression plasmid for the ShRNA was gradually eliminated. These results are most compatible with hepatocyte mitogenesis being driven primarily by the stores of existing HGF, and with the new HGF synthesized after 3 h post PHx, having a smaller role to play for this part of the regenerative process. The impact of the new HGF may be more relevant to other cell populations expressing c-met, such as biliary cells and endothelial cells. The triggering of new HGF synthesis in lungs after PHx suggests a hitherto not fully appreciated systemic response in support of liver regeneration. Only norepinephrine [74] and IL6 [75] have been shown to directly stimulate synthesis of new HGF in mesenchymal cells, and they both rise in the blood after PHx [52, 76]. It should also be noted that mice with hepatocyte-targeted elimination of MET develop hepatic fatty change and fibrosis and have deficient regenerative responses [77]. Mice systemically deficient in uPA also have a compromised regenerative response [78].

EGF, TGFα(alpha) and the EGFR Ligand Family of Proteins

Most of the members of the EGFR ligand family have been shown to be mitogenic in hepatocyte cultures [55, 57]. Injection of EGF by itself to unoperated rats, leads to hepatocyte DNA synthesis [58, 79]. Mice with hepatocyte-targeted transgenic expression of TGFα(alpha) have very enlarged livers [80] and develop hepatocellular carcinomas [81]. EGF itself is produced in exocrine glands. Removal of the salivary glands (a major site of EGF production) in male mice is associated with decreased regeneration [82]. EGF is also produced by Brunner's glands of the duodenum, histologically similar to salivary glands, but underlying the duodenal mucosa [83]. Norepinephrine and epinephrine, rising in the blood after PHx (see below) are known to stimulate production of EGF from Brunner's glands [83]. EGF is taken avidly by liver and is deposited in periportal sites [84]. There is no measured increase in EGF in the plasma after PHx. However, the EGFR is phosphorylated in tyrosine sites with the exact same time kinetics as cMET [70]. There is literature documenting interactions and cross-talk between MET and EGFR in several studies [85], though a direct interaction has not been reported in liver regeneration.

TGFα(alpha) expression increases in hepatocytes within 2–3 h after PHx and lasts for most of the regenerative response [86].

Since hepatocytes express TGF α(alpha) and EGFR, it has been proposed that an autocrine loop between TGF α(alpha) and EGFR contributes to the signaling leading to hepatocyte proliferation. On the other hand, mice with systemic elimination of TGF α(alpha) do not suffer any major abnormalities (none reported in liver) and complete liver regeneration normally [87]. However, mice with systemic elimination of amphiregulin (another member of EGFR ligand family), do have defective regeneration [88, 89]. HB-EGF, another member of the same family, enhances hepatocyte proliferation during liver regeneration especially in mice subjected to a limited hepatectomy [90]. HB-EGF expression changed in proportion to the amount of tissue resected whereas that of HGF and TGF α(alpha) did not [90].

Intracellular Signaling Events

It is widely accepted that the final event of the early, pre-S-phase, in hepatocytes after PHx (a time known as "priming" [91, 92], roughly corresponding to G1 phase of the cycle), is the activation of Cyclin D1 and its move to the nucleus [93]. This represents the culmination of multiple events following activation of receptors of both mitogenic growth factors (see above) as well as non-mitogenic cytokines (see below). Several cell cycle associated genes including c-jun, c-fos, c-myc, p53, and p21 show enhanced expression at different times in the first 1–10 h after PHx [94]. Activation of Stat3 is also enhanced within 1–3 h after PHx [76]. NFkB is activated as well (from 2 to 4 h after PHx) [95, 96]. β(beta)-catenin migrates to the nucleus within 15 min after PHx [97], an event that may be related to Wnt activities, but also to enhanced tyrosine phosphorylation by cMET and EGFR [98]. Mice genetically deficient in β(beta)-catenin or rats injected with β(beta)-catenin antisense exhibited delayed liver regeneration in response to hepatectomy [99–101]. Mice genetically deficient in IL6 have deficient activation of Stat3 and delayed regenerative response [76]. Mice deficient in TNF α(alpha) receptor 1 have defective activation of NFkB and delayed regenerative response [102]. In both situations, regeneration eventually completes as evidenced by restoration of the liver weight. Changes in hepatocyte associated transcription factors include a decrease in the ratio of C/EBP α(alpha) to C/EBPβ(beta), but no measurable changes in HNF4 [103]. Another signaling system involved with hepatocyte proliferation and liver regeneration is the Notch/Jagged signaling pathway. The Notch intracellular domain (NICD) migrates to hepatocyte nuclei within 15 min after PHx with subsequent activation of HES1 gene. Notch and Jagged do co-localize on hepatocyte plasma membranes during days one and two after PHx. Treatment of liver with ShRNA expressing cDNA plasmids directed against either Notch or Jagged1 causes a decrease in hepatocyte DNA synthesis during regeneration [104]. Genetically induced elimination of Notch on the other hand is associated with spontaneous hyper-proliferation of hepatocytes in unoperated livers, but the response of hepatocytes to PHx is decreased as well [105].

The onset of liver regeneration is associated with rapid changes in hepatocyte gene expression [106, 107]. In addition to genes related to cell cycle, there are others of no such obvious connection (e.g. IGF binding proteins) [108]. These changes are rapid and more than 100 new genes are expressed in the first hour after PHx. However, it is remarkable that despite the rather cataclysmic events associated with cell division, there is little apparent impact on overall hepatic functions. Most of the proteins synthesized by the liver change very little in the peripheral blood and their rate of synthesis is minimally affected, if at all. Albumin, coagulation factors, serum amyloid proteins, complement components, etc. are very little affected during liver regeneration.

Auxiliary Mitogens

This term reflects a group of molecules which, independent of each other, have been found to cause delay in liver regeneration when their signaling is interfered. The list, originally small, has expanded through the years. There have been many instances in which such discoveries were associated with pronouncements to the effect that liver regeneration was caused by "a" specific factor. We have to understand that multiple extracellular signaling agents converge upon hepatocytes very soon (within minutes) after PHx and that each one plays an important role, but none alone either causes or fatally disrupts (after its elimination) the regenerative response. However, this should not undermine the significance of these auxiliary mitogenic signals. Regeneration is a very complex response. While there are compensatory signaling pathways to overcome specific signaling blocks under normal circumstances (as with experimental animals), this is most often not the case in catastrophic human diseases associated with massive hepatic necrosis. In these situations, any less than optimal regenerative response can make a significant difference in the balance between life and death of the organism. Thus, the role of the auxiliary mitogens in optimizing the regenerative response is of extreme importance, and it should be further studied in relation to the pathways associated with liver regeneration and liver failure [109].

TNF α(alpha)

This cytokine is produced in liver by hepatic macrophages. TNF α(alpha) levels in the plasma rise quickly after PHx [102, 110, 111]. Mice deficient in TNF α(alpha) receptor 1

(TNFR1) have delayed liver regeneration and defective activation of the transcription factor NFkB [95, 110, 111]. Antibodies against TNF α(alpha) delay liver regeneration and interfere with activation of Jun kinase [112]. The findings are enigmatic because administration of TNF α(alpha) to normal rats and mice is associated with hepatocyte apoptosis and levels of TNF α(alpha) are elevated in liver failure [3, 113]. However, these findings are consistent with the effects of TNF α(alpha) in other tissues. TNF α(alpha) is enhancing pro-mitogenic pathways in cells stimulated towards mitogenesis, primarily by enhancing activation of NFkB. As its name implies, it can also activate apoptotic pathways in normal cells [114]. The outcome of TNFR1 signaling in part depends on increased concentration of reactive oxygen species [115] and on other factors regulating the dissociation of NFkB from IkB, controlled by the kinase IKK [116, 117]. In general, activated NFkB confers resistance against apoptosis induced by a variety of agents [118]. TNFα(alpha) by itself is not mitogenic in hepatocyte cultures in the absence of serum, but it does enhance the mitogenic effects of HGF and EGF and, when administered in vivo in animals submitted to a limited hepatectomy, it also enhances their in vivo mitogenic effects [119]. Studies utilizing adenovirus vectors expressing a form of IkB resistant to IKK (NFkB "super-repressor and thus preventing NFkB activation) showed that PHx is followed by massive hepatocyte apoptosis [113]. On the other hand, selective targeted expression of the same IkB "super-repressor" form, in an inducible system of gene expression, showed that suppression of NFkB and PHx were not associated with apoptosis [120]. Thus, the mechanism of action of TNFR1 in relation to liver regeneration may be more complex, with other studies suggesting that enhanced processing of TGF α(alpha) via TACE [121], as well as stimulation of IL6 synthesis [102], might also contribute to its effects.

Interleukin 6

Mice with systemic genetic elimination of Interleukin 6 (IL6) had deficient response to PHx in one study [76], whereas a subsequent study showed that liver weight was restored in $IL6^{-/-}$ mice as in the wild type animals [27]. However, in both studies, there was delayed activation of the transcription factor STAT3. This transcription factor, a very important component of the JAK/STAT signaling system in hepatocytes, contributes to the signaling cascade coordinating the transition of hepatocytes through the cell cycle [122, 123]. In addition to IL6, its activation also seems to be dependent on the norepinephrine [124] (see below). Mice with genetic elimination of STAT3 have rather small defect in regeneration, but this may be due to compensatory effects exerted by increased levels of STAT1 [123]. IL6 is not a mitogen for hepatocytes in culture, but it is a mitogen for bile duct epithelial cells [125]. IL6 is a well known trigger of the acute phase response [126] and there are several proteins associated with the acute phase response that increase shortly after PHx [47]. IL6 levels in plasma also increase after PHx [76]. There is a presumption that IL6 is produced by Kupffer cells, but the possibility that other hepatic cell types, including hepatocytes, also produce IL6, has not been ruled out. Overexpression of both IL6 and its soluble receptor causes periportal hyperplasia in hepatic lobules, but has not been associated with hepatic neoplasia [127]. However, in a different study, excretion of large levels of IL6 from Chinese hamster ovary (CHO) cell lines producing excess IL6 under constitutive promoters, was associated with massive hepatic enlargement when the CHO cells formed tumors in mice [128].

Bile Acids

A recent study demonstrated that mice with genetic elimination of the transcription factor FXR had deficient liver regeneration [129]. Depletion of bile acids also had a negative effect on the regenerative process. Bile acid levels do rise after PHx, peaking at 3–6 h. The study demonstrated that the FXR-bile acid pathway is relevant to optimizing the regenerative response [129]. The pathways associated with this are not clear.

Norepinephrine

Levels of norepinephrine and epinephrine rise in the plasma after partial hepatectomy [130]. In primary cultures of hepatocytes in chemically defined serum-free medium, norepinephrine enhances the mitogenic effect of EGF [131] and HGF, and it decreases the mito-inhibitory effects of TGFβ(beta)1 [132, 133] and Activin, the latter effect mediated via induction of Smad7 [134]. These effects are exercised through the alpha-1 adrenergic receptor [131]. Activation of this receptor in both normal hepatocytes and hepatoma cell lines results in activation of STAT3 via involvement of Src kinase and EGFR [124]. The effects of norepinephrine on liver regeneration are even more global. Norepinephrine induces expression of HGF from fibroblasts [74]. Since norepinephrine increases rapidly after PHx, it may be involved in the enhanced expression of HGF in the lung, seen after PHx [72]. In addition, norepinephrine and epinephrine also enhance production of EGF from Brunner's glands of the duodenum [83]. The combination of effects at

both the level of the mitogenic receptor signaling as well as enhanced production of HGF and EGF suggests that the involvement of norepinephrine in liver regeneration is rather crucial. Blockade of the alpha-1 adrenergic receptor by the specific blocker prazosin delays regeneration by 72 h [130]. The same effects are also observed following sympathetic denervation of the liver [130]. Recent studies have also shown that in addition to its origins from sympathetic neurons, norepinephrine may be locally available to hepatocytes from the stellate cells, which have now been shown to produce norepinephrine [135, 136].

Insulin

Insulin exerts multiple effects on many aspects of hepatocyte biochemistry and function, especially in the areas of carbohydrate and lipid metabolism. Insulin, produced by the pancreatic islets, is made available directly to the liver via the portal circulation, prior to entering the systemic circulation. Portacaval shunt (diverting portal vein flow to the inferior vena cava) causes hepatic atrophy [137]. This is reversed when insulin is injected directly into the liver, and this reversal of atrophy is associated with hepatocyte mitosis [138]. Insulin is required for hepatocyte viability in primary culture, though it is not a mitogen per se [139]. In its absence, the mitogenic effects of both EGF and HGF are substantially diminished [55]. Insulin receptor appears to be a modulator of hepatocyte metabolic responses, by activating of PIK3CA, AKT, mTOR, [47] and other pathways. Recent studies have shown that there is a drop in plasma glucose after PHx and that administration of glucose has suppressive effects on liver regeneration, associated with increased expression of C/EBPα(alpha), p21 and p27, and decreased expression of FoxM1. These effects may be mediated through activation of p21 [140]. It is not clear whether plasma insulin plays a role in these events.

Termination of Liver Regeneration

Most of the studies in the field have focused on pathways leading to initiation of liver regeneration. As a consequence, not as much is known about the pathways leading to termination of liver regeneration. These pathways must be very carefully orchestrated. They accomplish the remarkable endpoint of returning the liver to the original mass with a high degree of precision! Whatever the pathways may be, they do serve that role very effectively. We can hypothesize that a critical determinant of the size of the liver is the flow through liver of the portal circulation, and that when a certain proportion between portal flow and live size is reached, further growth stops [1]. While this is a self-fulfilling truism, it does not guide to further speculation as to which, if any, component of the portal blood (or the amount of the flow) is important. Furthermore, proliferation of hepatocytes is almost entirely over at the end of day two, post-PHx in the rat (day 3 in the mouse). However, liver mass is still expanding at those time points due to proliferation of non-parenchymal cells and enlargement of the proliferated hepatocytes. Thus, the proliferation kinetics of hepatocytes and the end of their active growth occur before the original proportion between portal vein flow and hepatic mass is restored. In view of that, testable hypotheses can be easier, generated by exploring the role of specific signals known to have growth inhibitory effects on hepatocytes and exploring how they affect proliferation of specific cell types and liver regeneration as a whole.

Transforming Growth Factor Beta 1 (TGFβ(beta)1)

This multifunctional cytokine is produced in the liver by stellate cells. Hepatocellular carcinomas (as most cancers) also produce TGFβ(beta)1 [141], but it is not clear whether this reflects a type of epithelial-mesenchymal transition (seen in many tumors), or whether hepatocytes can also produce TGFβ(beta)1 under specific circumstances. TGFβ(beta)1 is a member of a family of three TGFβ(beta) molecules, each from a separate coding gene, and all expressed in the liver [141]. It is not clear whether the members of TGFβ(beta) family have different functions. TGFβ(beta)1 binds to decorin, a protein present in the hepatocyte pericellular space and anchored to hepatocyte plasma membrane by a GPI linkage [142, 143]. Immunohistochemical stains localize TGFβ(beta)1 on hepatocytes and it appears to be removed from hepatocytes when they enter the cell cycle [25] (see section "Histologic Changes and Cellular Proliferation Kinetics" above). Plasma levels of TGFβ(beta) also rise rapidly after PHx, with the same time kinetics as HGF [43]. This may reflect mobilization of TGFβ(beta)1 following remodeling of the pericellular matrix. Circulating TGFβ(beta)1, if not attached to its LAP protein, is bound by α(alpha)-2 macroglobulin and transferred to LRP receptors on hepatocytes for degradation [144].

The effects of TGFβ(beta)1 on specific cell types differ. It enhances production of extracellular matrix by mesenchymal cells (fibroblasts, stellate cells, etc.) [145]. It facilitates formation of capillaries resulting from tube formation by endothelial cells [146]. It also inhibits proliferation of hepatocytes and most other epithelial cells [132]. The effects of TGFβ(beta)1 are mediated after binding to TGFβ(beta)R2. More details on TGFβ(beta) signaling are provided in Chapter 23. The complex then binds to TGFβ(beta)R1 and activates its serine-threonine kinase function (known as ALK5).

This generates a signaling cascade involving Smad proteins, which migrate to the nucleus and transmit relevant messages altering expression of specific genes [147].

The second half of liver regeneration (loosely defined as days 3 to 7 after PHx) is a time when hepatocyte proliferation stops; there is also extensive endothelial cell proliferation and formation of sinusoids lined by fenestrated endothelial cells; in addition, there is synthesis and restoration of the extracellular matrix degraded immediately after PHx. Since TGFβ(beta)1 is known to affect such types of events, and since TGFβ(beta)1 expression is at its highest levels in the second half of the regenerative process, it is logical to postulate that the enhanced expression of TGFβ(beta)1 is involved in the eventual termination of regeneration and the restoration of histologically intact liver tissue at the lobular level. Such considerations, plausible as they are, are derived primarily from extrapolation and not from direct evidence. Expression of TGFβ(beta)1 begins to rise at 2–3 h after PHx [148] (about the same time as the enhanced expression of HGF, see above). It remains expressed at high levels for more than 72 h after PHx. This does not seem to affect proliferation of regenerating hepatocytes, reaching their peak of proliferation in that time frame. Hepatocytes isolated from regenerating liver are more resistant to the effect of TGFβ(beta)1 [132]. There are several contributing factors that probably facilitate this resistance. Expression of TGFβ(beta)1 receptors (TGFβ(beta)R1 and TGFβ(beta)R2) on hepatocytes decreases during liver regeneration [149]. In addition, there is less immunoreactive TGFβ(beta)1 around hepatocytes during regeneration [25]. Furthermore, norepinephrine, known to inhibit the mito-inhibitory effects of TGFβ(beta)1, rises rapidly in the plasma during the first two days after PHx and also perhaps plays a role in promoting "resistance" of the regenerating hepatocytes to the mito-inhibitory effects of TGFβ(beta)1 [130]. The origin of the rising TGFβ(beta)1 have been examined in a study involving cell isolation and it appears that all main cell types including hepatocytes are involved in TGFβ(beta)1 production [150].

Despite all expectations from the above studies, mice with hepatocyte-targeted transgenic expression of TGFβ(beta)1 do not seem to have problems with liver regeneration [151]. Additionally, genetic elimination of TGFβ(beta)RII also does not seem to prolong liver regeneration, unless activin is also inactivated by administering follistatin [152]. Activin itself is also a mito-inhibitor for hepatocytes. However, it is known to be produced by hepatocytes [153]. Potential interactions between activin and TGFβ(beta)1 towards the later stages of liver regeneration may include effects on termination of proliferation and tissue building. Regardless of its potential role in termination of liver regeneration, TGFβ(beta)1 seems to also play a role in keeping hepatocytes in G_0 stage of quiescence. Inhibition of TGFβ(beta) receptor II or the activin receptor II in rats with dominant negative constructs resulted in stimulation of proliferation of hepatocytes in normal (not subjected to PHx) animals [154, 155]. The results suggest that TGFβ(beta)1 and perhaps activin, under normal conditions, exercise a continued effect on hepatocytes maintaining a state of proliferative quiescence. The stimulation of hepatocyte proliferation when this effect was removed, however, also suggests that mitogenic stimuli present in the immediate environment of the hepatocytes continually exert an opposing pro-mitogenic effect which is balanced by TGFβ(beta)1. Candidates for that role, among others, maybe HGF and EGF. The former is present in the immediate periportal hepatic ECM, bound to glycosaminoglycans. The latter is continually produced by the Brunner glands of the duodenum and made available to the liver through the portal circulation.

Extracellular Matrix

Initiation of liver regeneration is associated with remodeling and breakdown of many ECM components, mediated by a proteolytic cascade involving uPA, plasmin and MMPs (see above). The process is reversed at the end of regeneration, with re-synthesis of many ECM components (see above). The re-synthesis of ECM proteins may be part of the signaling process leading to the termination of liver regeneration. Support of this hypothesis comes from hepatocytes in primary culture, in which hepatocytes enter into cell proliferation under the influence of HGF and/or EGF [55]. However, they lose most of their hepatocyte specific differentiated functions and there are associated changes in hepatocyte-specific transcription factors. Addition of extracellular matrix in the form of type I collagen gel or a (high in laminin and glycosaminoglycans) extract of EHS mouse sarcoma leads to restoration of hepatocyte differentiation with simultaneous cessation of cell proliferation [55, 156]. It should be noted (see "Intracellular Signaling Events", above) that during regeneration, despite the involvement of hepatocytes in the complex process of cell proliferation, the hepatocyte-specific patterns of differentiation are overall maintained, in contrast to cell culture. It can be speculated that the complete absence of matrix in hepatocytes cultures (other than a dry film of type I collagen) may not be sufficient for providing signaling required to maintain differentiation, in contrast to the in vivo situation, in which (despite matrix remodeling) there is sufficient ECM still remaining for signaling related to differentiation.

Signaling of ECM through integrins, in addition to cooperative signaling with growth factors and their receptors, proceeds primarily through two proteins, namely focal adhesion kinase (FAK) and integrin linked kinase (ILK) [157, 158]. The latter forms a complex with proteins PINCH and parvin and establishes complex association and signaling

through the cytoskeleton, and it may be even directly associated with intranuclear signaling events [159]. Elimination of ILK in liver was used as a surrogate approach for interfering with ECM signaling. Acute elimination of ILK was associated with massive hepatic necrosis [160]. Gradual elimination through genetic approaches (expression of Cre recombinase under the albumin promoter) was compatible with embryonic development. However, there was a wave of spontaneous hepatocyte proliferation after birth resulting in altered matrix deposition and liver enlargement [161]. The results suggest that ILK and ECM/integrin signaling have control over what constitutes normal liver size. Furthermore, when these mice were subjected to PHx, the final liver weight was much larger than liver weight prior to PHx by about 60%, due to prolonged hepatocyte proliferation [21]. The data demonstrate that ECM and integrin signaling are important in termination of liver regeneration. Of interest, excessive growth of the ILK deficient livers was not only seen after PHx but also in response to chemical mitogens such as phenobarbital [162].

In addition to direct signaling contributions, ECM is also involved by maintaining proper proportions of concentrations of heparin-binding growth factors (e.g. HGF and HB-EGF) and growth inhibitors (such as TGFβ(beta), bound to decorin). Reconstitution of ECM may be resulting in rebinding of excess growth factors and inhibitors and thus allowing hepatocytes to exit the cycle and stay in G_0 phase of quiescence.

Glypican 3

Glypican 3 (GPC3), a component of pericellular matrix, is linked to hepatocyte plasma membranes by GPI linkage. Its expression is highly upregulated in hepatocellular carcinomas [141, 163]. However, loss-of-function mutations of GPC3 in humans are associated with the Simpson-Golabi-Behmel syndrome, in which there is enlargement of internal organs and bone structures. The same is observed with GPC3 loss-of-function mutations in mice [164]. GPC3 expression becomes elevated after day 3 post PHx, and associations of GPC3 and its binding partner CD81 are seen on hepatocytes and non-parenchymal cells at the end of their proliferation kinetics [46]. Inhibition of expression of GPC3 in hepatocytes in primary culture leads to prolonged cell proliferation. The latter finding establishes the fact that at least in hepatocytes, GPC3 is associated with inhibition of growth.

Yes-Associated Protein

Several recent publications have established yes-associated protein (YAP) as a key protein involved in liver size regulation. Hepatocyte targeted over-expression of YAP is associated with massive hepatic enlargement and development of hepatocellular carcinomas within 10 weeks after birth [19]. Mst1 and Mst2 are two related kinases, which are homologous to the *Drosophila* Hippo, pathway. These two kinases, in association with an adaptor protein known as SAV1 phosphorylate and activate kinases Lats1 and Lats2, which in turn associate with another adaptor, Mob and phosphorylate YAP in the nucleus. Phosphorylated YAP leaves the nucleus. YAP itself is a coactivator transcription factor of many genes associated with cell cycle. Critical to this chapter is the finding that systemic genetic elimination of both MST1 and MST2 (resulting in increase of YAP in the nucleus) affected specifically the liver as the only organ, and caused massive hepatomegaly and hepatocellular carcinoma [20, 165]. These findings are extremely important and their significance is not yet fully developed in relation to liver biology. The specific impact on only the liver size when MST1 and 2 were systemically eliminated, establishes a unique importance of the Hippo kinase homologues and YAP protein in regulation of the hepatic size. It is of interest that during liver enlargement and enhanced regeneration in hepatocyte-targeted elimination of ILK (see above), there is increase of YAP protein in the nucleus and decrease of phospho-Yap in the cytoplasm of hepatocytes [21]. The same is true with the enhanced growth response to phenobarbital in the same mice [162]. Conversely, in mice with over-expression of GPC3 in hepatocytes and suppressed liver regeneration, there is decrease of YAP in the nucleus and increase of phospho-YAP in the cytoplasm. These findings suggest that the Hippo/MST1&2/YAP system is at least under partial control by integrins and ECM signaling and that GPC3 (through pathways yet to be identified) is also playing an important role in YAP regulation. Studies related to this system are undoubtedly going to be of importance for understanding of issues related to liver size regulation.

Exchange of Growth Regulatory Signals Between Hepatic Cell Types During Liver Regeneration

As mentioned above, of the different hepatic cell types, hepatocytes are the first to enter into DNA synthesis. Proliferation of the other hepatic cell types (non-parenchymal cells: stellate cells, biliary epithelial cells and sinusoidal endothelial cells) occurs following hepatocyte proliferation [22]. Stellate cells and epithelial cells can be seen proliferating as early as 24 h after PHx whereas endothelial cells have a longer time span for proliferation and restoration of sinusoidal vascular architecture [28]. It is now clear that replicating hepatocytes

synthesize several growth factors capable of stimulating proliferation of adjacent non-parenchymal cell types. These include platelet derived growth factor (PDGF) [166] and fibroblast growth factors 1 and 2 (FGF1 and 2) [167, 168]. Both of these are mitogenic to stellate cells and endothelial cells. Hepatocytes also synthesize Angiopoietins 1 and 2 [169] as well as vascular endothelial growth factor (VEGF) [67, 170, 171]. These are also mitogenic for sinusoidal endothelial cells. TGFα(alpha), a member of the family of ligands of EGFR, is also synthesized by replicating hepatocytes [86]. Though TGFα(alpha) is mitogenic for endothelial cells, hepatocytes also express EGFR and it has been speculated that hepatocytes may establish an autocrine loop between EGFR and TGFα(alpha). However, this has not been verified and mice genetically deficient in TGFα(alpha) do not have defects in liver regeneration [87]. This would imply that such autocrine loop does not exist; it may also imply that other compensating members of the EGFR ligand family known to be involved in liver regeneration (amphiregulin, HB-EGF, and EGF) compensate for the loss of this autocrine effect, also suggesting that this autocrine loop is dispensable or can be replaced by paracrine loops from the other members of the EGFR ligand family. Hepatocytes are also on the receiving end of mitogenic signals. Stellate cells synthesize HGF [172] and endothelial cells stimulated by VEGF in VEGF R1 do the same [67], creating a potential for a mutual growth-promoting feedback between replicating hepatocytes and sinusoidal endothelial cells. However, hepatocytes cease most of their proliferation by post-PHx day two (in the rat) and three (in the mouse), a time when proliferation of endothelial cells is barely beginning. This suggests that the bidirectional growth stimulating interactions between hepatocytes and endothelial cells, while possible in principle, may not be occurring in reality. Synthesis of norepinephrine, a recently discovered property of stellate cells [135], also has many ramifications for regulation of both production of growth factors (EGF and HGF) as well regulation of the responsiveness of their receptors (see section "Termination of liver regeneration" above). TGFβ(beta)1 is produced by stellate cells, by Kupffer cells, and hepatocytes [150] and it effects a variety of processes from formation of vascular endothelial tubes to stimulation of synthesis of ECM, and thus is a key regulator for this process. Though its mitoinhibitory effects seem to be "resisted" by regenerating hepatocytes via down-regulation of the TGFβ(beta)1 receptors (see section on "Transforming Growth Factor beta 1", above), its importance for the overall regenerative process appears very significant. TNFα(alpha) and IL6 are produced by Kupffer cells. In view of the involvement of these two cytokines in regulation of activation of NFkB and IL6, Kupffer cells are also important contributors to the mitogenic signaling for hepatocytes. Injury of the hepatic macrophages by use of gadolinium chloride causes delays in regeneration [119, 173]. Biliary cells also participate in cell proliferation during liver regeneration. In fact they show histochemical evidence of DNA synthesis (e.g., Ki67 positive nuclei) with almost the same kinetics as hepatocytes. Studies with primary cell cultures of biliary epithelium have shown that it shares the same mitogenic receptors with hepatocytes, namely EGFR and MET. However, IL6 is a mitogenic substance for biliary cells in culture, in contrast to hepatocytes [125].

Clinical Implications of Liver Regeneration

As mentioned in the beginning of this chapter, liver regeneration after PHx has allowed the study and mechanistic analysis of the regenerative stimuli controlling liver regeneration and growth, independent of involvement of other signaling pathways associated with the inflammatory events associated liver disease [3]. We now understand that there are two main mitogenic receptors for hepatocytes, namely the EGFR and the HGF receptor (MET). All others tested in vivo, or in vitro, are not effective (with mild effects seen by FGF family members) [55, 60]. We also understand that auxiliary mitogens are of great importance, and they include TNFα(alpha), IL6, norepinephrine, insulin, bile acids, and possibly serotonin (see section on "Auxiliary mitogens," above). This does not imply that other such signals may not exist that will be discovered in the future. As the recent evidence regarding YAP protein suggests, there is much room for future discoveries. In order to understand the pathways associated with hepatocyte proliferation in the context of liver disease, we need to concentrate on what signaling pathways associated with inflammation may do that would impinge (inhibit or enhance) the pathways already understood as controlling hepatocyte proliferation. Relevant considerations in this topic follow.

Generation of Growth Enhancing or Inhibitory Signals as Part of the Inflammatory Process

Both macrophages and polymorphonuclear leukocytes are known to produce HGF [174]. Macrophages are also known to produce TGFα(alpha) [174]and TGFβ(beta) [149]. The balance of these signals in relation to hepatic tissue damage by toxins and viruses has not been fully studied and it may vary between specific settings. The same signals are associated with well known biologic effects of acute and chronic inflammation in other tissues, and are generally viewed as related to local wound healing [175].

Effects of Viruses and Toxins on the Capacity of Hepatocytes to Replicate

This is also an area which should receive further attention. Though it is intuitively assumed that infection with viruses and exposure to toxins is likely to be inhibitory to cell proliferation, this may not be the case. As an example, Hepatitis C virus (HCV) utilizes CD81, the partner of GPC3, to enter into hepatocytes [176, 177]. The latter, also known as "target of the anti-proliferative antibody" is associated with growth regulation in many cell types, including hematopoietic cells and astrocytes [178], and it is known to be associated with integrins, including the α(alpha)3-β(beta)1, the main hepatocyte associated integrin [179]. HCV may be acting as agonist of CD81 and thus be a positive long term regulator of hepatocyte growth. This may have effects on hepatocyte gene expression and potentially be of relevance with the well known promoting effects of HCV on hepatic neoplasia. Furthermore, as discussed independently in Chap. 38, the HCV genome directly regulates several signaling pathways that are of relevance in hepatic pathobiology. Hepatitis B virus (HBV) often inserts next to genes related to cell cycle, and thus may be the source of clones of hepatocytes with enhanced proliferation [180]. Similarly, many different components of the HBV are also known to influence cell signaling as discussed in Chap. 37. Overall viral studies in hepatocytes are hampered by the lack of well defined culture systems capable of sustaining viral infection. On the other hand, there are opportunities for further studies using histopathologic material and immunohistochemically demonstrable cell proliferation markers (e.g. PCNA and Ki67). Such studies are needed to answer these questions. Commonly encountered toxins such as acetaminophen and alcohol seem to have a uniformly inhibitory effect on hepatocyte proliferation and associated with hepatocyte apoptosis or necrosis. It is not paradoxical, in view of the discussion on the "hepatostat" above, that agents which inhibit proliferation or cause necrosis of a portion of hepatocytes enhance proliferation of the residual cells by stimulating the same signaling pathways as liver regeneration after PHx. This is perhaps the single most important message from the study of liver regeneration, namely that loss of hepatocytes regardless of any cause triggers the established regenerative mechanisms to restore cell loss. While this result is desirable in restoration of acute injury, if it becomes a prolonged situation as with chronic liver injury, it leads to distortion of hepatic architecture (see below). It is also likely that proliferating hepatocytes may more likely succumb to cell death from the effects of toxins, and this may create scenarios of vicious cycles in the liver, in which the very attempts of hepatocytes to restore hepatic cell loss makes them more vulnerable to the toxins which caused the loss. It is also well known from earlier studies that proliferating hepatocytes are much more susceptible to neoplastic transformation induced by carcinogenic chemicals [181].

Cirrhosis as a Chronic Regeneration Process

Cirrhosis is a result of chronic injury to the liver by agents that are primarily known to cause hepatocyte loss. While there are multiple types of cirrhosis based on viral, toxic, metabolic or vascular etiologies, it is generally applicable that there is no etiology of cirrhosis that is not associated directly or indirectly with some degree of localized or panlobular hepatocyte damage and death. This is definitely the case with some of the most common etiologies of cirrhosis, associated with chronic viral infections or caused by alcohol. Loss of hepatocytes on a continued basis is a hallmark of pathogenesis leading to cirrhosis. At the beginning of the process, loss of hepatocytes may be related to the main offending etiologic agent. However, at the end stage, vascular alterations such as arteriovenous shunts at the lobular level causing decreased availability of blood to hepatocytes confound the deleterious effects of the etiologic agents and add on to the causes of hepatocyte death [182–184]. Another hallmark of cirrhosis is the increased deposition of extracellular matrix by stellate cells and portal fibroblasts that become activated during development of cirrhosis. A more detailed account on pathogenesis of fibrosis and cirrhosis is provided in Chap. 30. Also, a detailed account on stellate cell (Chap. 5) portal fibroblasts (Chap. 31) is also included in this textbook. Liver regeneration after PHx is also associated with both remodeling and re-synthesis of ECM. The pathways associated with ECM changes during normal liver regeneration (involving urokinase, MMPs, etc. in the beginning of regeneration, and re-synthesis of ECM at the end) are all also relevant in cirrhosis [185, 186]. During normal liver regeneration, there is maintenance of a balance between ECM degradation and synthesis. However in cirrhosis, due to altered performance ("activation") of stellate cells, an imbalance between ECM degradation and deposition leads to a net gain of ECM, which results in an alteration of the hepatic architecture. Simple histochemical studies have shown that there is a high rate of proliferation of hepatocytes in cirrhotic liver [187]. This is a compensatory reflex (i.e. liver regeneration related) in which the hepatocytes of the cirrhotic liver proliferate in order to restore total hepatocyte mass (as governed by a need defined by the "hepatostat", discussed in the beginning of this chapter). While the role of activation of stellate cells in cirrhosis is currently at the center stage of research, the impact of the signals from the continually replicating hepatocytes on the stellate cells (see section "Exchange of Growth Regulatory Signals Between Hepatic Cell Types During Liver Regeneration," above) also

deserves further exploration. It is the cumulative impact of such signals during the later stage of liver regeneration that induce synthesis of ECM by stellate cells and restore hepatic microarchitecture. Thus, replicating hepatocytes signaling on stellate cells may contribute to the development of the end-stage cirrhotic histologic changes by continually stimulating stellate cells towards synthesis of extracellular matrix, as at the second half of the regenerative process. Another area which deserves further study is the stereotactic relationship between hepatocytes and stellate cells. Under normal conditions, hepatocytes have intimate contact with stellate cells. Under conditions of chronic hepatocyte loss, since the associated injurious agents are mostly hepatocyte-specific, it is likely that a large number of stellate cells may be left "orphan" from hepatocytes. The biochemical and signaling disturbances of stellate cells associated with such a permanent change have not been studied, due to lack of suitable models. However, at a minimum, it has been shown that at the time of hepatocyte death, mere phagocytosis of fragments of dying hepatocytes may lead to stellate cell activation [188, 189]. This may also set the scenario for a vicious cycle. HGF has cytoprotective effects on hepatocytes [190, 191]. It is also known that activated stellate cells produce less HGF [192]. Thus, a decrease in production of a major cytoprotective agent for hepatocytes is seen precisely when such cytoprotection is needed the most, i.e. in the face of chronic exposure to hepatocyte-damaging agents. This may further enhance hepatocyte susceptibility to injuring agents and exacerbate their effects, accelerating development of cirrhosis. HGF has specifically been shown to ameliorate chronic liver injury and prevent or reverse development of cirrhosis in animal models [190, 191].

Overall Conclusions

Hepatic growth biology has benefited from well defined studies on hepatocyte cultures, surgical models leading to liver regeneration, and genetic models of mice in which signaling pathways can be neatly delineated. Newer approaches with nucleic acid constructs aimed to interfere with specific signaling pathways are also likely to be helpful. Now that considerable fundamental knowledge on signals related to both initiation and termination of regeneration has been accumulated, it is appropriate to capitalize on such knowledge to better understand how such signals are affected in the setting of liver disease. Mobilization of such signals may lead to desirable effects on repair. However, distortion of these signals, particularly in the setting of chronic liver disease may lead to the opposite result, namely irreparable liver damage. The underlying pivots that determine the balance between these two outcomes can only be understood on the basis of defined studies of liver growth biology, as exemplified liver regeneration after PHx. The body of knowledge gained so far on hepatocyte growth biology (and its corollary, pathways to hepatocyte death) will be of paramount importance for development of rationally based future therapeutic approaches for host of liver diseases.

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Chapter 19
Senescent Liver

Nikolai A. Timchenko

The decline of cellular functions is a main characteristic of aging. Although the senescent liver preserves its functions relatively well, there are a number of alterations which create conditions for the development of age-associated diseases and which cause problems with drug therapy in the elderly. Aging liver is characterized by morphological alterations of hepatocytes and sinusoids, by alterations in functions of Kupffer cells, by steatosis, and by chronic inflammation. One of the most significant age-associated alterations in the liver is the reduction of its regenerative capacity. Aging causes alterations of many signal transduction pathways in the liver which change expression and activities of transcription factors and chromatin remodeling proteins. The age-associated alterations in the liver cover several levels of regulation of gene expression including transcription, translation, and posttranslational modifications. Senescent livers increase expression of the histone deacetylase 1 (HDAC1) and accumulate multi-protein C/EBPα-HDAC1 and HDAC1-C/EBPβ complexes, which occupy and silence E2F-dependent and C/EBP-dependent promoters. This epigenetic silencing of the promoters seems to play a critical role in the inhibition of liver regeneration. The age-associated appearance of the multi-protein complexes is the result of multiple alterations in translation and posttranslational modifications of HDAC1, C/EBPα, and CUGBP1. Decline of growth hormone (GH) and GSK3β with age is also a critical event in the development of senescent phenotype in the liver.

Introduction

It is now recognized that aging changes expression of senescence-specific genes and alters signal transduction pathways, leading to significant alterations in the biology of many tissues. It has been shown that telomere shortening is the main reason for the senescence of human cultured cells and for the reduced lifespan [1]. The mechanisms of senescence in animals and humans are much more complicated and involve the senescence of individual tissues as well as alterations in communications between different tissues. Recent studies have revealed a significant role of epigenetic regulation in the development of senescent phenotype in cultured cells and in organismal aging. It has been shown that chromatin structure is dynamic and is the subject of extensive remodeling, and that chromatin remodeling is critical for cellular and organismal aging [2–4]. The decline of cellular functions in vertebrates seems to be associated with alterations in the circulatory system. It has been shown that the systemic environment of young organisms contains certain factors, which support normal cellular functions of the tissues and which might correct dysregulations of some biological processes observed in tissues of old animals. Elegant work from Rando's group with parabiotic pairs has clearly shown that a young systemic environment rejuvenated skeletal muscle, liver, and other tissues in old mice [5]. GH is one of the components of the circulatory system that supports cellular functions on tissues in young organisms. The decline of GH with age plays a critical role in the reduction of tissue function. In addition to age-associated changes of the systemic environment, aging also affects each tissue individually. These age-associated changes in the systemic environment and in individual tissues lead to the development of age-associated diseases and aberrant body homeostasis. Although the most dramatic age-associated alterations are observed in musculoskeletal and cardiovascular systems, the senescent liver is also characterized by many serious abnormalities. This chapter summarizes recent findings which advance our knowledge of the molecular mechanisms by which aging changes liver biology, with the focus on the alterations of molecular pathways responsible for the reduction of the regenerative capacities of the senescent liver.

Liver Functions and Age

Liver size is reduced with age, and in patients over 65 years, it is around 30% smaller than in people under 40 years [6, 7]. In addition to the reduction of liver size,

N.A. Timchenko (✉)
Department of Pathology, Baylor College of Medicine,
Houston, TX, USA
e-mail: nikolait@bcm.tmc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_19, © Springer Science+Business Media, LLC 2011

analyses of many normal subjects of different age revealed that the hepatic blood flow is decreased with age [8, 9]. Despite these alterations, the senescent liver preserves its functions on relatively high levels until it receives challenges such as surgical resections. One of the important functions of the liver is the production of proteins (albumin, transferrin, ceruloplasmin, etc.) for secretion into the blood. The initial studies of this liver function were focused on the examination of albumin since this protein is a major liver product. These studies have shown contradictory observations. Singh et al. have shown that the transcription of albumin genes is reduced in livers of old rats and that this reduction is associated with a high level of methylation of the albumin promoter at CCGG sequences [10]. On the contrary, observations by Shah and Mooradian have shown no age-related differences of the levels of albumin messenger RNA (mRNA) and protein in the liver despite the shortening of poly A tail of the albumin mRNA in livers of old rats [11]. Although investigations of the protein secretion in aged rodents generate contradictory data, the work with humans clearly shows that the concentration of albumin in blood is reduced with age [12]. Recent studies of healthy human subjects have confirmed the reduction of albumin in the blood of old individuals. Giovannini et al. have evaluated the concentrations of albumin in 92 patients of different ages before and after surgical resections and found that older patients have reduced levels of albumin both before and after surgeries and that this hypoalbuminemia is a marker of pathophysiologic frailty [13]. Additional examinations of blood parameters as the measurements of liver functions showed the reduction of albumin with age [14]. In agreement with these findings, it has been shown that age is a critical factor for the number of days required for serum albumin to return to normal levels after partial hepatectomy (PH) in humans [15]. A recent study of the liver function test (LFT) revealed that the age has a small but significant effect on LTFs [16]. These data are obtained by examination of 5,380 twin pairs with the range of age 18–81 years [16]. In support of these findings, examination of age-dependent alterations of liver functions in chimpanzees revealed a decrease in liver function including the reduction of albumin [17]. The liver is also responsible for the production of lipids and enzymes into the blood. Investigations of serum parameters of the blood in young and old patients showed that serum levels of cholesterol, high-density lipoprotein, and triglycerides increase with age. These age-associated alterations are summarized in several earlier reviews [18–22]. Taken together, various studies have convincingly shown that aging has small but significant effects on liver function.

Morphological Changes in Senescent Liver

Many morphological alterations are observed in the aging liver. However, the interpretations of some of these alterations in human livers are complicated since many samples have been taken from elderly subjects with liver disease. The most consistent results have been obtained in studies of old animals. The liver consists of a number of different cells which are differentially affected by aging. Although the size of hepatocytes is variable in young and old livers, the aging liver contains a much higher portion of macrohepatocytes with increased nuclei due to polyploidy [18, 22]. Chipchase et al. have found that livers of 2-year-old mice have abundant hepatocytes containing enlarged nuclei with increased ploidy, while livers of 3-month-old mice contain a much smaller portion of enlarged hepatocytes [23]. The number of enlarged hepatocytes reach up to 30% in livers of old mice. The premature liver polyploidy has also been described in DNA repair (Ercc1) deficient mice [23].

One of the main characteristics of the old livers is lipid accumulation in the hepatocytes [24]. The majority of studies have shown that lipids are accumulated in hepatocytes with age, leading to steatosis or fatty liver [24, 25]. Cree et al. have applied magnetic resonance spectroscopy to evaluate the age-dependent accumulation of liver fat and intramuscular lipids in patients of different ages and have found that the elderly had a significantly greater accumulation of liver fat [26]. In addition, these authors showed that elderly patients with fatty livers have increased insulin resistance [26]. Independent studies attest to the age-associated accumulation of lipids in hepatocytes [27, 28]. However, there are also reports that did not find any age-associated increase of lipid accumulation in the liver [29, 30]. Although the differences in these studies suggest that the "old" livers might not accumulate lipids, it is likely that the calendar and biological ages are not identical in some patients and that some "old" livers are phenotypically young. Independent of age, the accumulation of lipids in the liver leads to impaired glucose metabolism and to reduced insulin clearance [24, 31–33].

Age-dependent morphological alterations in the hepatic sinusoid have also been recognized. The liver is a highly perfused tissue receiving blood from the heart and the portal vein. The circulation of the blood within the liver is mediated by hepatic sinusoids. The sinusoids form a rich capillary network, which provides extensive endothelial surface area for interactions of liver cells with circulating factors including immune cells and other soluble macromolecules [34, 35]. The sinusoids contain several types of cells, most importantly, the liver sinusoidal endothelial cells (LSEC). The orchestrated work of these cells is required for endocytotic and host defense functions of the liver, especially in response

to toxicants [34]. Although early studies did not detect significant alterations in the LSEC in old age livers [36], recent studies clearly demonstrated multiple age-associated changes in the sinusoids [35, 37–39]. Particularly, these studies demonstrated age-associated reduction in the number of perfused sinusoids, which correlated with reduction of sinusoidal blood flow [38]. In addition, it has been shown that the expression of several markers of sinusoids, such as von Willlebrand factor, is increased in old age [35, 38–41]. It is interesting to note that caloric restriction, which extends life span, also corrects age-associated alterations in sinusoids [39]. Investigations of Kupffer cells in livers have provided contradictory results. While some studies have revealed an increase in the number and activity of Kupffer cells in old age [42], other reports have shown that volume densities of Kupffer cells in the livers of rat are the same at 2 and 24 months of age [43]. However, it is likely that aging reduces the ability of Kupffer cells to mount an effective immune response [35]. Thus, a large body of evidence shows that old age causes significant alterations in functions of the hepatic sinusoid, in activities of LSEC, and in expression of some endothelial antigens.

HCV Infections and Nonalcoholic Fatty Liver Diseases Are the Major Risk Factors for Hepatocellular Carcinoma in Elderly

Several liver diseases increase with age including hepatitis B virus (HBV) infections, hepatitis C virus (HCV) infections, nonalcoholic and alcoholic fatty liver diseases, and hepatocellular carcinoma (HCC) [18, 20, 44–46]. It has been shown that HCV infections are prevalent with age and it is expected that the burden of HCV infections in elderly persons will be significantly increased during next two decades [47]. A number of investigations showed the correlation between outcome of HCV and patients age [18, 47–49]. A recognized complication of HCV infection is the development of HCC in old patients. Investigations of HCC in 463 patients of different ages with chronic hepatitis C in Japan showed the age-dependent increase in the rate of HCC starting from 60 years age [50]. Another recent study of the incidence of HCC in patients with hepatitis C infections has examined 693 patients with HCV showed that elderly patients developed HCC more often than young patients [51]. Nonalcoholic fatty liver disease (NAFLD) is also being recognized as a risk factor for the HCC development in the elderly [18]. NAFLD refers to a wide spectrum of liver diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) [52]. In patients with type 2 diabetes, the prevalence of NAFLD might be as high as 70%. Given a burgeoning epidemic of diabetes in an aging population, NAFLD is expected to be a serious risk factor in the elderly. Examination of 34 NASH patients with HCC and 348 NASH patients without HCC revealed that old age and advanced fibrosis were the main risk factors for HCC, and that HCC was the major cause of mortality in NASH patients [53]. Recent studies of the methylation of 19 epigenetic markers in aging liver, chronic hepatitis, and HCC showed that methylation of DNA in HCC occurs in a gene-specific manner and that HCV infections accelerate the methylation process [54]. Methylation in HCC and HCV infected patients disrupts components of signaling pathways that are involved in tumor formation, such as p16, p53, and WNT/APC [54]. The development of HCC in HCV infected patients appears to be mediated by alterations in epigenetic regulation of gene expression. Hepatic steatosis, which is observed in NASH and HCV infected patients and in livers of old patients, is a risk factor for HCC [55]. Ikeda et al. have examined 2,215 patients with chronic viral hepatitis and found the appearance of cancer to be age-dependent [56]. Benvegnu et al. have examined 312 old patients with HBV and HCV and found HCC to be the most frequent complication in these patients [57]. Two recent reports have also confirmed age to be a risk factor in development of HCC [58, 59]. Thus, age is an important risk factor for development of HCC in HCV-infected patients and patients with NASH.

Aging Reduces Regenerative Capacity of the Liver

Liver is a unique tissue which is able to regenerate itself in response to injury and after surgical resection [60–63]. Liver regeneration after PH is a widely used experimental system for investigations of molecular mechanisms of liver proliferation and mechanisms by which aging inhibits liver regeneration. Liver regeneration in young animals is controlled by a complex cooperation of many signal transduction pathways which are described in several recent reviews [60–63]. Investigations of liver regeneration using genetically altered mouse models has now revealed that the deletion or modification of a single gene is insufficient to arrest liver regeneration completely and that there is much signal transduction redundancy in this event [61, 63]. It would be relevant to briefly review those critical steps in the regulation of liver regeneration in young mice that are affected by aging in old animals. Hepatocytes are normally quiescent cells. In response to PH, hepatocytes undergo one or two rounds of replication and restore the original size of the liver. The transition of cells from quiescence to proliferation requires activation

of S-phase and mitotic-specific genes, which are usually repressed in quiescent cells by E2F-Rb family complexes [64–66]. At the early stage of liver proliferation after PH, several genes of the cytokine network are activated and this activation is required for proper liver regeneration [60, 61]. The next step of liver regeneration includes activation of tyrosine kinase receptor, c-met, and IGF ligands, which in turn activate expression of transcription factors involved in liver regeneration. The important transcription factors activated by PH are c-jun, C/EBPβ, and CREM [60, 67–69]. At later time points after PH, the liver activates the expression of a group of proteins that are required for transition through S-phase and mitosis. These proteins include DNA polymerase α, c-myc, cdc2, and Fox M1B [70–75]. Under normal conditions, the quiescence of liver is mediated by C/EBPα, which inhibits cyclin dependent kinases, and by Rb-E2F complexes, which repress E2F-dependent promoters. In young livers, PH eliminates C/EBPα-dependent inhibition of liver proliferation by the reduction of C/EBPα mRNA and protein levels and by activation of Akt-PI3K signaling, which de-phosphorylates C/EBPα at S193 [76, 77]. The elimination of Rb-E2F complexes after PH is achieved by phosphorylation of Rb [64]. Thus, activation of liver regeneration after PH involves a complex cascade of alterations in gene expression which include activation of cell cycle proteins and elimination of growth inhibitory activities of Rb and C/EBPα proteins.

The reduction in the regenerative capacity of liver with age was discovered over 50 years ago. In experiments with PH in rat liver, Bucher et al. found that young animals had two sharp peaks of DNA synthesis after PH while livers of old rats showed one significantly reduced peak of DNA synthesis which occurred at much later time points after PH [78]. Many follow-up studies clearly demonstrated that the liver regeneration is significantly reduced with age. The results of these publications are summarized in a recent review [63] and are presented with more details in the section discussing epigenetic silencing of liver regeneration in the elderly. In addition to PH, liver regeneration in animals might be initiated by several stimuli including treatments with carbon tetrachloride (CCl_4) and with mitogenes such as the peroxisome proliferator, 1,4-*bis*[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) [60, 61]. Studies from the Columbano and Leda-Columbano groups have shown that molecular mechanisms of initiation of liver proliferation by TCPOBOP differ from those initiated by PH [79–81]. For example, although the activation of cyclin D1 is required for liver proliferation after PH, the loss of cyclin D1 does not inhibit the proliferative response of mouse liver to mitogenic stimuli [80]. One of the critical differences of liver regeneration after PH and TCPOBOP treatments is that liver regenerates identically well in young and in old mice after TCPOBOP treatments [82, 83]. A caveat to the studies is that the studies comparing liver proliferation after PH and after TCPOBOP were performed in young (2-month-old) and "old" (12-month-old) mice. Although the differences in the liver proliferation between these two age groups are easy detectable, 12-month-old mice are not really "old" and might not develop the complete set of the age-specific alterations which strongly inhibit liver proliferation in 22–24-month-old mice.

Telomere Shortening in Senescent Liver and Its Possible Contribution to the Inhibition of Liver Proliferation in Elderly

Studies of the aging phenotype in senescent human cultured cells have revealed a critical role of telomere shortening in cellular senescence [1, 84]. Since telomere shortening inhibits its proliferation of cultured cells, one of the hypotheses is that it might also be involved in the age-associated loss of regenerative response in old livers. Telomeres are short repetitive DNA elements (TTAGGG) which are located at the ends of chromosomes. During cell division, telomeres shorten after each round of DNA replication, leading to significant loss of the telomeres after many rounds of cell division. The length of telomeres is maintained by a telomerase reverse transcriptase (TERT) which adds TTAGGG to telomeric DNA using a telomerase RNA component [85]. It is important to note that telomere shortening is quite different in humans and in rodents. In humans, telomeres are approximately 15–20 kb in length while mouse telomeres are much longer and reach up to 150 kb [1, 84]. The most important difference between mice and humans is that the majority of human cells do not express hTERT, except specific germ lines and immortal cancer cells, while the majority of mouse tissues contain active TERT [84]. Therefore, the proliferation of human cells leads to the telomere shortening and growth arrest (replicative senescence) to protect cells from catastrophic death; while mouse and rat cells do not have significant telomere shortening within one generation and are using a different mechanism for cellular senescence [84, 85]. In agreement with this difference, mice with deletion of the telomerase RNA component can survive for many generations [86]. For humans, it has been shown that telomere length is reduced in livers of older patients [87, 88] suggesting that telomere shortening might be involved in the inhibition of liver proliferation in humans. Since telomeres are much longer in mice, they do not shorten significantly within one generation and do not seem to play a role in the inhibition of liver proliferation in old animals. Nevertheless, several reports revealed that the telomerase activity is important for proper liver regeneration. It has been shown that telomerase activity is increased in regenerating rat [89] and pig livers [90]. In addition, the liver-specific deletion of a

telomeric protein, TRF2, in mice leads to the inhibition of liver proliferation after PH [91]. In this case however, hepatocytes regenerate liver functions via endoreduplication and cell growth [91]. Thus, although telomerase activity is activated after PH, it is unlikely that telomere shortening is critical for the inhibition of liver proliferation in older animals [92].

Epigenetic Silencing of Liver Regeneration in Old Mice

The initial studies of molecular mechanisms which reduce liver regeneration in old animals have been focused on examination of single genes and on the comparison of their expression in young and old animals after PH. It has been found that the livers of old mice failed to activate c-myc [71, 93], DNA polymerase alpha [70], and FoxM1B [73, 75] to the levels observed in young mice after PH. Although these studies provided important information, further investigations revealed that the alterations of the cell cycle proteins in senescent liver are much more complex. A number of recent reports provide a strong support for the hypothesis that alterations of multiple signal transduction pathways and chromatin remodeling cause the inhibition of regenerative capacities of the liver. Comparison of protein–protein complexes in livers of old mice and rats identified a chromatin remodeling protein, Brm, to be increased with age and forming a multiprotein complex with transcription factors E2F4, C/EBP α, and tumor suppressor protein Rb [5, 71]. This age-specific C/EBPα-Brm complex occupies E2F-dependent c-myc, DHFR, and cdc2 promoters in livers of old mice blocking their activation after PH [71, 94, 95]. Consistent with this growth inhibitory role of C/EBP α-Brm complex, Brm knockout mice showed an increased rate of liver proliferation [96]. Since Brm is the chromatin remodeling protein, the appearance of the C/EBP α-Brm complex in aging liver suggested that this complex inhibited E2F-dependent promoters through epigenetic silencing. However, the precise mechanisms of this putative silencing of the chromatin have been not identified because the composition of C/EBP α-Brm complex appeared to be much more complex than originally suggested. Biochemical purification of the C/EBPα-Brm complex led to the identification of additional components like histone deacetylase 1 (HDAC1) and heterochromatin protein 1α (HP1α) [97]. HDAC1 is the catalytic unit of the complex which de-acetylates histone H3 at K9 on the E2F-dependent promoters leading to epigenetic inhibition of these promoters [97, 98] (Fig. 19.1).

In addition to alterations of transcription in the liver, aging also changes translation of the proteins by activation of RNA binding protein CUGBP1 and forming the translational CUGBP1-eIF2 complex [99]. HDAC1 mRNA is one of the targets of this translational complex [97, 98]. The CUGBP1-eIF2 complex binds to the 5′ region of HDAC1 mRNA and increases translation of HDAC1 mRNA in the livers of old mice [97]. The elevation of HDAC1 in livers of old mice also leads to the increased amounts of complexes of HDAC1 with another member of C/EBP family, C/EBPβ [100]. The C/EBPβ-HDAC1 complex is associated with C/EBPβ-dependent promoters and represses expression of these promoters [100–102] (Fig. 19.1). In agreement with the elevation of HDAC1 complexes in livers of old mice, Kawakami et al. reported decreased amounts of a target of HDAC1, histone H3 acetylated at K9 in livers of old animals [103]. Thus, the elevation of HDAC1 in livers of old animals suppresses gene expression through the interactions with C/EBPα-Brm and with C/EBPβ leading to epigenetic silencing of the E2F-dependent and C/EBP-dependent promoters (Fig. 19.1). It is interesting to note that the liver-specific elevation of HDAC1 in transgenic mice develops steatosis [104], which is the main characteristic of the aging liver [24]. It has been also shown that the ectopic expression of HDAC1 in livers of young mice alters expression of cell cycle genes [105]. Since C/EBPβ protein regulates expression of a number of genes involved in the regulation of lipids accumulation, it is likely that HDAC1-C/EBPβ complexes might be involved in the development of "fatty" phenotype in the liver of old mice. Because HDAC1 interacts with many transcriptional factors, it is likely that the elevation of HDAC1 in livers of old mice might repress promoters of other genes to which HDAC1 binds through the interactions with additional transcription factors (Fig. 19.1). In this scenario, the elevation of HDAC1 might be involved in several additional alterations in biology of senescent liver.

Mentioned earlier, livers of old mice proliferate well if the liver regeneration is initiated by TCPOBOP [82, 83]. Why is there such a difference in the ability of old liver to proliferate after TCPOBOP treatment and not after PH? In the light of recent data for epigenetic silencing of liver regeneration, it is likely that the activation of nuclear receptors by TCPOBOP leads to the removal of the epigenetic silencing by elimination of C/EBPα-Brm-HDAC1 and C/EBPβ-HDAC1 complexes.

Signal Transduction Pathways Which Change Epigenetic Control in Livers of Old Mice

The age-associated appearance of the C/EBPα-Brm-HDAC1 and C/EBPβ-HDAC1 complexes is caused by alterations of several pathways, which operate upstream of the individual components of the complexes. It has been shown that C/EBPα is hyper-phosphorylated at S193 in livers of old mice and that this phosphorylation is required for the interactions

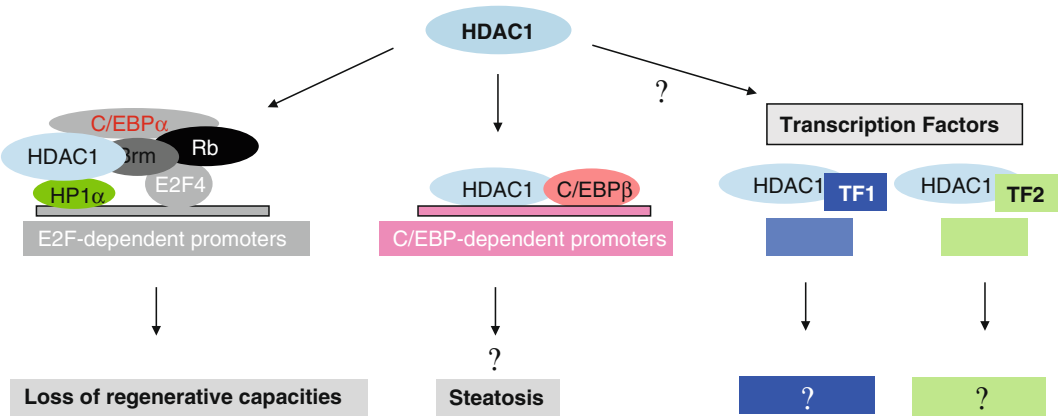

Fig. 19.1 Epigenetic silencing of liver regeneration in old mice. The increase of HDAC1 with age of the liver leads to accumulation of two complexes, C/EBPα-BRM-HDAC1 and C/EBPβ-HDAC1, which occupy and repress E2F-dependent and C/EBPβ-dependent promoters [71, 95, 97, 100, 101]. The silencing of E2F-dependent promoters inhibits liver regeneration after PH. Although the role of C/EBPβ-HDAC1 complexes in senescent liver is not known, these complexes might be involved in the development of steatosis. The possible interactions of HDAC1 with other transcription factors might be also involved in other age-associated alterations in the liver

of C/EBPα with Brm and HDAC1 [94, 95, 97]. Cyclin D3-cdk4 is the kinase which phosphorylates C/EBPα at S193. Senescent livers have higher expression of cyclin D3 through stabilization, which leads to activation of cdk4 and phosphorylation of C/EBPα at S193 [94, 98]. In the liver, stability of cyclin D3 is controlled by GSK3β-mediated phosphorylation of cyclin D3 at Thr283 [100–102]. In young livers, GSK3β binds to and phosphorylates cyclin D3 leading to a partial degradation of cyclin D3. Because the levels of GSK3β decline with age, cyclin D3 is de-phosphorylated at Thr283 and stabilized [100]. It has been shown that senescent liver reduces GSK3β by repression of the GSK3β promoter via elevation of C/EBPβ-HDAC1 complexes [100–102]. In agreement with these findings, recent observations have shown that the reduction of GSK3β by specific inhibitors or by siRNA effectively induces senescence phenotype in human liver-derived Chang cells [106]. A number of reports have shown that the reduced proliferative capacities of old livers and epigenetic alterations in livers of old mice are associated with the reduction of GH [63, 75, 95]. Elegant work from Costa's group has shown that GH is one of the components of young systemic environment which corrects liver regeneration in old mice [75]. It has been later shown that the normalization of GH levels in old mice is sufficient to reduce cyclin D3 and to eliminate the C/EBPα-Brm-HDAC1 and HDAC1-C/EBPβ complexes [95, 97]. Moreover, examination of GH-deficient Little mice showed that the GSK3β-cyclin D3 pathway is altered in young Little mice the same way as in WT old mice [100]. These data show that the decline of GH with age leads to the alterations on cyclin D3-GSK3β pathway and to appearance of multiprotein complexes in the liver causing epigenetic silencing of cell cycle genes and inhibition of liver regeneration (Fig. 19.2).

In livers of old mice, cyclin D3 is elevated in nucleus and in cytoplasm [95, 99]. While in nucleus, cyclin D3-cdk4 phosphorylates C/EBPα, the cytoplasmic accumulation of cyclin D3 leads to phosphorylation of RNA CUG binding protein CUGBP1 [95]. CUGBP1 has been discovered as RNA binding protein which is involved in development of myotonic dystrophy type 1, DM1 [107–110]. Further studies showed that CUGBP1 is also involved in the development of the aging phenotype in adipose and liver tissues [97, 99, 111]. In the liver of old mice, cyclin D3-cdk4-mediated phosphorylation of CUGBP1 at S302 increases the interactions of CUGBP1 with eukaryotic translation initiation complex eIF2 and formation of the translational CUGBP1-eIF2 complex [99]. The CUGBP1-eIF2 complex has several targets in the liver. Particularly, CUGBP1-eIF2 binds to the 5′ regions of HDAC1 and C/EBPβ mRNAs and increases translation of these proteins [97–99]. It is interesting to note that a recent report has described the accumulation of the CUGBP1-eIF2 complexes in patients with myotonic dystrophy type 2 (DM2) which have expansion of RNA CCUG repeats [112]. Consistent with these findings, the CUGBP1-eIF2 complex is elevated in transgenic mice expressing the mutant CCUG repeats [112]. These observations suggest that the patients with DM2 might develop age-associated alterations in the liver at younger age. Since the CUGBP1-eIF2 complex increases translation of HDAC1 and C/EBPβ in livers of old mice [97, 99], it would be important to examine if these proteins form HDAC1-C/EBPβ complexes in DM2 patients and if epigenetic control is altered in livers of DM2 patients.

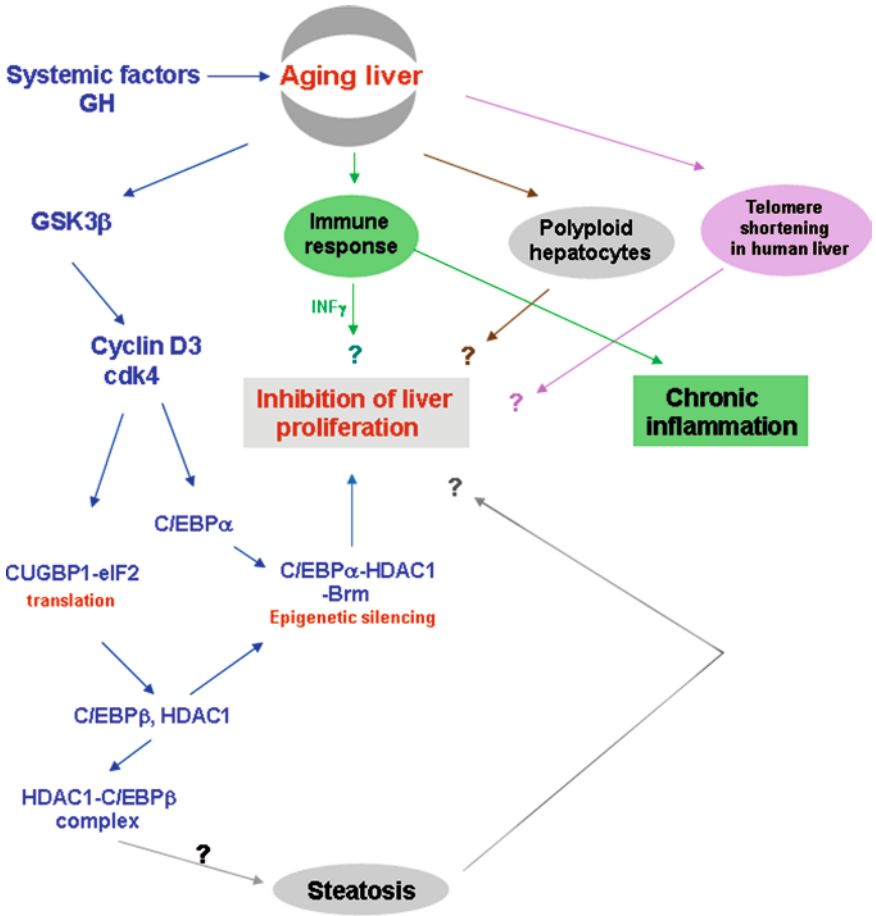

Fig. 19.2 Age-associated changes of biology of the liver and alterations of signal transduction pathways in senescent liver. The diagram summarizes recent data showing alterations of signal transduction pathways in senescent livers. The young systemic environment rejuvenates old livers [5] suggesting that systemic factors in circulating systems are responsible for maintenance of "young" phenotype of the liver. It has also been shown that a component of systemic environment, growth hormone (GH), corrects liver regeneration in old mice. *Blue arrows* and pathways show alterations in livers of old mice which are mediated by the decline of GH with age. Steatosis, alterations in the expression of genes of immuno response and polyploidy might also contribute to the inhibition of liver regeneration. Telomere shortening does not seem to be involved in the inhibition of liver proliferation in mice, but might contribute to the inhibition of proliferation in human liver

Additional Alterations that Might Contribute to the Decline of Proliferation in Senescent Liver

Although epigenetic repression of E2F targets by C/EBPα-HDAC1-Brm complex is the major mechanism of the inhibition of proliferative response of old livers, there are additional alterations in the liver which might be also involved in the inhibition. The accumulation of fat in hepatocytes of old mice might be involved in the inhibition of liver proliferation. It has been shown that liver regeneration is inhibited in leptin deficient ob/ob mice that have increased hepatic steatosis [113]. However, the correction of hepatic steatosis by prolonged administration of leptin or by food restriction does not improve liver regeneration in ob/ob mice [114] showing that steatosis is unlikely to be the major cause of the inhibition. Another well-documented alteration in the livers of old mice is the increased frequency of polyploid hepatocytes. Experimental work from Melton's lab showed that livers of 24-month-old WT mice contain a much higher number of polyploid hepatocytes than young animals and that Ercc1 mutants accumulate polyploidy hepatocytes at the age of 3 weeks [23]. Although the portion of polyploidy hepatocytes in senescent liver is relatively small, this polyploidy might also contribute to the inhibition of liver proliferation. The investigations of global gene expression by Darlington's lab in GH-deficient, long-lived Ames dwarf and Little mice have shown age-associated alterations in expression a number of genes [115, 116]. Particularly, these studies have revealed elevation of genes involved in xenobiotic metabolism in Ames dwarf and Little mice, suggesting that old GH-deficient mice are maintaining metabolic homeostasis at higher levels than WT animals of the same age [115, 116]. Further studies from this group have shown that old livers contain elevated expression of immune response genes [117]. Although no

alterations in expression of cell cycle genes have been detected in these investigations, the elevation of some genes in old mice might contribute to the inhibition of liver regeneration. One of these elevated genes is interferon gamma (INFγ). It has been shown that the overexpression of INFγ in livers of young mice inhibits hepatocyte replication after PH [118], suggesting that the elevation of INFγ in old mice might also contribute to the inhibition of liver proliferation. Further investigations of the role of the increase of inflammation-related genes in liver functions showed that aged liver exhibits a marked inflammation status which is accompanied by increased immune cell infiltration [117]. It is possible that these changes might be involved in the inhibition of liver proliferation.

Liver Surgery and Liver Transplantation in Elderly

Liver resections and liver transplantation are common ways to correct liver functions in individuals who suffered from liver diseases. The age of the liver transplant donors and recipients is quite important for the successful liver transplantation. The reduction of regenerative capacity of old livers is one of the problems for the recovery after surgeries in old patients and for survival of transplanted liver grafts from older human donors. The initial studies by Fortner and Lincer have examined the effects of age on the operative mortality rate and have shown that the patients below 50 years had 0.7% of mortality; while patients after 64 years had 11.1% of mortality [119]. Further studies have confirmed these observations [15, 18]. The multivariate analysis of the impact of 52 donor characteristics in 5,150 liver grafts patients has been performed by Cuende et al. to show that increasing age of the donors has a negative effect on liver graft survival [120]. The studies on 301 independently transplanted patients have showed similar findings [121]. Berenguer et al. have examined 522 cirrhotic patients after liver transplantation and, in agreement with previous data, have shown that main cause of death after liver transplantations to be high donor age [122]. Rull et al. have evaluated the role of donor age in the liver functions and final outcome after liver transplantation by analyzing 228 liver graft transplantations and found that the donor age over 65 years was associated with worse prognosis in terms of immediate liver functions and long-term survival [123]. Several recent reports also confirm the above findings [124–127]. In addition to investigations of transplantations in humans, Koh et al. have performed a detailed examination of the impact of donor age on the growth and liver functions of young recipient rats after liver transplantation from donors of different ages. These studies showed that transplantation of the liver from 11-weeks-old donors did not have any negative effects on the liver functions and survival; however, 52-week-old grafts transplanted into 11-week-old recipient resulted in deficient liver growth and a decline in serum albumin [128]. Although the majority of these studies revealed that the age of donor is one of the major risk factors for liver transplantation, there are examples of successful graft survival using old liver donors [129–131]. There might be several reasons for such exceptions. It might be that the successful transplantation of old liver is associated with young systemic environment of the recipients, which is known to rejuvenate old livers [5]. It is important to note that, in some cases, the calendar age does not correlate with biological age of the patients suggesting that some "old" donors did not develop age-associated changes and the liver functioned rather as the "young" tissue. The interpretations of successful and unsuccessful transplantations as functions of age are complicated because of different health conditions of recipients.

Development of Approaches to Improve Liver Regeneration and Graft Survival in Older Patients

Given the problems with liver regeneration and graft survival after liver transplantations, it is important to develop therapeutic approaches for the correction of liver regeneration in elderly. As discussed above, recent studies have revealed that the inhibition of liver proliferation in old organisms is mediated mainly by epigenetic silencing. Several reports suggested that treating with GH might be one approach to correct the proliferative capacity of old livers [75, 95]. GH can eliminate epigenetic silencing of E2F-dependent promoters by disrupting the C/EBPα-Brm complex and GH can eliminate silencing of C/EBPβ targets by reduction of C/EBPβ-HDAC1 complexes [95, 97, 98]. Despite the fact that this approach looks promising, the precise strategy for the treatments with GH needs to be further developed. It has been shown that GH treatments increase onset of diabetes and liver proliferation in quiescent livers without surgical resections [132], suggesting that the latter may lead to development of liver tumors. On the other hand, these studies applied relatively long-time exposures to GH. It is likely that examination of shorter treatments with GH might provide better conditions for correction of liver proliferation without significant side effects. Another promising approach to correct proliferation in old livers has been suggested by experiments with TCPOBOP. Columbano and Ledda-Columbano have found that aged livers proliferate well if proliferation is initiated by the activation of nuclear hormone receptors using the mitogene TCPOBOP [82, 83]. It might be possible to establish conditions under which TCPOBOP might correct liver proliferation in

the elderly. Recent examinations of the role of GSK3β in liver proliferation showed that expression of GSK3β is reduced in the liver with age and that the correction of GSK3β in livers of old mice also corrects liver regeneration [100]. The genetic modulations of the GSK3β in liver can also be considered as a potentially promising approach for correction of liver regeneration in elderly.

Concluding Remarks

Aging changes the biology of the liver at different levels. The main alterations include: (1) small but significant reduction of liver functions; (2) changes in the liver morphology including polyploidy of hepatocytes, steatosis, and alterations in sinusoid, as well as alterations of functions of Kupffer cells; (3) activation of immune response leading to chronic inflammation; (4) the decline of regenerative capacities of the liver; and (5) high rate of mortality after surgical resections and after transplantations of livers from old donors. Figure 19.2 shows liver pathways that are affected by age. The most critical, age-associated change of molecular pathways is epigenetic silencing of E2F-dependent and C/EBPβ-dependent promoters. So far, it has been shown that the epigenetic silencing of E2F-dependent promoters is a key event in the age-associated inhibition of liver regeneration. Since HDAC1 does not bind to DNA directly and displays its activity through interactions with transcription factors, the elevation of HDAC1 in livers of old mice could have a larger impact in gene expression. The age-associated epigenetic silencing is the result of alterations of several signal transduction pathways that finally lead to formation of the multi-protein complexes. The studies within the last few years have also emphasized a critical role of the decline of GH in the development of senescent phenotype in the liver. Figure 19.2 depicts the pathways (blue) which are affected by the reduction of GH in livers of old mice.

Acknowledgements I would like to thank Estela Medrano for the critical reading of the chapter and for useful recommendations. This work is supported by NIH grants GM55188, CA100070, and AG20752.

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Chapter 20
Signaling Pathways in the Liver

Abigale Lade and Satdarshan Pal Singh Monga

Introduction

As molecular executors, proteins are at the heart of tissue homeostasis. They are necessary for determining the state and fate of a cell and ultimately the basis of health of the tissue. This chapter is a generalized but succinct introduction to many signaling pathways that are of relevance to liver physiology and pathology. While these pathways will be discussed in separate chapters based on their relevance to specific states of the liver, this chapter will only introduce the readers to the important components and functioning of the relevant pathways with a brief mention of their known roles in liver pathobiology. The listing of the signaling pathways is in alphabetical order and does not represent their relative importance in the liver.

The proteins that are the components of various signaling pathways dictate the molecular basis of health and disease. This also applies to the liver. Signal transduction is the process by which a cell responds to changes in extracellular or intracellular conditions through the conversion of an external stimulus into a change in cell behavior. Signaling events typically involve molecular cascades in which multiple protein players are activated in succession, ultimately converging on changes in specific cell activities, such as alterations in gene expression, cytoskeletal rearrangement, cell death, or entry into the cell cycle.

Signaling cascades typically commence with the binding of a ligand to a receptor. Ligands can be molecules of various classes, including growth factors, cytokines, chemokines, hormones, neurotransmitters, and extracellular matrix components. They may be soluble factors secreted from cells nearby (paracrine signaling), from distal cells, which release them into the blood stream (endocrine signaling), or from the same cell receiving the signal (autocrine signaling); or may involve interactions between a cell and non-secreted factors of the extracellular matrix (matricrine), or a membrane-bound receptor on an adjacent cell (juxtacrine). Receptors for the ligands may be either extracellular or intracellular. Extracellular receptors include receptor tyrosine kinases, G-protein coupled receptors (GPCRs), integrins, and toll-like receptors, all of which pass through the cell membrane and transmit signals across it by changing their conformation, and consequently activity, upon ligand binding. Intracellular receptors are located within the cell, and are activated by internal factors, such as small lipophilic molecules that are able to traverse the cell membrane or intracellular secondary messenger molecules downstream of other signaling activities. Intracellular receptors include hormone receptors, peroxisome proliferator activator receptors, and IP_3 receptors. Following is an overview of the key signaling pathways with relevance to liver biology.

β(Beta)-Catenin Signaling

The importance of the Wnt/β-catenin signaling pathway in liver is becoming increasingly evident. β-Catenin is unique in that it has dual roles in cells. As a structural component of adherens junctions, it links the actin cytoskeleton to the transmembrane adhesion protein E-cadherin. Also, as an effector of the canonical Wnt signaling pathway, it translocates into the nucleus to transactivate expression of target genes by displacing the inhibitor Groucho from T-cell factor/lymphoid enhancement factor (TCF/LEF) family transcription factors. Activation of the signaling activity of β-Catenin occurs downstream of extracellular binding of Wnt family glycoproteins to their transmembrane Frizzled (Fz) receptors (Fig. 20.1). The Wnt family is comprised of 19 secreted proteins that are activated by lipid modification and act as morphogens in the context of embryogenesis, cancer, and normal tissue physiology. Wnt proteins act as ligands for receptor proteins in the Frizzled family, which, in conjunction

S.P.S. Monga (✉)
Division of Experimental Pathology, Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA
e-mail: smonga@pitt.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_20, © Springer Science+Business Media, LLC 2011

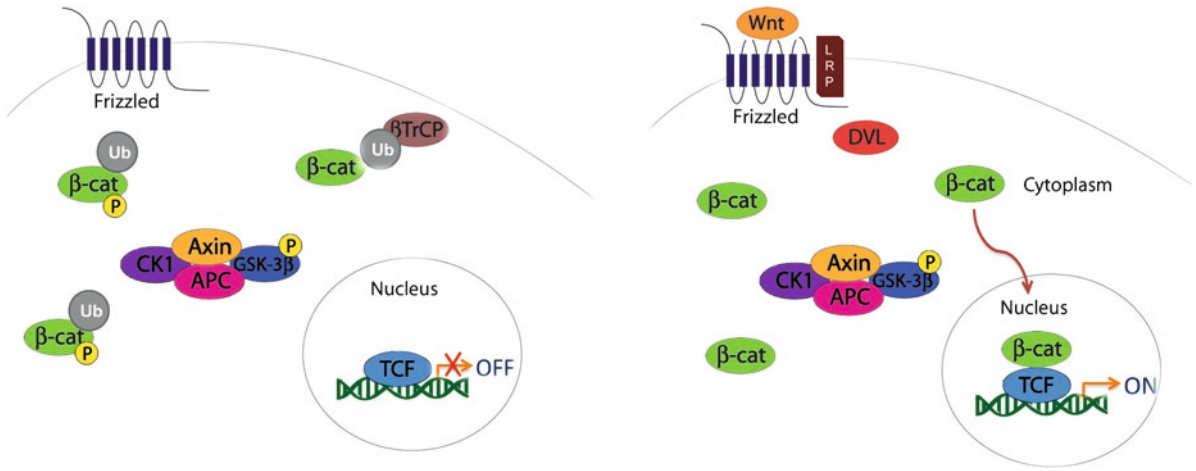

Fig. 20.1 The Wnt/β-catenin signaling pathway in absence of Wnt signal or in "off" mode (*left*) results in phosphorylation of β-catenin at specific serine/threonine residues in its amino-terminal by the degradation complex that results in its recognition by the β-transducin repeat containing protein (βTRCP) for ubiquitination and proteosomal degradation. In the presence of Wnt signal in an "on" mode (*right*), β-catenin translocates to the nucleus and binds to TCF family to induce target gene expression

with co-receptor LDL related protein 5/6 (LRP5/6), triggers the cascade that involves Disheveled (Dvl) and components of the β-catenin degradation complex, thus resulting in their inactivation. This inhibits serine/threonine phosphorylation of β-catenin, and prevents its recognition by a ubiquitin ligase, β-transducin repeat containing protein (β-TrCP), which normally targets β-catenin for constitutive degradation by the proteasome. Specifically, recognition of β-catenin by β-TrCP is dependent on phosphorylation of specific residues on β-catenin located in exon-3 (Ser 33, Ser 37, Thr 41, and Ser 45) by a protein complex composed of Axin, glycogen synthase kinase-3β (GSK3β), Casein kinase-1α (CK1α), and adenomatous polyposis coli gene product (APC), which occurs in the absence of Wnt signal or in the presence of upstream inhibitors of the Wnt pathway such as Fz-related proteins, Dickkopf, and others. Stabilization of β-catenin permits its nuclear translocation, thereby promoting Wnt pathway target gene expression through interactions with TCF family proteins, other relevant co-activators, and histone acetyl transferases to eventually alter cell proliferation, survival, differentiation, and migration.

Phosphorylation of several tyrosine residues by both receptor and non-receptor tyrosine kinases alters the activity of β-catenin, including Y86, 142, 654, 670, and others. Phosphorylation of Y654 by kinases such as c-src, Bcl-Abl, Met, EGFR, and ErbB2, decreases the affinity of β-catenin for E-cadherin, leading to disassembly of the adherens junctions complex, decreased cell–cell adhesion, and translocation of β-catenin into the nucleus to promote cell proliferation [1–7]. Likewise, Fer or Fyn phosphorylation of β-catenin at tyrosine 142 regulates its affinity for α-catenin, its other key adherens junction binding partner [8].

β-Catenin-independent Wnt signaling pathways also exist, the chief examples being the "non-canonical" planar cell polarity (PCP) and Wnt/calcium signaling pathways. Not only do these cascades operate independently of β-catenin, but they also actively suppress canonical β-catenin activity [9, 10]. Non-canonical Wnt signaling cascades utilize Wnt and Fz proteins, and while they are highly complex and incompletely understood, evidence is emerging that the particular Fz protein that is activated dictates whether canonical or non-canonical signaling occurs downstream [11].

Roles for β-catenin have been established for nearly every aspect of liver biology including embryogenesis, organogenesis, regeneration, zonation, and maintaining tissue and organ homeostasis. It has critical responsibilities in the regulation of multiple cellular functions, including lineage specification, differentiation, proliferation, survival, maintenance of redox state, morphogenesis and metabolism [12].

As is so often the case, the importance of a pathway in liver physiology means its dysregulation is often seen in pathological liver conditions. Hepatoblastomas, hepatocellular carcinomas (HCCs), cholangiocarcinomas, focal nodular hyperplasias, and hepatic adenomas all have been linked to β-catenin dysregulation, as have liver fibrosis and steatohepatitis [12, 13].

ERBB/EGFR Family

Another pathway with particular relevance to liver function is that activated by the epidermal growth factor receptor (EGFR) or ErbB family. EGFR family proteins are cell-surface

receptor tyrosine kinases of which there are four members, designated as EGFR (ErbB1, HER1), ErbB2 (HER2, neu), ErbB3 (HER3), and ErbB4 (HER4). Typically, binding of epidermal growth factor (EGF) ligands to the extracellular region activates these receptors. The extracellular ligand-binding region of EGFR proteins is comprised of two leucine-rich repeat domains denoted by L1 and L2 [14] which are interspersed with two cysteine-rich, beta-helix containing domains [15] (referred to as CR1 and CR2). The intracellular portion of the receptor contains a kinase domain. Ligand binding induces receptor oligomerization, and triggers autophosphorylation of cytosolic tyrosines, which can then recruit adaptor proteins or kinases involved in major signaling pathways including the ras/Raf/MAP kinase, JAK/STAT, Akt/PI3 kinase/mTor, src/NFκB, Pak1/rac, Wnt/β-catenin, and PLC pathways [16]. In addition to these pathways, EGFR signaling exhibits crosstalk with integrin [17], PKC [18], STAT [19], and steroid receptor signaling [20], and is also affected by VEGFR and PDGFR RTK pathways [21].

EGFR ligands are expressed as inactive precursors. They become anchored to the membrane and are activated by membrane-associated proteases, which remove regulatory domains and release the receptors from the cell surface [22]. The four EGFRs have distinct sets of binding partners. ErbB2 lacks ligand binding activity, but can heterodimerize with other ligand-bound EGFRs to become active. ErbB3, on the other hand, while capable of ligand-binding, does not possess kinase activity and must associate with ErbB2 or ErbB4 to activate signaling cascades. ErbB3 and ErbB4 bind different sets of a family of EGFR ligands called neuregulins; ErbB4 binds the four neuregulin family members NRG1β, NRG2β, NRG3, and NRG4, while ErbB3 binds NRG1α and NRG2α. Finally, ErbB1 binds both soluble and heparin-bound (HB) EGF, TGFα, the androgen receptor (AR), betacellulin (BTC), and epiregulin (EPR) [23].

As a pathway linked to inflammation, survival and growth signaling, EGFR is poised to mediate many key aspects of liver biology. In rats, ErbB2 is expressed by liver progenitors and persists through postnatal liver development, and ErbB3 expression occurs both in developing and adult liver [24]. Perinatal deletion of EGFR in mice results in impaired liver regeneration [25], as does the inhibition of EGFR signaling via infection of adult mice with a virus encoding the EGFR pathway inhibitor suppressors of cytokine signaling (SOCS) [26], and these impairments appear to stem from defective G1-S cell-cycle progression and stress response [25], possibly mediated through STAT3 activation [26]. Given the capacity of EGFRs to promote liver growth, it is perhaps not surprising that many studies have identified links between overactive EGFR signaling and hepatocellular and biliary carcinomas [27–40]. Recently, EGFR expression was also shown to be increased in hepatoblastomas of both fetal and embryonal types [41]. Overall, a picture emerges from studies of this pathway that EGFR signaling regulates hepatocyte proliferation and survival in response to stress.

Hepatocyte Growth Factor (HGF) Signaling

Hepatocyte growth factor (HGF) was discovered independently in several systems and was shown to promote a diverse array of context-specific effects including cell migration [42–44], growth [44–48], morphogenesis [49], and inhibition of tumor growth [50] before molecular characterization revealed a single protein behind these effects [51–53]. Structurally, HGF is related to the blood protein plasminogen, which is the precursor of the clotting protein plasmin. Along with apolipoprotein, HGF and plasminogen share an N-terminal plasminogen activation peptide domain, a serine protease modality, and "kringle" domains that are loop-in-a-loop structures formed by disulfide linkages between four cysteine residues [54]. Cleavage of the 728 amino acid HGF-precursor produces an α-chain comprised of residues 1–494 and a β-chain comprised of residues 495–728. Heterodimerization of these chains produces an active ligand capable of activating its tyrosine kinase receptor.

The receptor for HGF, c-Met, is a heterodimer consisting of a 50-kDa extracellular ligand-binding subunit linked to a 140-kDa transmembrane subunit containing an intracellular kinase domain. The active, 185-kDa dimer undergoes autophosphorylation at residues Y1234 and Y1235 to activate its kinase domain [55, 56]. Phosphorylation of residues Y1349 and Y1356 allows c-Met to recruit various SH2/SH3-domain containing signaling and adaptor proteins [57] including growth factor receptor-bound protein 2 (Grb2), Shc transforming protein 1 (Shc), GRB2-associated binding protein 1 (Gab1), Signal transducer and activator of transcription 3 (STAT3), phosphatidylinositol 3-kinase (PI3-kinase), phospholipase C gamma (PLCγ), as well as Src, V-crk sarcoma virus CT10 oncogene homolog, and the related Crk-like (CrkL) [57, 58].

Recruitment of different signaling proteins by c-Met-bound adaptors allows for the activation of a constellation of signaling pathways. One example is the activation of the Ras-MAPK and PI3-kinase/AKT pathways that promote cell migration, invasion, and branching morphogenesis [59–61]. Another is the Crk/Crkl recruitment of C3G and DOCK180 to activate Rap1- and Rac1-mediated regulation of cytoskeletal rearrangement, cell spreading, and cell adhesion [62]. A third example is the induction of tubule morphogenesis and anchorage-independent cell growth promoted by Stat3 [63, 64]. In addition to these mechanisms that activate signaling, Grb can also induce ubiquitinylation and targeting of Met for proteasomal degradation by recruitment of the ubiquitin ligase c-Cbl [65, 66].

Though its name suggests its primary role in the liver is to promote hepatocyte growth, HGF itself can actually inhibit proliferation of transformed rat liver cells in vitro [62] and in vivo [61], and further work has revealed that it is expression of c-Met rather than HGF, which is critical for mitogenesis [67]. However, during development, HGF and c-Met are expressed more highly in liver than any other tissue [68], and both $HGF^{-/-}$ and $c\text{-}Met^{-/-}$ mice die in utero, and exhibit decreased liver parenchymal cell mass and a failure of hepatocyte differentiation [69–71], thus pointing to a role for HGF/Met signaling in prenatal liver growth. Of note, the earliest discovery of HGF came from studies in which it was isolated from the conditioned medium of cultured rat HCC cells, as a factor that had mitogenic effects on normal rat hepatocytes. Met was first detected as an oncogene in a osteosarcoma cell line transformed by carcinogen treatment [72, 73] until further characterization revealed it to be a chromosomally-rearranged fusion between Met and Tpr [74, 75], missing the Met residue Y1003 that is necessary for its negative regulation [75]. However, though overexpression of HGF or Met is seen in some HCCs [76–82], further studies assessing the effect of activation of this pathway on transformed cells in vivo or in vitro conversely suggest anti-tumorigenic effects including growth inhibition [78, 83, 84] and promotion of apoptosis [78], and no correlation is observed between Met expression and tumor size [67, 76, 85]. Interestingly, mice lacking Met in hepatocytes showed a paradoxical increase in HCC in response to chemical hepatocarcinogenesis [86]. Critically, patient studies do not address whether Met mutations exist in the analyzed samples that could affect the downstream signaling outcomes. In some cases, rather than playing a causative role in HCC development, the upregulation of these proteins may be secondary to tissue damage or cellular stress associated with cancer. HGF is among the first proteins upregulated after liver injury, and active Met increases concordantly [87], as well as the protease responsible for its activation [88]. While the mechanism leading to these changes is still being elucidated, hypoxia promotes transcriptional activation of Met [89, 90], as do inflammatory mediators such as IL-1 α, IL-6, TNF-α [91], and prostaglandins [90], and this may account for the increases seen in cancers. Given their upregulation in response to liver injury and ability to promote hepatocyte growth, it is no surprise that extensive research has revealed HGF and Met to be critical in supporting liver regeneration. Of note, Met also can inhibit apoptosis by binding the Fas receptor and hindering the trimerization necessary for its activation [92], another function that would facilitate a role in responding to tissue injury. Furthermore, Met phosphorylation of β-catenin at residue Y654 induces its translocation to the nucleus along with activation of target gene expression, some of which promote cell cycle entry [1, 7, 93]. In the widely-utilized 2/3 partial hepatectomy (pHx) rodent model of liver regeneration (please see Chap. 18 for additional details on the process of liver regeneration), hepatocytes have been shown to increase utilization of existing HGF stores between 0 and 3 h after hepatectomy, and then, once these are depleted, they drastically increase their synthesis from hour 3 to hour 21 after pHx [94]. Activation of Met by tyrosine phosphorylation is seen between 30 and 60 min after partial hepatectomy [95]. Mice with hepatocyte-specific [96] or conditional Met deletions [97] exhibit severe impairments in regeneration. While the precise context-specific outcomes of Met activity are still being investigated, Met clearly has a critical role in many aspects of liver physiology.

Hedgehog Signaling

Hedgehog signaling instructs patterning of embryos through specification of cell fate, and regulates proliferation and stem cell maintenance in adult tissues [98]. The pathway is named after the three (sonic hedgehog, shh; Indian hedgehog, ihh; and desert hedgehog, dhh) secreted ligands of the Hedgehog family that bind the seven-transmembrane receptor Patched. Hedgehog ligands are expressed as 45 kDa precursors, and then autoproteolyzed to a 19 kDa form to which a palmitoyl group and cholesterol group are attached before secretion [99]. Binding of Hedgehog proteins inhibits the activity of Patched, releasing its inhibition of Smoothened and thus results in an increased activity of the transcription factor Gli3 (Fig. 20.2). In the absence of Hh, Gli3 is targeted for cleavage by the proteasome, generating a C-terminally cleaved fragment that functions as a transcriptional repressor. Active Hh signaling results in translocation of transcriptionally active Gli proteins into the nucleus and expression of target genes such as *Gli* and *Ptc* [100].

Hedgehog signaling plays a role in liver development, as well as homeostasis, regeneration and pathologies including alcoholic liver disease [101], hepatic [102] and biliary [103–107] fibrosis, HCC [108–110], hepatoblastoma [108–114] and cholangiocarcinomas [115]. Hedgehog expression is often increased in HCCs [108, 112] and hepatoblastomas [109, 116], and inhibition by the hedgehog antagonist cyclopamine decreases tumor growth, Hedgehog target gene expression, and promotes apoptosis [110]. Shh expression is seen throughout the foregut endoderm at embryonic day E8.5, and then disappears in the liver bud during the onset of liver specification at E9.0–E9.5. Its expression appears once more at E11.5, appearing to drive cell proliferation with a subsequent decline thereafter. This is apparently critical for hepatocyte differentiation [100]. Interestingly, loss of expression of Ihh also occurs at E11.5, with its expression increasing throughout prenatal development and then decreasing to a

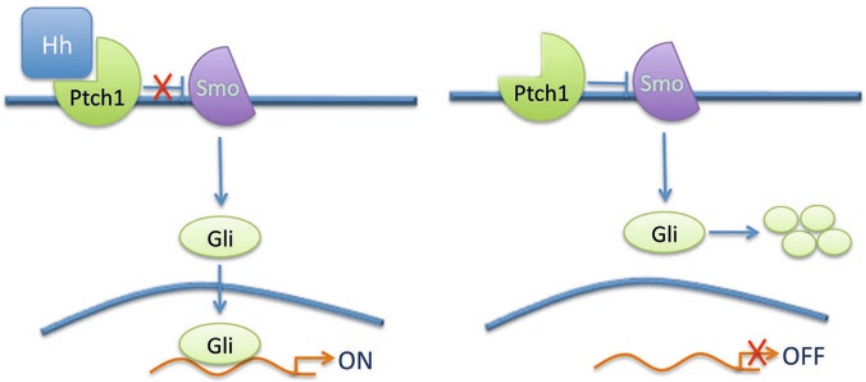

Fig. 20.2 The hedgehog signaling pathway, which in presence of the hedgehog ligands results in target gene expression (*left*), and in the absence of ligands results in activation of smoothened with resulting degradation of Gli3 (*right*) and thus lack of target gene expression

lower level that appears to be maintained postnatally [100]. Cholangiocarcinoma growth appears to be stimulated by Hedgehog signaling [115].

JAK/STAT Pathway

JAK-STAT signaling is initiated by binding of cytokine signals, such as growth factors, interferons (IFN), and interleukins, to their transmembrane receptors, ultimately leading to activation of Stat transcription factor family proteins. A classical example of this signaling is demonstrated by the IL-6/Stat3 interaction in the liver biology. The Jak, or Janus kinase receptor-activated tyrosine kinase, family consists of JAK1, JAK2, JAK3, and Tyk2. The Jak proteins act as mediators of signaling by binding to receptors and becoming activated in response to changes in conformation triggered by ligand binding (Fig. 20.3). Jak then phosphorylates tyrosine residues on the receptor to create a binding site for Stat, which then becomes phosphorylated and translocates into the nucleus. STATs possessing SH2 domains and capable of binding these phosphotyrosine residues are recruited to the receptors, and are themselves tyrosine-phosphorylated by JAKs. They are then able to bind other STATs via their SH2 domains, and result in homo- or hetero-dimers capable of activating gene transcription. JAK-independent, direct phosphorylation of STATs by EGFR and other receptor tyrosine kinases, the cytoplasmic kinase src, also occurs.

JAK-STAT pathway is regulated by two major classes of proteins: SOCS and PIAS or protein inhibitors of activated STATs. SOCS proteins bind and inhibit JAKs or STATs [117] and prevent STAT activation, and PIASs use various inhibitory mechanisms, such as blocking STAT binding sites on promoters, recruiting transcriptional co-repressor proteins, and/or promoting protein sumoylation to alter cellular trafficking [118]. Additionally, protein phosphatases regulate

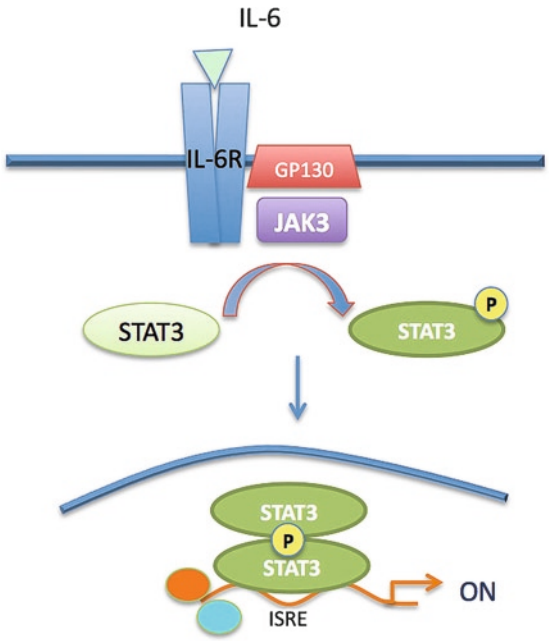

Fig. 20.3 The IL-6/JAK/STAT signaling pathway shows activation of JAK3 in response to IL-6 stimulation, which leads to Stat3 phosphorylation and nuclear translocation, where it interacts with co-factors to turn on target genes containing interferon stimulated response elements (IRSE)

JAK-STAT signaling by dephosphorylating receptors or STATs [119].

Seven Stat proteins exist in mammals, designated STAT1, 2, 3, 4, 5A, 5B and 6, each activated by a different set of cytokines. STAT1 activation occurs via binding of IFN α/β and γ, and plays roles in cellular responses to viral infection, tumor activity, inflammation, and promotes apoptosis. Similarly, STAT2 is also activated by IFN α/β and IFNλ, and also mediates antiviral responses. STAT3 responds to IL-22 as well as IL-6 family cytokines, and participates in acute phase response in addition to playing a key role in liver

regeneration, glucose homeostasis, and lipid metabolism. IL-12 activates STAT4, whose role is currently undefined, but may promote ischemia/reperfusion injury of liver. STATs 5A and 5B, activated by growth hormones, regulate expression of genes involved in growth and metabolism. Finally STAT6, activated by IL-4 and IL-13, promotes and mediates ischemia/reperfusion injury [120].

IL-6/STAT3 activity possesses particular relevance to liver physiology and pathology, and thus has been extensively studied in that context [121] (Fig. 20.3). During liver regeneration in rats treated with PHx, levels of IL-6 in circulation increase within the first hour [122]. Resident liver macrophages, and possibly hepatocytes themselves produce this cytokine, which acts not as a hepatic mitogen, but rather promotes biliary cell proliferation and function [123, 124]. Although increases in IL-6 occur early in regeneration, work done with liver-specific IL-6 pathway knockout animals suggests that this pathway is not responsible for initiating regeneration. Also, IL-6 deficient mice reveal the role of the interleukin-6/glycoprotein 130/signal transducers and activators of transcription-3 pathway in mediating liver steatosis and injury [125] and prevention of alcohol-induced liver damage and cirrhosis [123]. In addition to the aforementioned activities, activation of JAK/STAT pathway is common in HCC. One recent study found elevated Jak/Stat pathway activity in all HCCs relative to non-tumor adjacent tissue and normal livers [126], thus underscoring a need to better understand and target this pathway in liver cancers.

MAP Kinase Pathway

A major signaling pathway controlling a diverse set of cellular functions, the mitogen activated protein kinase (MAPK) pathway includes five distinct classes of MAPKs: MAPK/ERK kinase/extracellular regulated kinase (MEK/ERK), c-Jun N-terminal kinase (JNK), p38, ERK5, and ERK3 [127, 128]. The pathway acts downstream of activation of many growth factor receptors, and can effect cell proliferation, adhesion and differentiation through both cytosolic and nuclear targets. Growth factors such as EGF, vascular endothelial growth factor (VEGF), insulin, neurotrophins, and inflammatory cytokines activate MAPKs.

The MAPK/ERK kinase/extracellular related kinase (MEK/ERK) pathway is activated by receptor tyrosine kinases, which recruit the SH2-containing protein Grb2, to intracellular phosphotyrosine residues after activation by ligand binding (Fig. 20.4). Grb2 associates with a Ras-activating protein called Sos, which is activated when Grb2 binds activated RTKs, and thus can activate Ras. The activity of Ras, a GTPase, is dependent on binding to GTP (guanosine triphosphate) and can thus be modulated by GTPase activating proteins (GAPs) which inactivate Ras by promoting GTP hydrolysis, and guanine nucleotide exchange factors (GEFs) that activate Ras by catalyzing the exchange of a GDP molecule for a GTP. Active Ras proteins recruit Raf kinases to the membrane, enabling them to activate MEK-1 and -2 by phosphorylation, which then activate ERK-1 and -2. ERKs dimerize upon activation and can further phosphorylate cytosolic or nuclear targets, generally involved in proliferation or differentiation.

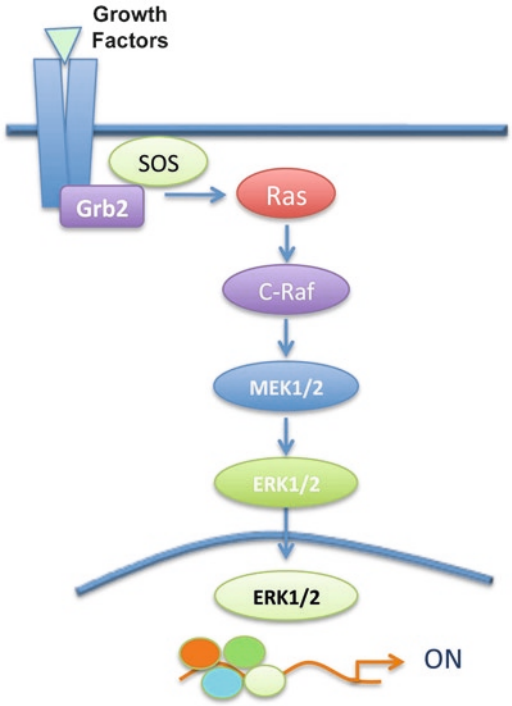

Fig. 20.4 The MAP kinase pathway is stimulated by certain growth factors that activate receptor tyrosine kinases, which lead to signaling through adapter molecules such as Grb2 and eventually lead to activation of ERK1/2, which induces target gene expression

A second MAP kinase signaling cascade is the JNK pathway, which is typically activated by GPCR, or cytokine signaling, or cell stress. This cascade proceeds through activation of MEK4 or MEK7, either of which may phosphorylate ubiquitous JNK family members JNK-1, -2, or -3, allowing their translocation to the nucleus. Once in the nucleus, they can activate transcription factors such as STAT3, ATF-2, c-Jun, and HSF1 and thereby regulate processes such as heat shock response, apoptosis, proliferation, and differentiation.

A third class of MAP kinases, activated by GPCRs, cytokines and cell stress, as well as some hormones, are the p38 family kinases. The four p38 family members α, β, γ, and δ are targeted by MEK3 and MEK6 and also target substrates such as eukaryotic initiation factor 4e (eIF4e) and MAPK interacting kinases Mnk1 and Mnk2 to regulate diverse

cellular processes including cell differentiation, proliferation, apoptosis, and inflammation.

Evidence exists for roles for multiple MAP kinase pathways in liver physiology as well as several hepatic pathologies. The roles of the most relevant growth factor receptors upstream of these pathways will be discussed separately, but activation and dysregulation of MAP kinase pathways bear some discussion. Sustained activation of ERK is seen in liver during embryogenesis [129], and activity of p38 kinase regulates hepatocyte proliferation during rat liver development [130]. Dysregulation of the JNK pathway is often a characteristic of HCCs [131–135], and Ras activation is commonly increased in HCC relative to adjacent non-tumor tissue [126]. Implications of MEK/ERK pathway aberrations in HCC are revealed by evidence that inhibition of ERK1/2 activation by RNAi knockdown of the upstream kinase MEK1 abrogated growth of human hepatocarcinoma cell lines both in vitro and in mouse tumor xenografts [136]. Additionally, p38 kinase activity is suppressed in HCC [137].

Notch Signaling

Another pathway with a critical role in liver function is the Notch signaling pathway. Notch signaling regulates tissue growth and differentiation [138]. It is activated via juxtacrine interactions, in which a ligand on the surface of one cell binds a receptor on a neighboring cell. The Notch receptor is expressed as a precursor that is cleaved to form an extracellular N-terminal fragment and a transmembrane/intracellular C-terminal fragment that assemble to form a functional, heterodimeric, transmembrane receptor [138]. Ligands for the Notch receptor are designated "DSL" ligands, as they include the Delta and Serrate/Jagged family members in Drosophila and mammals, and LAG-2 in *C. elegans*. DSL ligands are type I transmembrane proteins containing up to 36 extracellular EGF-like repeats that bind a corresponding EGF-like repeat domain on Notch. Upon ligand-receptor binding, Notch changes conformation to expose an intracellular cleavage site and is cut by a furin-like convertase to produce a soluble intracellular fragment referred to as NICD (*N*otch *I*ntra*c*ellular *D*omain) (Fig. 20.5). NICD is transported into the nucleus and participates with CSL family transcription factors to promote expression of genes such as Hes1, Hey1, and PDGFRB [138, 139]. In terms of the role of this pathway in liver, Notch signaling via Notch2 underlies the commitment of hepatoblasts to biliary epithelial cells, and absence or inhibition of the pathway leads to impaired intrahepatic biliary development including that seen in Alagille syndrome [140–146]. Notch pathway aberrations have also been linked to HCC [147–149] as well as hepatoblastoma [41, 150, 151].

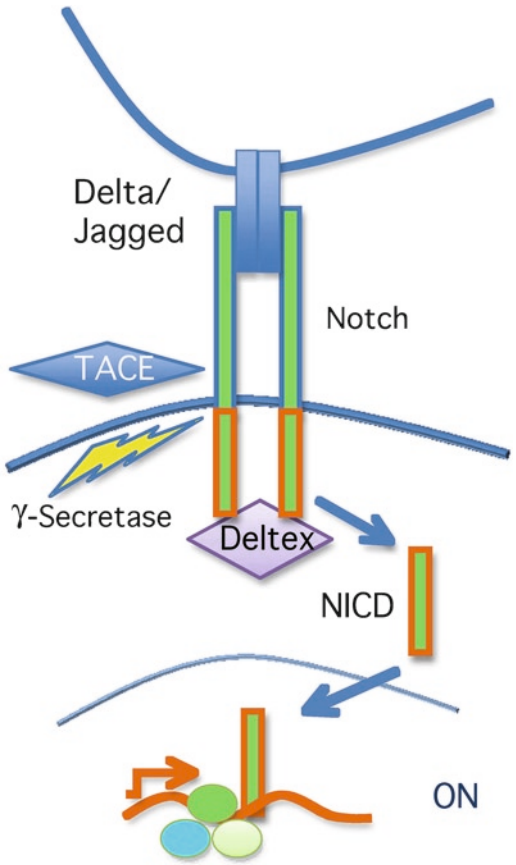

Fig. 20.5 The Notch signaling pathway is activated when signal from a neighboring cell in the form of a membrane bound ligand such as jagged/delta binds to its receptor notched that leads to proteolytic cleavage and the release of the intracellular domain, which enters the nucleus and transactivates specific target genes

PDGF/PDGFR Signaling

Platelet-derived growth factor (PDGF) receptors are prototypical receptor tyrosine kinases activated by binding by extracellular growth factors (for a recent review, see [152]). Receptors in this family include PDGFRα and PDGFRβ, and VEGFR1, 2, and 3, all of which are comprised of five extracellular Ig loops and a split intracellular tyrosine kinase domain that becomes active upon ligand-stimulated receptor homo- or hetero-dimerization. The activated kinase domains target the receptor itself, creating phosphotyrosine residues that serve as binding sites for downstream signaling molecules, many of which contain SH2 (Src homology 2) domains. Major signaling pathways triggered by PDGFR activation include: activation of PI3-Kinase, leading to cytoskeletal reorganization, migration, proliferation, and cell survival; PLCγ activation that leads to release of intracellular calcium stores and activation of the kinase PKC, and ultimately promotion of cell growth and motility; and the Ras-MAPK pathway, which activates

transcription of target genes involved in cell growth, differentiation, and motility. In addition, PDGFRs can interact with integrins, linking PDGFR signaling to focal adhesions and action to regulate cytoskeletal remodeling.

PDGFR ligands include four PDGFs denoted PDGFA, B, C, and D; four VEGFs designated VEGFA, B, C, and D, and one additional growth factor, the placenta growth factor (PlGF) [152]. PDGF-A and PDGF-B are expressed and secreted by most tissues, but as inactive propeptides that must be activated intracellularly by cleavage of N-terminal pro-domains by furins or other convertases. PDGF-C and D are activated extracellularly, by plasmin or tPa in the case of PDGF-C, or plasmin alone the in the case of PDGF-D. An additional level of regulation of PDGF activity is instituted by variable inclusion of a C-terminal, a positively-charged extracellular matrix-binding domain that promotes extracellular retention. This inclusion is regulated by alternative splicing in the case of PDGF-A and proteolytic processing in the case of PDGF-B (though the responsible protease has not been definitively identified).

Several studies provide evidence for the promotion of liver fibrosis by PDGFR signaling [153–156]. More recently, work has been done revealing a link between this pathway and HCC in preclinical and clinical studies and additional characterization is underway [157–159].

Peroxisome-Proliferator-Activated Receptors (PPARs)

As the name suggests, peroxisome-proliferator-activated receptors (PPARs) were first identified as proteins activated by peroxisome proliferation in Xenopus [160]. PPARs are a class of nuclear receptor proteins which exist as three isotypes: PPARα, PPARγ, and PPARβ/δ with distinct patterns of tissue-specific expression. PPARα is expressed by liver, heart, kidney, muscle, and adipose, where it orchestrates fatty acid uptake and lipoprotein metabolism; PPARγ, expressed by adipose, macrophages, heart, muscle, kidney, pancreas, retina, spleen, and large intestine, regulates insulin response and differentiation of adipocytes, finally, PPARβ/δ has the highest expression in brain, adipose tissue, and skin and lower expression in most other tissues. Upon ligand binding, these proteins can heterodimerize with retinoic x receptor (RXR) and adopt a conformation capable of binding to peroxisome proliferator hormone response elements in promoters of the target genes. Depending on association with co-activators and co-repressors, this binding may promote expression or repression of targets.

While PPARα agonists promote hepatic proliferation and may contribute to development of HCC [161, 162], hepatocyte-specific overexpression of PPARα alone does not appear to be sufficient to induce HCC [163]. Defects in PPARα activity lead to severe hepatic steatosis in fasting animals, underscoring the role of this pathway in fatty acid oxidation in liver [164–167].

PI3-Kinase/AKT Pathway

The PI3-kinase (PI3K) pathway is classically recognized as a pro-survival signaling pathway due to its ability to activate PKB/Akt, but can also promote cell proliferation, differentiation, motility, intracellular trafficking, and protein synthesis in different contexts.

PI3-Kinases themselves are divided into three classes based on structure and function. Class I PI3Ks are the most well-studied, and promote the generation of phosphatidylinositol-3-phosphate (PI3P), phosphatidylinositol (3,4)-bisphosphate ($PI(3,4)P_2$), and phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) by phosphorylation of the hydroxyl group at the third position of the inositol ring of phosphatidylinositol lipids [168]. These modified lipids then serve as second messenger molecules triggering the downstream effects of the pathway. This is chiefly mediated through phosphorylation of PDK1 and eventually of Akt, which in turn acts as upstream effector of a host of proteins including mTOR, GSK3β, MDM2, IKKα, and others to regulate diverse biological activities (Fig. 20.6).

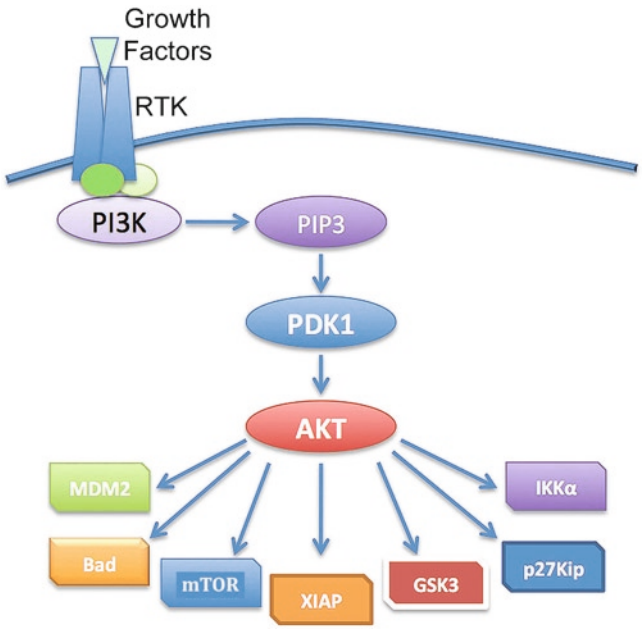

Fig. 20.6 The PI3 kinase pathway is activated upon binding of a growth factor to a receptor tyrosine kinase (RTK), which induces phosphorylation and activation of PI3 kinase, and eventually induces activation of AKT, which can have an effect on a wide variety of downstream molecules to induce biological response in a stage- and tissue-dependent context

Within class I, PI3-kinases are subdivided into Class IA and Class IB based on the proteins that activate them and the adaptor proteins they recruit. Active class IA PI3-kinases function as heterodimers of a p110 subunit with catalytic activity and a p85 subunit with a regulatory domain [168]. Three p110-subunit isoforms exist: p110α, p110β, and p110δ, encoded by *PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively. Three isoforms exist for p85 subunits as well: p85α, p85β, and p55γ encoded by *PIK3R1*, *PIK3R2*, and *PIK3R3*, respectively, of which alternative splicing of *PIK3R1* can generate shorter isoforms p55α and p50α. Activation of Class IA PI3-Kinases is downstream of receptor tyrosine kinase activity.

Class IB PI3-kinases are activated by G protein-coupled receptors (GPCRs) and consist of heterodimers between a p110 γ catalytic subunit and a p101 regulatory subunit, or one of the p101 homologues p84 or p87PIKAP (PI3Kγ adaptor protein of 87 kDa) [169]. The second class of PI3-kinases differs from Class I in many ways. These Class II kinases contain a unique C-terminal C2 domain and include PIK3C2α and PIK3C2β, which are expressed in all tissues, and the PIK3C2γ isoforms expressed exclusively in liver, and are activated by receptor tyrosine kinases, integrins, and cytokine receptors. While the outcomes of their activation is less well understood than that of the Class I kinases [170], it is known that they phosphorylate PtdIns and PtdIns(4)P, localize to clathrin-coated pits and lack adaptor subunits, and it is believed that they may regulate membrane trafficking and/or endocytosis [169].

Finally, class III PI3Ks exist as heterodimers between a 100 kDa Vps34 catalytic subunit and a p150 adaptor subunit, and regulate mTor activity and autophagy, with outcomes ultimately affecting cell growth and survival. This class of PI3Ks phosphorylate phosphatidylinositol only.

PI3 kinases can be activated by a number of kinases, including both receptor tyrosine kinases and non-receptor tyrosine kinases. The RTKs known to phosphorylate PI3 kinases include EGFRs, fibroblast growth factor receptors (FGFR), platelet-derived growth factor receptors (PDGFR), insulin-like growth factor receptor-1 (IGFR-1), IFN receptor, integrin receptor, and VEGFRs. These receptors get activated by growth factors and directly activate Class I PI3K p85 subunits, while the downstream MAP/MEK/ERK pathway protein Ras activates the p110 subunit.

Activation of PI3Ks can also occur via protein kinase C, which is activated by release of intracellular calcium stores, as well as other non-receptor tyrosine kinases such as Src and Shp1, and kinases Rac and Rho involved in cytoskeletal remodeling and regulation of intracellular calcium release. Once active, PI3Ks phosphorylate membrane-associated phospholipids, converting them into second messengers. In the case of Class I PI3Ks, phosphorylation of a hydroxyl group on PI(4,5)2 creates PI(3,4,5)P3, which activates proteins containing pleckstrin homology domains (PH), such as protein kinase B/Akt, guanine nucleotide exchange proteins (GEFs), GTPase-activating proteins (GAPs), Tec family tyrosine kinases, and adenosine diphosphate (ADP)-ribosylating factor 6 (ARF6) as well as proteins containing FYVE, Phox (PX), C1 or C2 domains [168].

The pro-survival activity of the PI3 kinase pathway immediately suggests a possible role in promoting liver regeneration and cancer, and indeed, several studies have investigated PI3K activity in both. In regenerating livers, the PI3K pathway is rapidly activated and inhibits apoptosis [171, 172]. PI3-kinase promotes invasion and metastasis in HCC [173], and Akt is increased in some HCCs and correlates with disease progression and prognosis [174–178]. Interestingly, increased Akt expression is also seen in cholangiocarcinomas, but existing evidence points to a correlation between Akt and positive prognosis in that context [179, 180]. More analysis needs to be done to better understand the meaning of the opposing PI3-kinase/Akt pathway changes in liver cancers of different origins, but it seems clear that this pathway has a meaningful role in regulation of liver cell growth, survival, and migration.

TGFβ Signaling

TGFβ superfamily proteins are growth factors that play roles in growth, differentiation, migration, adhesion, cell death, and homeostasis in multicellular organisms [181] The family includes over 30 members including TGFβ isoforms, growth and differentiation factors (GDFs Nodal and Activin), and BMPs (bone morphogenetic proteins). Beginning with their role in establishment of the first embryonic axes, TGFβ signaling is indispensible throughout the life of both invertebrate and vertebrate organisms [181]. TGFβ receptors exist as heterodimers of type I and type II transmembrane serine/threonine kinases that tetramerize upon ligand binding, allowing the type II receptors to phosphorylate the associated type I receptors. Different combinations of the seven type I receptors and five type II receptors found in mouse and human genomes result in different signaling outputs. While several pathways are activated by TGFβ receptors, the Smad pathway is the best-studied. C-terminal serines of the receptor-regulated Smads 1, 2, 3, 5, and 8 are targeted by TGFβ, allowing them to complex with the co-Smad, Smad4 to form a complex capable of transcriptional activation or repression. Briefly, TGFβ and other ligands such as Activins and Nodal utilize TGFβ receptors type II and type I, inducing their phosphorylation and activation, which in turn induces phosphorylation of receptor-activated Smads (Smad2/3) and their binding to co-smad (Smad4) (Fig. 20.7). This heteromeric complex translocates to the nucleus and interacts with histone

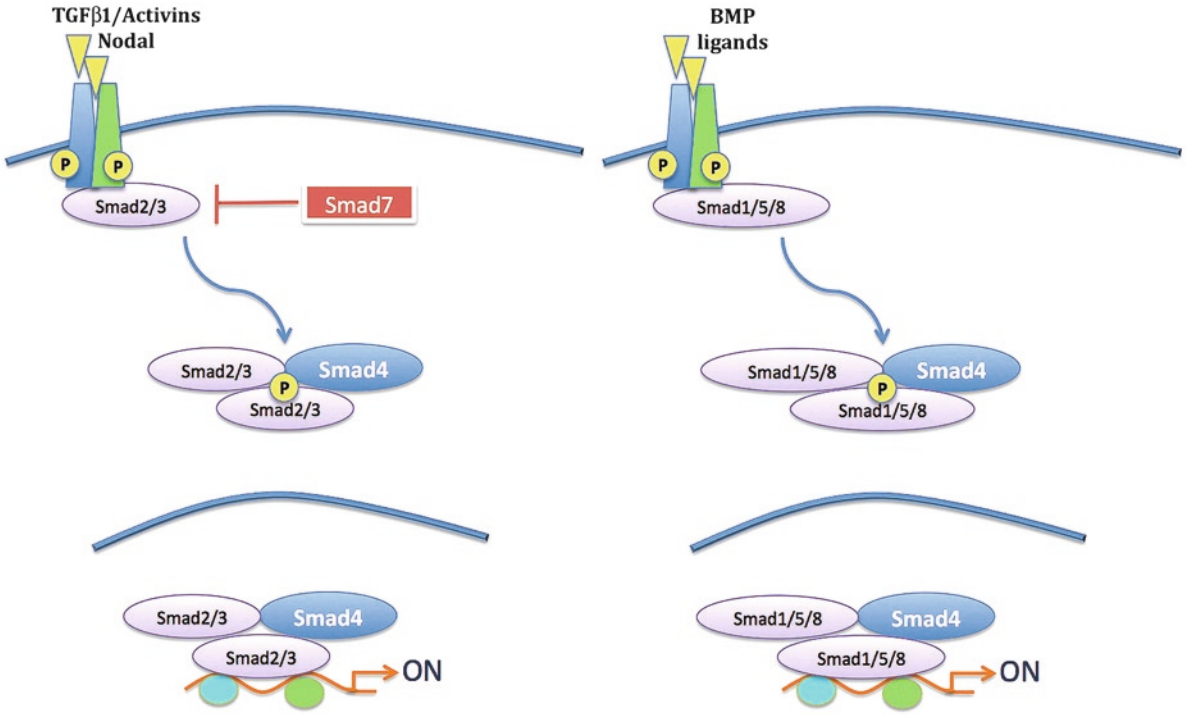

Fig. 20.7 The TGFβ pathway activation (*left*) occurs upon binding of ligands such as TGFβ1, Activins or Nodal to the TGFβ receptor type 2 and sequential activation through phosphorylation of receptor type I, smads 2 and 3 (inhibited by smad7), and smad4, eventually leading to the nuclear translocation of the complex of smads 2/3/4 and induction of target genes. In analogous situations, BMP ligands induce sequential activation of receptor type II, type I, smad1/5/8 and smad4 leading to the nuclear translocation of smads and target gene expression

acetyltransferases and transcriptional components to regulate target gene expression. Similarly, BMP ligands (2, 4, and 7) utilize receptors type II and I in the same way but induce phosphorylation and activation of Smad1/5/8. This also favors complex formation with Smad4 and induces nuclear translocation of the heteromeric complex to induce expression of distinct target genes in cooperation with transcriptional factors and co-factors (Fig. 20.7). Genes targeted by Smad complexes depend on the presence of transcriptional co-factors, which include FoxH1 and Mix family transcription factors [181]. TGFβ signaling can also activate the MAP kinase pathway, or pathways involved in epithelial-to-mesenchymal transition (via PAR6) or cytoskeletal rearrangement (via PAK2).

Links have been identified between loss of TGFβ expression by liver stem cells and development of HCC [182], and aberrant TGFβ activity has also been implicated in liver fibrosis [183].

TNFα/NFκB Signaling

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine best known for roles in inflammation and apoptosis, but which also plays roles in cell proliferation and differentiation [184]. TNF superfamily receptors are primarily type I transmembrane proteins characterized by extracellular cysteine-rich domains and intracellular regions containing either a death domain or sites for recruitment of TRAF proteins [184]. TNF ligands are type-II transmembrane proteins with an intracellular N-terminus and an extracellular C-terminus that is typically cleaved by a protease to allow differential binding to TNFRs. In total, the TNF superfamily of proteins is comprised of 19 ligands and 29 receptors. The pathway is named for the first-identified member, TNFα, which was so named after it was found to promote necrosis of transplanted tumors in mice [185]. The signaling pathway initiated by TNFα that leads to cell death has since been well-characterized, and related signaling pathways have also been described in increasing number and detail [182]. Binding of TNFα to TNF receptor 1 (TNFR1) triggers the recruitment of TNFR1-associated death domain protein (TRADD), TRAF1 (TNF receptor-associated factor 1), and receptor-interacting protein (RIP). A more detailed account and a diagram depicting the pathway are included in Chap. 24. TRADD binding to TNFR1 can lead to three major outcomes: activation of NF-κB, promoting transcription of pro-survival target genes, initiation of the stress-associated Jun kinase (JNK) signaling pathway, which is generally pro-apoptotic, but can promote proliferation or differentiation, and recruitment of Fas-associated protein

with death domain (FADD), which can recruit caspase 8 in sufficient concentrations to trigger its autoproteolytic activation and subsequent activation of the apoptotic cascade. The overall outcome of TNF signaling in a cell is dependent on various factors such as levels of reactive oxygen species and other pro- or anti-inflammatory cytokines.

Roles are emerging for the TNF pathway in cytoskeletal rearrangement in a wide range of processes such as cell adhesion, cell migration, tissue morphogenesis, membrane blebbing, axonal growth cone collapse, and cell polarity (reviewed in [186]) TNFα may be responsible for the promotion of fatty liver by alcohol consumption [187], and, emerging evidence suggests that certain TNFα alleles may increase predisposition to alcoholic liver disease [188]. Additionally, TNFα plays a key role in driving liver regeneration [189], and in particular, activation of NFκB downstream of TNFα signaling seems to be critical in the regenerative response [190, 191].

The NFκB arm of TNFα signaling possesses additional relevance to liver biology. NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a family of transcription factors that regulate immune response in the context of various types of cell stress that include cytokine signals, UV radiation, oxidative stress, and infection. NFκB proteins are regulated by the constitutive binding of the inhibitory protein IκBα, which can be phosphorylated by IκB kinase (IKK), ubiquitinated, and proteasomally degraded to activate NFκB in response to cell stress. NFκB is then free to translocate into the nucleus and promote expression of target genes involved in stress response, including cytokines, chemokines, p53, pro-survival and pro-apoptosis proteins [192]. The NFκB protein family consists of five members (RelA/p65, RelB, c-Rel, p105/p50 and p100/p52) that contain a Rel homology domain (RHD) involved in dimerization, DNA binding, and nuclear localization [193].

Mice lacking the NFκB family protein, p65, die at embryonic day 16 with massive liver degeneration due to apoptosis, pointing to a key role for the NFκB pathway in late fetal liver development [194]. At this time in development, the liver increases its size threefold owing to a massive rise in hepatocyte proliferation and a high resistance to apoptosis, though it is unclear whether this resistance is mediated by NFκB transcriptional activity [195]. Studies utilizing conditional p65 knockouts report that it is not critical in postnatal development, though it does confer resistance to TNFα induced apoptosis [196].

Given the importance of NFκB proteins in regulating cell survival in the context of inflammation and stress, it is perhaps unsurprising that they can be oncogenic [197]. Inflammation is a significant contributor to the development of HCC [198], and sustained activation of NFκB activity is observed in many human HCC patients. However, interestingly, both overactivity and inhibition of NFκB contribute to HCC in mouse models [199–201].

Conclusion

Underlying every physiological process are signal transduction events that allow cells to respond to changes in their internal or external environment by modifying cell behavior. Such pathways control physiological liver processes, such as specification, growth, differentiation, metabolism, and homeostasis, but also are implicated in disease states such as cancer and fibrosis. This introduction gives a broad overview of some major signal transduction pathways and their relevance to liver biology, to serve as a foundation for the more in depth discussions to follow.

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Part III
Applied Liver Biology

Chapter 21
Hepatocyte Transplantation

Mirela-Patricia Sirbu-Boeti, Kyle Soltys, Alejandro Soto-Gutierrez, and Ira J. Fox

Introduction

The scientific foundation for clinical hepatocyte transplantation was developed over the last 40 years by extensive laboratory work in animals. The first attempts at human hepatocyte transplantation were performed by Mito et al. who used autologous hepatocytes to treat patients with chronic liver disease [1]. Habibullah et al. [2] later transplanted allogeneic fetal hepatocytes to treat patients with fulminant hepatic failure. The first attempted treatment of a metabolic disorder was performed in patients with familial hypercholesterolemia using autologous retrovirus-transduced hepatocytes by Grossman et al. [3]. Several years later, allogenic hepatocytes were used to treat a different metabolic liver disorder with some success in a patient with Crigler-Najjar syndrome type I [4].

While modest success has been documented in treating metabolic disease, data concerning the efficacy of hepatocyte transplantation for hepatic failure in humans have been difficult to interpret. Although clinical experience has failed to show that transplanted cells lead to liver regeneration, as found in animal experiments, hepatocyte transplantation remains partially effective as a treatment to bridge patients to orthotopic liver transplantation, potentially reducing the risk of organ transplantation in patients with clinical complications. Although the numbers of patients treated and the lack of controlled clinical trials are limited, liver cell therapy has been applied in many medical centers around the world [5]. While hepatocyte transplantation has proven beneficial to some degree, development and application in humans is also constrained by the availability of human hepatocytes. Before hepatocyte transplantation can become conventional therapy for liver failure or liver-based metabolic disorders, several additional hurdles must be overcome.

I.J. Fox (✉)
Department of Surgery, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: foxi@upmc.edu

Indications for Human Hepatocyte Transplantation

Hepatocyte transplantation has been used as an alternative therapeutic option for patients with liver-based metabolic diseases. Inborn errors of metabolism are rare disorders with a collective incidence of one in 1,500 persons [6]. Normally, such patients must adhere to very specific and limited diets and may require additional interventions and medications throughout the day to avoid metabolic crises and chronic complications. Medical interventions may also affect the quality of life. The only definitive treatment available at this time is orthotopic liver transplantation, which offers up to 90% long-term survival in children with metabolic diseases [7]. However, orthotopic liver transplantation still has associated risks of morbidity and mortality [5]. Inborn errors of metabolism have been an important target for liver cell therapy due to the less variable clinical condition of the patients and the availability of objective measures to unequivocally determine the efficacy of treatment. Only rarely is the situation immediately life threatening and there are often-acceptable moderating therapies so that transplantation can be performed electively.

Acute or fulminant liver failure is a rapidly progressive condition where the only effective therapy is liver transplantation. Hepatocyte transplantation has been considered as an experimental option for these patients as well. Viral hepatitis, idiosyncratic drug reactions, acetaminophen, and mushroom ingestion are common causes of acute liver failure although on many, if not most, occasions the cause is unknown. Acute liver failure can also be encountered in patients following extensive liver resections where a marginal hepatocellular mass is left to sustain the metabolic needs of the patient [8, 9]. It can occur following a relatively small liver resection, after any procedure in a patient with preexisting liver disease, or it can occur in patients suffering primary allograft nonfunction after liver transplantation (Table 21.1) [10–12]. Delay in availability of a functioning new-liver allograft, rapid deterioration of the patient, and the existence of contraindications for re-transplantation, lead to

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_21, © Springer Science+Business Media, LLC 2011

Table 21.1 Human liver cell transplantation

Liver disease	No. of patients	Injection of hepatocytes	Type of transplantation	Results	References
Metabolic					
Familial cholesterolemia	5	Intraportal	Autotransplant. Gene therapy (transduction with LDL receptor gene)	Cholesterol decreased in patients, transgenic expression <5% ≥4 months	[3]
α1 AT-deficiency	1	Intraportal	Allotransplant	OLT after 4 days	[10]
Crigler Najjar type I	5	Intraportal/ hepatic artery	Allotransplant	Decrease of serum bilirubin. Patients ultimately required OLT or APOLT	[4, 30, 43, 69, 96]
Urea cycle defect	5 OTC deficiency	Intraportal/ umbilical vein	Allotransplant	Decrease of ammonia levels, improvement of neurological outcome for up to 1 year; most patients underwent OLT or APOLT	[17, 54, 97, 98]
	1 ASA lyase deficiency	Intraportal	Autotransplant	Metabolic control was achieved together with psychomotor catch-up	[99]
Glycogen storage 1a	1	Intraportal	Allotransplant	Satisfactory correction of hypoglycemia, 30–40% decrease of blood triglyceride concentration for more than 9 months	[5, 57, 94]
Factor VII deficiency	3	Intraportal	Allotransplant	Reduced administration of exogenous Factor VII for up to 6 months. Patients ultimately required OLT	[100, 101]
Infantile refsum disease	1	Intraportal	Allotransplant	Total bile acids and abnormal dihydroxy-coprostanoic acid markedly decreased in the patient's serum, pipecholic acid decreased by 40%	[31]
Progressive familial intrahepatic cholestasis	2	Intraportal	Allotransplant	No clear benefit was seen. OLT	[39]
Acute liver failure					
Drug induced	20	Intraportal, intraperitone-alIntrasplenic (splenic artery)	Allotransplant	Ammonia reduction, improvements in encephalopathy. One patient with complete recovery after intraportal cell transplantation alone and two patients with complete recovery after intraperi-toneal transplantation with fetal hepatocytes	[2, 5, 60, 98, 102]
Viral	8	Intraportal, intraperitone-alIntrasplenic (splenic artery)	Allotransplant	One patient completely recovered after cell transplantation alone	[5, 10, 60, 74]
Idiopathic	6	Intraportal, intraperitone-alintrasplenic (splenic artery)	Allotransplant	Significant ammonia reduction, measur-able improvements in encephalopathy. Two patients with complete recovery. One patient died at day 1 posttransplant	[2, 5, 60, 102]
Mushroom Poisoning	1	Intraportal	Allotransplant	Full recovery	[103]
Posthepatectomy	1	Intrasplenic (splenic artery)	Allotransplant	Patient died at day 2 posttransplant	[10]
Acute fatty liver during pregnancy	1	Intraperitoneal	Allotransplant	Complete recovery within 7 days	[104]
Chronic liver failure					
Cryptogenic	1	Intraportal	N/A	Decreased levels of ammonia. OLT at 1 week	[5]
Idiopathic fibrosis	1	Intraportal	N/A	Decreased levels of ammonia and encephalopathy score. OLT at 6 weeks	[5]

(continued)

Table 21.1 (continued)

Liver disease	No. of patients	Injection of hepatocytes	Type of transplantation	Results	References
Total parenteral nutrition and sepsis	1	Intrasplenic (splenic artery)	Allotransplant	Hyperammonemia and encephalopathy improved. The effect was transient	[56]
α1 AT-deficiency	2	Intrasplenic (splenic artery)	Allotransplant	Significant decrease of hyperammonemia. OLT at day 2 and 4	[10, 56]
HCV	1	Intrasplenic (splenic artery)	Allotransplant	Unclear	[60]
Ethanol	5	Intraportal/ intrasplenic (splenic artery)	Allotransplant	Unclear	[10, 60]
Multicausal cirrhosis	10	Intrasplenic (splenic pulp)	Autotransplant	No clear benefit	[1]

OLT orthotopic liver transplantation; *APOLT* auxiliary partial orthotopic liver transplantation; *HCV* hepatitis C virus; *α1 AT-deficiency* alpha-1 antitrypsin deficiency; *OTC* ornithine transcarbamyl; *ASA* argininosuccinate; *ICH* intracranial hypertension

death in these patients [13]. Hepatocyte transplantation may provide rapid support for such failing livers, providing time for either complete recovery, or as a bridge to organ transplantation.

The scenario associated with end-stage chronic liver disease is more problematic. Besides functional hepatocyte abnormalities, the hepatic architecture contributes to the altered liver function. Moreover, intrahepatic portal-to-portal venous shunts may prevent efficient exchange of oxygen and nutrients to hepatocytes. Thus, the benefit of transplanting hepatocytes into the liver without restoring normal liver architecture is questionable.

In general, the potential advantages of hepatocyte transplantation over orthotopic liver transplantation include the following:

- Preservation of the native liver.
- Reversibility of the cellular transplant, should complications of immune suppression occur.
- Lack of interference with subsequent liver transplantation if needed.
- Simplicity of the minimally-invasive procedure and low procedural morbidity and mortality.
- Lower cost.
- Availability of unused cadaveric donor organs or resected hepatic grafts from reduced liver transplants as sources of hepatocyte grafts.
- Possible use of small numbers of hepatocytes from multiple donors for a single recipient.
- Possible use of hepatocytes isolated from a single liver for multiple recipients.
- Potential for hepatocyte banking for later use.
- The possibility of performing repeat hepatocyte transplantation with low morbidity.
- The possibility of ex-vivo modification of hepatocytes with gene-targeted techniques.

Despite the numerous advantages to cell therapy for liver diseases, there are important issues that need to be overcome: the lack of predictability in hepatocyte engraftment, function, and expansion following transplantation and apparent failure of long-term hepatocyte survival after transplantation.

Hepatocyte Preparation: Source, Isolation, and Quality

Cell Sources for Human Hepatocyte Transplantation

One of the major factors limiting the clinical advancement of human hepatocyte transplantation is a shortage of mature, functioning human hepatocytes. This is due to the extensive and growing use of whole livers for transplantation. Currently, donor hepatocytes are primarily obtained from livers rejected for orthotopic liver transplantation. Recent studies have shown that with current hepatocyte isolation protocols, it may be possible to obtain adequate numbers of viable human hepatocytes from many livers not suitable for whole liver transplant [12, 14, 15]. Livers are generally rejected for whole-organ transplantation based on biopsy findings of macrosteatosis, fibrosis, cirrhosis, and extended warm ischemic times [16]. Successful isolation of functioning hepatocytes from steatotic livers has been recently reported [14, 15]. To date, it has not yet been established whether unfractioned hepatocytes isolated from cirrhotic livers can be utilized for

hepatocyte transplantation, and further investigation is needed to determine the engraftment and functional capabilities of cells recovered from cirrhotic livers [5]. Autotransplantation of hepatocytes recovered from resected sections of the liver has been reported in an attempt to treat liver failure from cirrhosis in the past [1].

Unused segments of livers from reduced grafts used in pediatric recipients may provide a useful, untapped source of hepatocytes [17]. An unlikely but possible future source of hepatocytes might also include cells recovered from liver segments resected for nonmalignant disease or for malignancy, if, at some point, complete removal of tumor cells can be accomplished by selection using magnetic beads [18]. More likely, organ perfusion techniques might be able to recover good quality hepatocytes in the near future from livers injured from prolonged warm ischemia [19].

Despite their high capacity to proliferate in vivo, hepatocytes cannot be cultured in vitro for long periods of time. Due to advances in telomere biology and aging research, prolonged hepatocyte cultures have been accomplished using genetically modified, nontumorigenic, immortalized hepatocyte cell lines [20–22]. Reversible immortalization has been accomplished using the Cre-lox recombination and excision system using genes encoding hTERT and the SV40 large T antigen [21, 23]. The disadvantage of these immortalized cells for clinical application is poor differentiated function [24] and high tumorigenic potential [25].

Fetal liver cells have also been proposed as an alternative source of functional hepatocytes for transplantation and it is discussed in greater detail in Chap. 16 [2, 26]. Selection of fetal cells for transplantation has been accomplished in animals by sorting for liver progenitor cells [27]. Fetal liver cells appear to have some advantages for transplantation compared to adult liver cells: high proliferation capacity, potentially diminished immunogenicity, high capacity for integration in the liver, and greater tolerance for cryopreservation [24, 28, 29]. However, their metabolic functions may be less mature when compared to freshly isolated human hepatocytes and they may be more useful for treating specific inborn errors of metabolism [24]. Since fetal hepatocytes for clinical use would have to come from aborted fetuses, which are performed in the vast majority of cases during the first trimester of pregnancy, the engraftment potential and function of early gestational age fetal liver cells will need to be investigated. To this end, a 1-year-old-girl with biliary atresia was recently treated by hepatic progenitor cell infusion through the hepatic artery. Following transplantation, the bilirubin in this child decreased and hepatobiliary scintigraphy showed improved liver cell function [30]. Whether this improvement is related to cell therapy will need to be determined by further prospective controlled studies.

Finally, based on recent scientific advances, autologous cells could become a source of hepatocytes, should hepatocytes become safely generated in vitro from stem cells. Mesenchymal stem cells have shown potential for hepatic differentiation [31] and sustained efforts are now being directed toward establishing improved efficiency in generating induced pluripotent stem cells and differentiation of induced pluripotent cells into hepatocytes [32].

Hepatocyte Isolation and Storage

A standardized protocol of hepatocyte isolation is the mainstay for large-scale clinical application of liver-cell therapy. Since first used in 1969, collagenase perfusion has remained the cornerstone of hepatocyte isolation from whole livers [33, 34]. The traditional two-step collagenase perfusion technique has been further developed aiming to improve hepatocyte viability [12]. Enzymatic digestion of the liver yields a mixture of cells containing approximately 90% hepatocytes and 10% nonparenchymal cells. The cells are then passed through a mesh and centrifuged at low *g*-forces to isolate only the hepatocytes. In contrast to "regular" hepatocytes, which cannot be expanded in primary culture, small hepatocytes have been reported to have a higher potential for proliferation. However, their utility is limited by their small numbers [35].

A three-step perfusion procedure has been reported to yield an average of 2.6×10^6 viable cells per gram of whole liver tissue [12, 36]. Cell yield from infant donor livers has been considerably higher; however, the relationship between donor age and isolation efficiency has not been consistent [37, 38]. While some authors have found no correlation between hepatocyte viability and cold ischemia time, reducing both cold and warm ischemia time before processing has proven to be beneficial by others [37, 39]. Donor variables that can negatively impact hepatocyte viability include use of non-heart-beating donors [39] and severe steatosis [38]. Using current techniques, isolation of hepatocytes from cirrhotic livers is very inefficient [12]. Special, hand-made multiperfusion systems may be required for successful hepatocyte isolation from resected liver segments, which can yield approximately 10^6 human hepatocytes per gram of tissue with viability from 55 to 86% [40].

Like whole-liver transplantation, transplantation of fresh hepatocytes must be done as soon as possible after isolation, since metabolic function and engraftment efficiency deteriorates even at 4°C. Cryopreservation and storage of isolated hepatocytes have been described in the literature [41, 42]; however, viability and engraftment of thawed cells have been inconsistent and less than that of freshly isolated hepatocytes [43–45]. Current research also focuses on augmenting hepatocyte viability after isolation. The isolation procedure exerts significant stress on cells by disrupting cell–cell and

cell–matrix interactions. Use of antiapoptotic molecules and matrices has been investigated with mixed success [46].

Although hepatocyte transplantation has been performed with cell viability as low as 50% [13], it is generally preferred that transplantation be performed only if cell viability is greater than 60% [39]. This level of viability maximizes the functional hepatocyte mass and minimizes portal vein induced pressure changes.

Assessment of Hepatocyte Quality for Transplantation

Isolation, cryopreservation, and storage procedures require conditions of "good manufacturing practice" for clinical application. This requires use of HEPA filters, positive pressure rooms, and aseptic handling of tissues and cells using comprehensive quality control processes. Organ recovery, by definition, is a clean-contaminated procedure that can result in contamination by organisms in the bile and upper digestive tract. In one study, bacterial contamination after organ recovery was detected 37.5% of the time in the preservative solution used to transport donor livers into cell isolation units [47]. After tissue processing, this contamination was reduced to approximately 5% in the final hepatocyte product. The most common organism recovered was coagulase-negative staphylococci, a skin contaminant. Preparations of these hepatocytes were used for cell transplantation with no evidence of sepsis in recipients [47].

Cell viability, attachment efficiency, and metabolic testing have been used as objective criteria for assessment of isolated hepatocyte quality. The viability of hepatocyte suspensions is usually estimated by vital staining with trypan-blue exclusion. Overnight plating efficiency of isolated hepatocytes can also be used as an estimate of viability, and has been associated with engraftment potential [48]. DNA content of plate-attached cells after 50 min roughly quantifies adherence capacity to fibronectin/collagen-coated dishes [49], and metabolic function can be assessed by urea and albumin synthesis, drug-metabolism, MTT assay, and other measures of protein synthesis [48]. Cytochrome P450 activity by isolated hepatocytes can be assessed following a 30-min incubation with a cocktail of selective substrates (CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A4) [49]. However, these tests have not been used reliably to predict the percentage of hepatocytes that engraft [50]. While not used routinely, retrospective assessment of engraftment following transplantation of an aliquot of fresh donor cells is probably the most accurate measure available to assess the quality of the cell preparation. Unfortunately, this information is only available after the decision to transplant cells has already been made.

Enhancement of hepatocyte function can theoretically be accomplished with genetic manipulation of the cells prior to infusion into the recipient. Transplantation of genetically modified autologous hepatocytes was evaluated in a clinical trial of patients with familial hypercholesterolemia. After isolation from the left lateral sector of the patient's own liver, LDL-receptor gene deficient hepatocytes were genetically modified in vitro using a replication-defective recombinant virus that transferred expression of the gene encoding the LDL receptor. The genetically modified hepatocytes were then transplanted back into the patient. Unfortunately, the results of this first human trial of liver-directed gene therapy were limited by low expression of the transferred gene [51].

Hepatocyte Transplantation: Location and Engraftment

It is estimated that the human liver consists of approximately 250×10^9 cells, organized into 10^6 hepatic lobules, each containing approximately 250,000 liver cells. Because 65–70% of these cells are hepatocytes, the estimated total number of hepatocytes in a human liver is thought to be approximately 175×10^9 cells [52]. For a single enzyme deficiency, such as that found in Crigler-Najjar syndrome, a relatively small number of hepatocytes may be needed to improve the bilirubin level. It is believed that the number of transplanted hepatocytes should represent 5–10% of the liver cell mass ($200–400 \times 10^6$ cells/kg, body weight). It is also estimated that 5–20% of the liver mass may be required to support liver failure in adults, or $10–15 \times 10^9$ cells [18]. Unfortunately, the actual hepatocyte volume required to restore enzyme activity or reverse liver failure is largely unknown.

The liver appears to be the most accommodating site for hepatocyte transplantation due to its unique hepatic organization and its mechanism for biliary drainage, access to hepatotrophic factors, and interaction with nonparenchymal liver cells. Injection of hepatocytes directly into the portal vein has been used in patients with a normal hepatic structure, a situation encountered in patients with inborn metabolic liver diseases and those with acute liver failure. There are different methods of accessing the portal circulation: (1) percutaneous transhepatic portal-vein catheterization; (2) transjugular transhepatic portal-vein catheterization; (3) insertion of a catheter through a patient's umbilical vein (Fig. 21.1); and (4) open insertion of a catheter through a branch of the mesenteric vein [53–55]. Investigators have shown that immediately after injection into the portal vein, transplanted hepatocytes are deposited in the portal region and the sinusoidal spaces. However, the capacity of the sinusoidal spaces limits the number of hepatocytes that can be transplanted. Therefore, direct hepatocyte delivery into the portal system

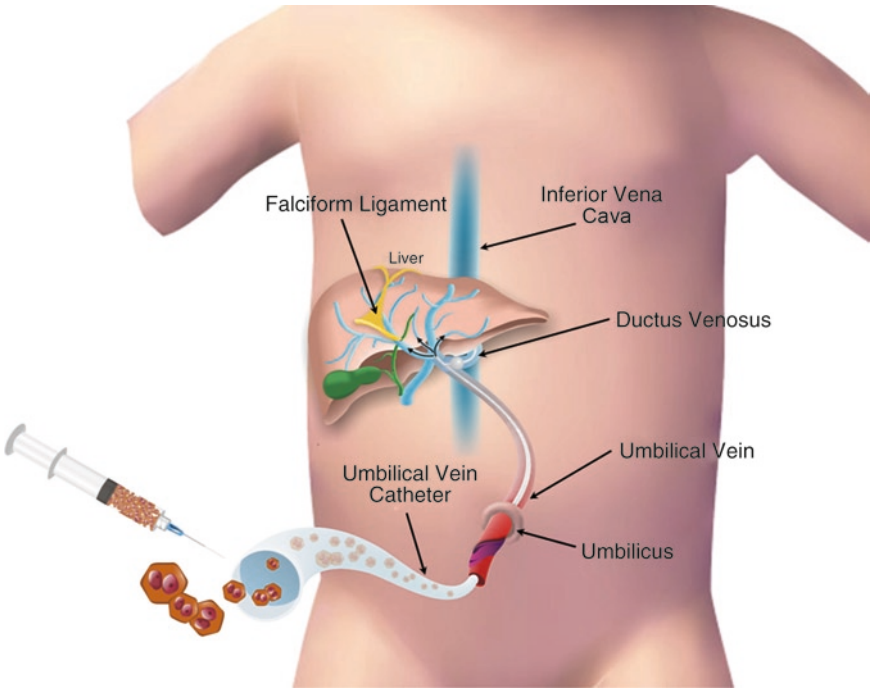

Fig. 21.1 Hepatocyte transplantation through the umbilical vein. In an infant, access to the portal circulation can be obtained through the umbilical vein. The vein extends from the umbilicus to the left portal vein. To transplant hepatocytes, a catheter is placed through the umbilicus and passed into the portal circulation. If patent, the ductus venosus needs to be temporarily occluded during the hepatocyte infusion to avoid translocation of cells through inferior vena cava into the systemic circulation

can induce portal hypertension and portal-vein thrombosis [4, 56–58]. The risk of portal-vein thrombosis after cell infusion is higher in parenchymal liver disease than in metabolic disorders due to the associated inflammation and fibrosis with resultant lower portal blood flows [11].

To minimize the risk of portal hypertension and thrombosis, heparin can be added to the cell suspension and the number of cells should be limited to approximately 100×10^6/kg of body weight [59]. Portal-vein pressure and flow monitoring should be carried out during the infusion with prompt discontinuation of infusions until portal pressures return to near baseline. A moderate increase in pulmonary pressure and transient pulmonary dysfunction with hypoxemia has been reported in one patient who received hepatocytes. This likely represented secondary hepatocyte translocation into the lung parenchyma [11]. Examination of lung tissue in the laboratory has confirmed the presence of hepatocytes in the alveoli and blood vessels following intraportal infusion; however, macroscopic infarction has not been documented [13, 60]. A significant percentage of the liver mass can be infused over a prolonged period of time and several investigators have used multiple hepatocyte infusions to maximize the total number of cells engrafted. Questions about the timing of repeat infusions and the efficacy of infusions over a period of time have yet to be answered.

Following infusion through the portal vein, most hepatocytes undergo blood-flow-mediated translocation to the hepatic sinusoids. Entrapment within periportal sinusoids is mediated by passive sinusoidal occlusion and receptor-mediated interactions between the transplanted hepatocytes, hepatic endothelial cells, and hepatic matrix cell-surface protein interactions with sinusoid matrix ligands [61]. Hepatocytes are very large in comparison to the sinusoid lumen, thus, entrapment of the donor cells in the sinusoids takes place. The hepatocytes then integrate into liver parenchyma by disrupting the sinusoidal endothelium and entering the space of Disse. Translocation of transplanted hepatocytes into liver cords and restoration of the normal sinusoidal barrier requires up to 7 days [62]. It is believed that only a fraction of transplanted cells survive the early posttransplantation period and a maximum of 20–30% of transplanted hepatocytes, under ideal conditions, ultimately engraft into the liver parenchyma [58]. Pharmacologic disruption of endothelial integrity and sinusoidal dilatation has been shown experimentally to improve cell engraftment [63, 64]. Once infused hepatocytes engraft, they must become functional. One study found hepatic function as early as 48–72 h after transplantation, based on significant improvement in aminopyrine and caffeine clearance [13]. While immediate hepatocellular function is not essential after hepatocyte transplantation in

patients with liver-based metabolic disease, lack of immediate function could be problematic in patients with acute liver failure.

Cirrhosis limits hepatocyte engraftment because of its abnormal circulation to regenerative nodules, sinusoidal endothelial changes that result in an increased endothelial barrier for donor hepatocyte engraftment, intra- and extrahepatic portosystemic shunts that permit translocation of hepatocytes into the pulmonary circulation, and portal hypertension that increases the risk of portal thrombosis [28]. Investigators have injected hepatocytes into the splenic artery via a transfemoral hepatic artery catheterization in the face of cirrhosis with limited success [5, 13].

In most human studies of hepatocyte transplantation, donor cell engraftment appears to approach only 1% of the liver mass. However, in animal models, replacement of >90% of host hepatocytes can be accomplished by using a relatively small number of transplanted cells in a process referred to as "liver repopulation" [65]. In this setting, the transplanted cells have a growth advantage over endogenous hepatocytes. Several laboratories have developed strategies to induce preferential proliferation of engrafted hepatocytes in settings where this may not occur naturally [66]. An approach that might be developed for clinical application, consists of preparative hepatic irradiation, which reduces mitotic capacity of the host hepatocytes [67, 68] and treatment with factors that promote hepatocyte expansion. Inhibition of hepatocyte cell cycle with hepatic irradiation appears to increase liver repopulation by transplanted hepatocytes without affecting native hepatocellular function.

Injection of hepatocytes into the peritoneal cavity is simple and has been considered an option in patients where transplantation into the liver is not feasible or might be dangerous. This is because the peritoneal cavity is easily accessible, can accommodate a large number of cells, and there is no risk of embolism of hepatocytes to the lungs [2, 13, 56, 69]. Habibullah treated a number of patients with acute liver failure by intraperitoneal transplantation with some success [2]. Unfortunately, hepatocytes engraft poorly unless they are encapsulated or microencapsulated in semipermeable membranes [70–72]. Whether this approach will be utilized with success using techniques allowing hepatocyte attachment to the peritoneum remains unanswered at this time.

Posttransplantation Hepatocyte Engraftment and Immune Response

When hepatocytes are transplanted into ectopic locations, such as the spleen, definitive histologic evidence of transplanted hepatocytes can be obtained and nuclear scanning can help confirm the presence of engrafted hepatocytes. Engraftment in the liver cannot be easily assessed histologically or by nuclear studies at this time. Hepatocytes isolated from male donors and transplanted into female patients may be detected on liver biopsy by fluorescent in situ hybridization (FISH) [13]. Real time PCR analysis of the Y chromosome is also useful and permits detection as low as 0.25% engraftment [73]. DNA extraction from liver biopsies and assessment of donor and host cells can be accomplished by similar nucleic acid determination [74]. For liver-based metabolic disease, measurement of enzyme activity on liver biopsies can be definitive for documenting hepatocyte engraftment.

Similar to vascularized allografts, allogenic hepatocytes are subject to both antibody and cell mediated rejection. Gene products of the major histocompatibility complex are the primary targets involved in the host immune response. Presentation of these alloantigens on the surface of antigen presenting cells is an early event in the recognition and proliferation of alloantigen-specific lymphocytes and production of antibodies [75, 76]. Unstimulated hepatocytes do not express class II major histocompatability complex antigens, but hepatocyte preparations may occasionally be contaminated by immunogenic antigen presenting cells or Kupffer cells [77]. In cases of viral hepatitis, hepatocytes can express aberrant major histocompatability complex class II antigens and can function as antigen presenting cells [78].

Transplantation of hepatocytes from animals that differ genetically at the major histocompatability complex results in sensitization and rapid disappearance of allografts in 4–7 days [79]. Hepatocytes are rejected by $CD4^+$ and $CD8^+$ mediated immune responses and also destroyed by the innate immune system [80]. Granulocytes and Kupffer cells destroy even syngeneic hepatocytes early after transplantation [81] and granulocytes have been documented as surrounding transplanted cells 24 h after their infusion [82]. Portal microvascular occlusion by cell emboli also induces ischemic injury, oxidative stress, and impaired cell viability, which cause both a local inflammatory response and subsequent stimulation of Kupffer and stellate cells, resulting in further cell loss. Survival of transplanted hepatocytes has been augmented with granulocyte-depletion and pretreatment with gadolinium chloride, which significantly impairs Kupffer-cell function [83].

Although infusion of allogenic or xenogeneic cells through the portal circulation has been shown to induce donor-specific tolerance in rodents and pigs [84, 85], this tolerance has not been readily duplicated following infusions containing hepatocytes. This may be due to the fact that hepatocyte preparations do not contain significant numbers of antigen presenting cells [86]. Whether cotransplantation with donor bone marrow cells and transient immunosuppression, which induces tolerance in some studies, will allow intraportally transplanted hepatocytes to induce tolerance is not known. The immune response to cellular grafts has also

been altered by gene transfer using recombinant adenoviruses containing the genes encoding immunosuppressive cytokines. Transduction of donor cells with a number of genes has effectively blocked allograft and xenograft rejection [87]. Adenovirus mediated expression of CTLA4Ig, a fusion protein of the extracellular domain of CTL4A and the Fc portion of the IgG2a molecule, downregulates the immune response to cellular grafts by costimulatory blockade [88, 89]. Unfortunately, there is little understanding of the mechanisms responsbile for rejection of transplanted allogenic hepatocytes in humans, making optimization of immunosuppressive regimens difficult.

The most common immunosuppressive medications used for solid organ transplantation consist of calcineurin inhibitors (CNI), such as tacrolimus or cyclosporine, and steroids. Conventional immunosuppression with cyclosporine, azathioprine, tacrolimus and antilymphocyte serum can prolong donor hepatocyte survival in laboratory animals [90]. Induction therapy using IL-2 receptor antibodies (e.g., basiliximab, daclizumab) [91] or antithymocyte globulins (e.g., ATG Fresenius or Thymoglobulin) may also be considered. Depending on the patient's underlying disease and existing comorbidities, minimal immunosuppressive regimens are favored. In addition, reduction or replacement of CNIs may be possible using agents such as mycophenolate mofetil (MMF) or sirolimus [92]. As there are limited data regarding the immunosuppression required for hepatocyte transplantation, it is not known whether requirements after hepatocyte transplantation may be different than those required after solid organ transplantation.

Finally, unlike whole organ transplantation, transplantation of hepatocytes does not easily allow real-time modulation of immunosuppression because there are no current techniques available to document rejection of hepatocytes. The utility of liver biopsy in hepatocyte transplantation is limited by the consequences of sampling errors. In addition, documentation of donor-hepatocyte loss leaves few therapeutic options available for reversal of the rejection process. Cessation of immunosuppression may be indicated in patients who received hepatocyte transplantation and completely recovered from acute liver failure. For these patients, withdrawal of immune suppression will depend on liver histology that indicates recovery of host hepatocellular mass [74].

Overview of Current Clinical Experience

Promising results with hepatocyte transplantation in preclinical animal models of human disease have led investigators to attempt to reproduce these results in humans. In clinical trials, hepatocyte transplantation has offered some measurable clinical benefit in a variety of settings [93].

Studies in patients with acute liver failure and chronic liver disease have been difficult to interpret. Some studies have demonstrated modest improvement in neurologic and biochemical parameters, but there has been limited evidence of altered survival (Table 21.1). In addition, if hepatocyte transplantation proves to be effective in improving the physiologic abnormalities associated with chronic liver disease, portal hypertension will need to be controlled by surgery or TIPS, and surveillance for the development of hepatocellular carcinoma will need to be augmented.

Due to the high variabililty in the natural history of acute liver failure, evaluation of the success of potential therapies in this population is difficult. Prognosis can be affected by the multiple etiologies, confounding standard support treatments, and spontaneous recovery rates approach 40% [94]. Although there is some suggestion that the transplanted hepatocytes provided a benefit to some patients, convincing evidence for engraftment and function of transplanted cells has not been obtained. Part of the problem could be that relatively small numbers of liver cells were transplanted in many of the patients.

Treatment of liver-based metabolic disease with hepatocyte transplantation has met with more success (Table 21.1). Immediate correction of metabolic activity as a result of transplantation in these patients has been documented in a variety of diseases. After 4–8 weeks, metabolic and clinical effects have been reported and have been observed for up to 2 years after cell transplantation [5]. However, for long-term engraftment and function, transplanted hepatocytes may require a selective survival advantage over the recipient's cells. In many metabolic diseases, the liver-based deficiency does not influence the survival of host hepatocytes and transplanted hepatocytes cannot be expected to survive better than host cells. For other metabolic liver diseases, such as Wilson's disease, where there is a defect in the copper transporting ATPase ATP7B protein that causes copper to accumulate and leads to deterioration of hepatocytes [95], transplanted wild-type cells would have a selection advantage over the recipient's hepatocytes. In most settings, donor cells will not have a survival advantage over host hepatocytes, and strategies, such as preparative hepatic irradiation, may be required to improve outcome [68].

Conclusions

Laboratory studies and clinical trials spanning several decades have demonstrated that hepatocyte transplantation has great potential for the correction of a variety of liver disorders. An improved understanding of normal hepatocyte turnover, normal hepatic regeneration after injury, techniques to improve hepatocyte isolation, cell delivery, and hepatocyte engraftment will help to improve clinical translation.

Additional cell sources continue to be sought. Controlled clinical trials in patients with liver failure must contain standardized regimens for assessment of donor hepatocyte quality, delivery techniques, and immune-suppression regimens. Recipient characteristics need to be documented in detail as does the post transplant course. Documentation of engraftment in the liver must be recorded. For treatment of liver-based metabolic disorders, similar attention to standard approaches will be required, and for the most part, are in place. Additional efforts will be needed to understand impediments to long-term donor hepatocyte engraftment and function. This will need to be the major focus of future efforts. Coordinated effort of multiple investigators will be required to overcome the remaining hurdles before the enormous potential of this therapeutic strategy can be fully realized.

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Chapter 22
Hepatic Tissue Engineering

Jing Shan, Kelly R. Stevens, Kartik Trehan, Gregory H. Underhill, Alice A. Chen, and Sangeeta N. Bhatia

Introduction

Liver tissue engineering aims to provide novel therapies for liver diseases and create effective tools for understanding fundamental aspects of liver biology and pathologic processes. Approaches range from bio-mimetic in vitro model systems of the liver to three-dimensional implantable constructs. Collectively, these cell-based approaches endeavor to replace or enhance organ transplantation, which is the current standard treatment for liver diseases in most clinical settings. However, the complexity of liver structure and function as well as the limited supply of human hepatocytes pose unique challenges for the field. This chapter reviews advances in the field of liver tissue engineering within the context of current therapies for liver diseases, and clinical alternatives such as cell transplantation strategies and extracorporeal bioartificial liver devices.

Current Treatments for Liver Failure

Liver failure, representing the cause of death for over 40,000 individuals in the United States annually [1], can result from acute or chronic end-stage liver diseases. Current treatments for liver failure include administration of fluids and serum proteins, but these continue to be largely palliative. Liver transplantation is the only therapy proven to directly alter mortality, and therefore, remains the standard of care for liver disease patients. In order to maximize the therapeutic benefits of the limited supply of transplantable livers, a number of surgical techniques have been investigated, including the use of non-heart-beating donors or split liver transplants from cadaveric or living donors [2]. Partial liver transplants take advantage of the body's ability to regulate liver mass and the innate capability of mammalian livers to undergo significant regeneration [3]. However, although partial liver transplants have demonstrated some effectiveness, liver regeneration is difficult to regulate in clinical settings, and biliary and vascular complications are major concerns in these procedures [2]. Despite these surgical advances in expanding single donor livers into multiple grafts, the ballooning discrepancy between the number of livers available and the number of patients requiring liver transplants [4] indicates that organ transplantation alone is unlikely to fulfill the increasing demand for transplant-grade organs. Furthermore, patients who do receive transplants are subject to the costs and complications associated with major surgery as well as a lifetime of immunosuppressive regimens. Consequently, alternative approaches are actively being pursued. These include non-biological extracorporeal systems, such as hemoperfusion, hemodialysis, plasma exchange, and plasmapheresis over charcoal or resins [5–7]. They have shown only limited success, presumably due to the narrow range of functions supported by these acellular devices. Recapitulation of a more substantial number of the liver's purported 500+ functions will likely be needed to offer effective liver support.

Cell-Based Therapies

To provide the large array of known and currently unidentified liver functions, cell-based therapies have been proposed as an alternative to both liver transplantation and strictly non-biological systems [8]. These cell-based therapies range from approaches that provide temporary support, such as bioartificial liver (BAL) devices, to more permanent interventions, such as cell transplantation and implantable tissue engineered liver constructs (Fig. 22.1).

Extracorporeal devices primarily aim to offer transient support during liver regeneration or to serve as a bridge to transplantation. These devices process the blood of patients in a manner analogous to kidney dialysis systems. Substantial efforts have been invested in developing extracorporeal BAL devices containing hepatic cells to supply the multitude of

S.N. Bhatia (✉)
M.I.T., E19-502D; 77 Massachusetts Ave., Cambridge, MA 02139
e-mail: sbhatia@mit.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_22, © Springer Science+Business Media, LLC 2011

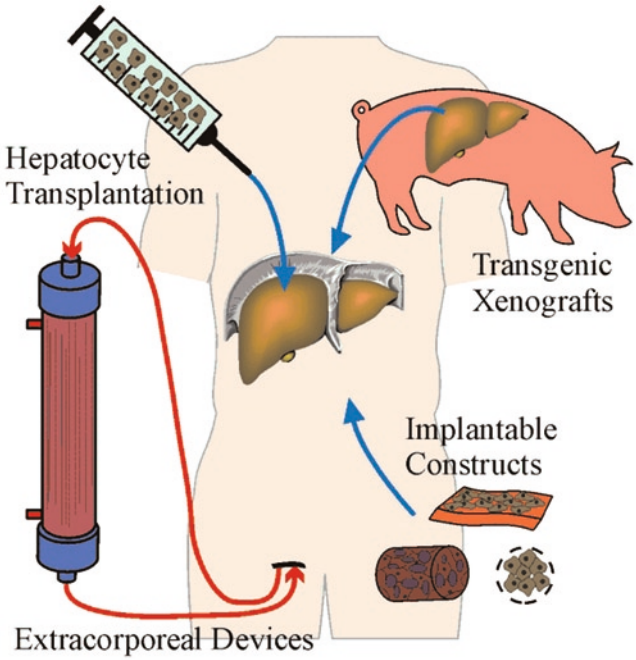

Fig. 22.1 Cell-based therapies for liver disease and failure. Extracorporeal bal devices provide temporary support by processing patient blood and plasma. more permanent interventions include tranplantation of isolated mature hepatocytes and hepatocellular constructs. Transgenic animal tissue have also been pursued. Reproduced from Allen et al., [5] used with permission

essential liver functions. There are four main categories of BAL devices [9, 10]: (1) hollow-fiber devices, (2) flat plate and monolayer systems, (3) perfusion bed or porous matrix devices, and (4) suspension reactors; each of these general designs exhibit innate advantages and disadvantages. Overall, a clinically useful BAL device must be scalable to therapeutic levels and exhibit key properties such as efficient bidirectional mass transfer and maintenance of cell viability and liver functions. Several BAL devices have been tested in clinical settings and researchers continue to improve device and trial designs. Ultimately, even if current BAL systems do not represent effective therapeutic options, information gained from these studies, along with advancements in cell sourcing and functional maintenance of hepatocytes *ex vivo*, promises to empower the next generation of devices.

In addition to temporary support, more permanent cell-based therapies are being actively developed to replace damaged or diseased liver tissue. One such approach is the transplantation of isolated hepatocytes, which has been demonstrated to be safe, and in some cases, effective, in both animal models and human trials [11–13]. A more comprehensive account of cell transplantation is provided in Chap. 21. Hepatocyte transplantation therapy is less invasive than organ transplantation [14] and could circumvent immunosuppressive regimens through the use of autologous cells. In rodent models, transplanted hepatocytes were further demonstrated to exhibit substantial proliferative capacity [12, 15–17]. This in vivo proliferation of transplanted cells is highly dependent on the presence of a "regenerative" environment, which can be provided by transgenic injury, partial hepatectomy (PHx), or the introduction of hepatotoxic agents prior to cell transplantation. The feasibility of hepatocyte transplantation is limited by the availability of appropriate cell populations, as only mature hepatocytes have been repeatedly and consistently shown to provide sufficient rescue of liver functions [18], and only organs deemed inappropriate for transplantation can be perfused to yield scarce supplies of these cells. This constraint of limited availability of highly functional hepatocytes is unfortunately universal to all cell-based approaches for liver disease treatment.

Another emerging therapeutic approach for liver failure is based on the development of implantable tissue engineered hepatocellular constructs. Similar to cell transplantation, this strategy relies on transplanted hepatocytes to perform liver functions. Tissue-engineering approaches further consider that hepatocytes are known to be anchorage-dependent; thus to maximize cell viability and functionality, hepatocytes are cultured *ex vivo* to form "organoids," immobilized on scaffolds, or encapsulated in aggregates prior to surgical implantation in a number of anatomical sites, including the spleen, liver, pancreas, peritoneal cavity and mesentery, and subcutaneous tissues [19, 20]. Proposed constructs have utilized scaffolds of various composition and architecture, both of which clearly influence hepatocyte survival and function. Despite advances in key aspects of hepatocyte maintenance in vitro, implantable systems remain largely experimental due to a number of obstacles that must be overcome before qualifying as a viable clinical modality. Specifically, hepatic tissue engineering shares many of the limitations of BAL devices and cell transplantation, but additionally faces challenges in establishing transplant vasculature and promoting transplant integration and remodeling. Details of these features will be discussed in later sections.

Cell Sourcing

Studies into cell-based therapies suggest great promise, but progress has been hindered by the propensity of hepatocytes to lose both phenotypic functions and the ability to proliferate in vitro [21, 22]. Thus, the continued elucidation of molecular mediators that regulate hepatocyte function and proliferation will be critical for the advancement of cell-based therapies and their routine use in clinics to treat compromised liver functions. In addition, the potential of alternative cell-sourcing approaches, based on stem cell differentiation and reprogramming, are active areas of investigation.

Mature Hepatocytes

Primary human hepatocytes are functionally the most robust cell type for cell-based therapies of liver diseases [8, 23]. Within their native microenvironments in vivo, human hepatocytes have phenomenal proliferative capability. Following resection of two-thirds of the liver through a surgical procedure known as PHx, the residual mature cell populations, comprised mainly of hepatocytes, are able to proliferate and restore lost liver mass [24]. Chap. 18 covers this topic in more detail. This full regenerative response can be seen after each of at least 12 sequential PHxs [25]. To demonstrate the clonogenic potential of the hepatocyte itself, mouse models were generated, in which livers were rendered incapable of supporting animal life through experimentally induced defects. Healthy hepatocytes injected into these compromised livers can proliferate, generate nodules of normal hepatocytes, and rescue the animals [26]. As low as 1,000 normal hepatocytes were found to be sufficiently therapeutic. Furthermore, cells from newly formed nodules of normal hepatocytes can be isolated and serially transplanted, through as many as four generations, to rescue other animals. Mathematical calculations based on this model predict that a single hepatocyte can undergo at least 34 cell divisions to give rise to 1.7×10^{10} cells, suggesting that a single rat hepatocyte can generate 50 rat livers of 300 million hepatocytes each [27].

Various attempts have been made in the last several decades to harness ex vivo this tremendous replication potential of mature human hepatocytes (Fig. 22.2). It is recognized that proliferating hepatocytes in vivo are presented a complex and dynamic mixture of soluble factors via the blood while maintained within an interactive support system of extracellular matrix (ECM) and non-parenchymal cells. Thus, early studies focused on providing select key components to in vitro culture systems, including humoral and nutritional supplements as well as ECM and supportive cell types [28]. To specifically promote hepatocyte expansion in vitro, primary cultures have been treated with serum and cytosol collected from livers that underwent PHx [29], and with more defined soluble factors including various growth factors [30, 31], sugars [30], amino acids [30], hormones [31, 32], vitamins [30, 33], serum proteins [30, 34], and trace metals [30, 34]. The effect of any individual supplement on hepatocyte proliferation can be difficult to determine directly, as the effect depends on the state of the hepatocyte, which is synergistically determined by the combination of all culture components [28]. Nevertheless, investigations have yielded a multi-factor media formulation, which can be used for moderate expansion of rat hepatocytes through a dedifferentiated bi-potential intermediate [30]. Non-soluble culture components such as different ECM [30, 35] and supportive cell types [35–38] have also been examined for mitogenic effects on hepatocytes. These include physiologic liver ECM proteins, and non-physiologic tumor-secreted protein mixtures in different configurations, in addition to co-cultures of hepatocytes with various intrahepatic and extrahepatic cell types, both live and dead. Many different combinations of culture components have been shown to support moderate expansion of rat hepatocytes although translation of these findings to human cultures has not been reported.

Human cells are critical for cell-based therapies due to substantial species-specific differences between animal and human hepatocellular functions including apolipoprotein expression, metabolic regulation of cholesterol, and phase I detoxification enzymes [39–41]. To overcome the growth limitations of primary human cells, investigations are underway to develop highly functional human-hepatocyte cell lines. A common approach is to introduce oncogenes through retroviral transduction. The Simian virus 40 tumor antigen gene (SV40 Tag) is a common immortalization agent, whose product binds to cell cycle regulator proteins Rb and p53 [42, 43]. Cell lines have also resulted from spontaneous immortalization of

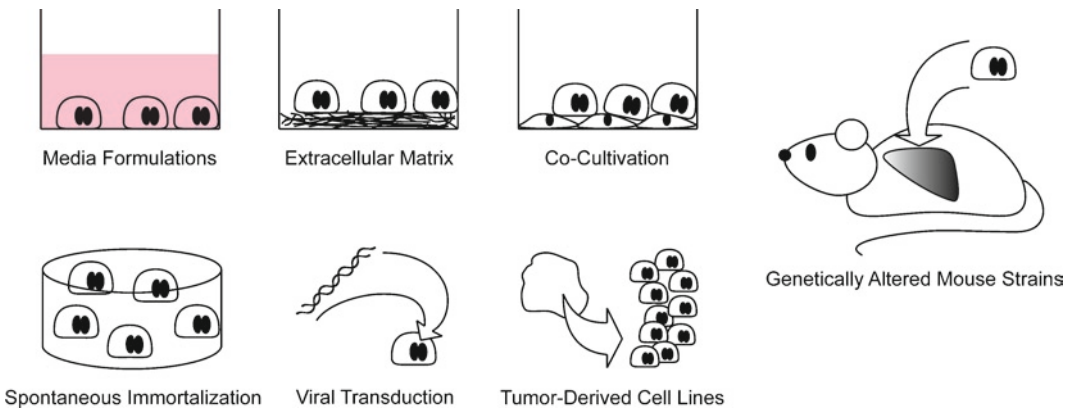

Fig. 22.2 Ex vivo expansion of mature human hepatocytes. Approaches have focused on providing essential culture components, generating hepatocyte cell lines, and leveraging in vivo regenerative environments

hepatocytes in co-cultures or collagen gel sandwich cultures [44], and additionally can be derived from liver tumors, as in the case of the HepG2 hepatoma cell line [45]. Although these cell lines are growth-competent, they introduce new safety concerns and typically underperform primary cells in terms of liver functions [46, 47].The principal safety concern is the transmission of oncogenic agents to the host, especially in the case of implanted cells. To address this, researchers have developed mechanisms to inactivate transduced oncogenes through temperature-sensitive SV40 Tag [48], Cre–*loxP*-mediated oncogene excision [49], and suicide genes such as herpes simplex virus thymidine kinase (HSV-tk) [50].

Another intriguing approach for human hepatocyte expansion, particularly as a model system, is the transplantation of human hepatocytes into genetically-altered mouse strains [17, 51–53]. This strategy takes advantage of the in vivo mitogenic environment, known to orchestrate many rounds of hepatocyte replication and can be generated through experimentally induced defects to host livers. Such defects can be produced by large amounts of urokinase, which can be abnormally over-expressed under the influence of the albumin promoter in hepatocytes [3, 12, 51, 54]. While effective as a hepatic xeno-repopulation system, these mice are fragile and present only a limited time window for transplantation. Alternatively, Grompe and colleagues have produced regeneration-inducing liver defects through an experimentally introduced deficiency in the catabolic enzyme fumarylacetoacetate hydrolase (Fah). After pretreatment with a urokinase-expressing adenovirus, Fah-deficient mice can be very receptive hosts to human hepatocytes [17]. Findings from these animal studies suggest that human hepatocytes do retain their considerable proliferation potential upon isolation and can expand given the appropriate stimuli. However, similar to the use of hepatocyte cell lines, the therapeutic utility of hepatocytes expanded in animal models is limited by safety concerns such as the transmission of pathogenic agents and the incorporation followed by expression of animal glycoproteins on human hepatocyte cell surfaces.

Ultimately, sustainable proliferation of highly functional human hepatocytes could generate patient-specific cell populations. These cells can be used to provide sufficient autologous materials for cell-based treatments, thus circumventing post-surgical immunosuppressive regimens. In vitro, the ability to expand human hepatocytes can enable drug therapies to be selected according to the characteristics of individual patients, thus minimizing adverse drug reactions.

Stem Cells and Progenitor Populations

Due to limitations in mature hepatocyte expansion in vitro, alternative cell sources are being pursued. These include various stem cell populations, which can self-renew in vitro and exhibit pluripotency or multipotency and thereby serve as a possible source of hepatocytes, as well as other non-parenchymal liver cells.

Studies have shown that embryonic stem cells can be induced to differentiate down the hepatic lineage in culture through the carefully orchestrated addition of various growth factors, and when supported by the appropriate ECM [55–57]. More recently, studies are also exploring in more scope and detail, the functional capacity of these differentiated populations, both in vitro and in vivo [58–60]. Such endeavors are being guided by improved insight into how different cell types are specified in embryonic development. This insight is typically gained through observations of cellular responses to individual inductive signals. Zaret and colleagues have further investigated how different inductive signals interrelate and have reported complex, dynamic signaling networks that could help explain incomplete cell programming in stem cell differentiation protocols [61].

In addition to embryonic stem cells, a wide range of fetal and adult progenitor cell types have been explored. Continuing investigations are focused on determining the differentiation potential and lineage relationships of these populations. Fetal hepatoblasts are liver precursor cells present during development that exhibit a bipotential differentiation capacity, defined by the capability to generate both hepatocytes and bile duct epithelial cells [62]. Furthermore, within the adult liver, a rare percentage of resident cells have been demonstrated to exhibit properties consistent with their designation as adult hepatic stem cells [63, 64]. It has been suggested that these cells represent precursors to adult progenitor cells, termed oval cells, which share phenotypic markers and functional properties with fetal hepatoblasts. In adult livers suffering certain types of severe and chronic injury, oval cells can mediate liver repair through a program similar to hepatic development [65, 66]. Various cell lines exhibiting characteristics comparable to fetal hepatoblasts and oval cells have been developed, e.g. lines derived from mouse E14 embryos by Weiss and colleagues. These bipotential mouse embryonic liver (BMEL) cells are proliferative, can be induced to be hepatocyte-like or bile duct epithelial-like in vitro [67], and can home to the liver to undergo bipotential differentiation in vivo within a regenerative environment [68].

Outside the liver, there may also exist multipotent stem/progenitor-like cells that are of therapeutic and biomedical interest [69]. For example, multipotent adult progenitor cells (MAPCs) derived from the bone marrow have been shown to generate hepatocyte-like cells in vitro [70]. Similarly, various mesenchymal stem cell preparations have been reported to give rise to cells exhibiting many characteristics of mature liver cells [71–74], including the ability to engraft in vivo; however, the extent of functional liver repopulation has been modest [69]. Other sources of extrahepatic liver cell progenitors include human amniotic fluid and membranes, which may contain cells capable of hepatic differentiation [75–79].

Reprogrammed Adult Cells

Fully differentiated adult cells, such as skin cells, were recently demonstrated to be reprogrammable to an undifferentiated, pluripotent state through forced expression of reprogramming factors Oct3/4 and Sox2, along with either Klf4 [80–83] or Nanog, and Lin28 [84]. These reprogrammed cells are termed induced pluripotent stem (iPS) cells and highly resemble embryonic stem (ES) cells, sharing many characteristics such as significant self-renewal capabilities in vitro and pluripotent differentiation potential. However, iPS cells offer an additional advantage of sourcing from adult somatic cells for the generation of patient-specific cell populations, potentially enabling therapies to be developed according to the characteristics of an individual patient. Work done by Duncan and colleagues, as well as other researchers, demonstrated that through iPS reprogramming and a subsequent multistep differentiation protocol, skin cells can give rise to hepatocyte-like cells, which not only exhibit a variety of hepatocyte-specific functions in vitro, but can also be induced to generate intact fetal livers in mice in vivo [85–87].

As a parallel strategy, work done by Melton and colleagues has demonstrated that it is also possible to directly reprogram one adult cell type into another, without an undifferentiated pluripotent intermediate. Similar to the use of master transcriptional regulators in the reprogramming to iPS cells, the expression of a key set of transcription factors in pancreatic exocrine cells in vivo induced conversion into cells that highly resemble β(beta)-cells [88]. These findings raise future possibilities for deriving hepatocytes directly from another adult cell type.

Ultimately, understanding the mechanisms governing the fates of stem and progenitor cell populations can empower the development of cell-based therapies. However, many challenges remain, including the ability to program differentiation completely. Furthermore, regardless of the cell source, phenotypic stabilization of hepatocytes ex vivo remains a primary issue. Accordingly, the development of robust in vitro liver models is an essential stepping-stone towards a thorough understanding of hepatocyte biology and improved effectiveness of cell-based therapies for liver disease and failure.

In Vitro Platforms and Applications

An important component of liver tissue engineering is the development of in vitro hepatocyte culture platforms. Such cultures can be used for applications aimed at studying fundamental hepatocyte biology, understanding and developing remedies for liver pathophysiology, and evaluating the liver metabolism and toxicity of pharmaceutical drug candidates. A summary of previously developed liver platforms is provided in Table 22.1. [89–104] When selecting a platform for a particular application, it is crucial to consider the necessary model criteria given the specific strengths and weaknesses of each approach.

Within these model systems, isolated primary hepatocytes are generally considered the most appropriate cell source; however, primary hepatocytes are notoriously difficult to maintain in culture due to a rapid decline in viability and liver-specific functions post-isolation [89–91]. Research has thus focused on providing the stimuli necessary to maintain the hepatocyte phenotype, and this research is gradually giving way to a systems-level picture of the molecular signals that furnish phenotypic stability of hepatocytes. In this section, we focus on methods that have been developed to stabilize

Table 22.1 Summary of liver platforms

Platform	Pros	Cons
Perfused whole organs, wedge biopsies, and precision-cut liver slices	More normal in vivo microenvironment and architecture Can use human liver samples [89, 95, 96]	Short-term viability (<24 h), limited nutrient/oxygen diffusion to inner cell layers, low availability, inter-donor variability [90]
Purified liver fractions (organelles, membranes) and single enzyme systems	Used in high-throughput systems to identify enzymes involved in the metabolism of new pharmaceutical compounds [97, 98]	Lack gene expression and full cellular machinery to execute liver functions
Cell lines from hepatoblastomas or immortalization of primary hepatocytes	Reproducible, inexpensive models of hepatic tissue [99–103]	Abnormal levels and repertoire of hepatic functions [91]
Isolated primary hepatocytes in suspension or cultured upon extracellular matrix	Functionally more representative of normal liver than cell lines. Can cryopreserve to overcome inter-donor variability	Difficult to maintain in culture due to dedifferentiation, low availability [89–91]
Engineered tissue models	Improved stabilization of primary hepatocytes and better reproduction of normal tissue architecture [92, 94, 104]. Can be translated to in vivo through implantation [93]	Models can be challenging and expensive to fabricate

the hepatocyte phenotype. We discuss in parallel how such models have been used in tissue engineering applications including drug development and disease modeling.

2-D Culture Platforms

Several parameters of two-dimensional culture can be modulated to enhance primary hepatocyte morphology, survival, and liver-specific functions. Three such parameters are culture medium, extracellular matrix, and heterotypic interactions with non-parenchymal cells.

Culture media supplemented with serum and physiological factors such as hormones, corticosteroids, growth factors, vitamins, amino acids, or trace elements [90, 105], as well as non-physiological factors such as phenobarbital and dimethylsulfoxide [106, 107], have been shown to modulate the hepatocyte phenotype. Hepatocytes have also been maintained in media without serum [108]. Investigators utilizing a co-culture system (co-culture configurations discussed in detail below), with endothelial cells in serum-free medium under high (95%) oxygen, recently demonstrated support of hepatocyte-gene expression and drug metabolism functions better than co-culture in serum medium at 21% oxygen [109]; furthermore, the oxygenated model s stabilized more quickly. Jindal et al., have additionally identified the amino acid proline as the key factor secreted by endothelial cells in co-culture responsible for mediating the acceleration of hepatocyte recovery [110].

ECM plays an important role in hepatocyte culture; ECM preparations of different composition and topology have different effects on hepatocyte morphology and function. For instance, the presence of collagen I on a substrate enhances hepatocyte attachment, although hepatocyte spreading on adhesive substrates is often associated with loss of liver-specific functions [111]. Culture of hepatocytes on a monolayer of "biomatrix," a complex ECM mixture extracted from the liver, has been shown to improve hepatocyte function over culture on a monolayer of pure collagen [111, 112]. To screen in greater throughput the effects of various ECM proteins on hepatocyte physiology, a microarray platform was developed by Flaim et al., [113]. This system enabled investigation of the synergistic impact of ECM combinations on hepatocyte function with potential implications for the cross-talk between integrin-signaling pathways initiated by various ECM molecules. However, monolayers of ECM are not the only means of presenting ECM molecules to hepatocytes. In the standard "double gel" configuration, hepatocytes are sandwiched between two layers of collagen gel. In this format, hepatocytes demonstrate desirable morphology and liver functions for approximately 1 week [90]; rat hepatocytes in particular show P450 induction and a contiguous, anastomosing network of bile canaliculi, indicative of polarized structures [22, 114]. Limitations of this format include the fact that phase I/II detoxification processes typically become imbalanced over time [115], and that an ECM gel above the hepatocytes may inhibit the diffusion of paracrine signals or other molecular stimuli in the culture medium. Additionally, surface modifications such as polyelectrolyte chemistries have been tested for effects on hepatocyte function in vitro [116, 117]. Specifically, Chen et al., developed a two-dimensional model consisting of polyelectrolyte multilayers that enables independent variation of both substrate mechanical compliance and ligand presentation. By enabling optimization of chemical and mechanical cues, such culture techniques could prove useful in rational design of culture platforms for applications such as tissue engineering.

Heterotypic interactions with non-parenchymal cells have been used successfully to preserve the viability, morphology, and function of hepatocytes from a range of species for several weeks. Through extensive studies beginning with initial work by Guguen-Guillouzo and colleagues [118], the "rescue" of hepatocytes within co-culture settings has been demonstrated by utilizing a wide variety of non-parenchymal cells from both within and outside the liver and across species barriers, suggesting that the mechanisms responsible for stabilization are conserved [119]. Overall, substantial experimental efforts continue to explore various co-culture systems as potential models of physiologic and pathophysiologic processes in the liver. Furthermore, the identification of the important mechanisms underlying stabilization within non-parenchymal co-cultures could provide the basis for the addition of key factors and increased functionality within hepatocyte-only culture platforms.

3-D Spheroid Culture

Certain substrates promote the aggregation of cultured hepatocytes into three-dimensional spheroids and can affect functionality [120–124]; this is potentially due to the retention of a 3-D cytoarchitecture, the presence of ECM surrounding the spheroids, and the formation of homotypic cell–cell contacts between neighboring hepatocytes [125]. On non-adhesive surfaces, for example, hepatocytes aggregate over 1–2 days first into smaller spheroids of ~50 μm, which over weeks, gradually fuse into larger 150–175 μm spheroids [111]. These spheroids have functions superior to standard collagen monolayer culture [126, 127]. On Matrigel (a laminin-rich basement membrane extract), hepatocytes also form spheroids that retain hepatic functions [128, 129] though it is difficult to pinpoint the cause of these effects due to the contamination of Matrigel with proteins, hormones, and growth factors [90, 130]. Further, Matrigel-based platforms

suffer from the gradual imbalance of phase I/II detoxification processes (CYP450 decline) over a few days in culture [115]. Upon transfer to static collagen surfaces, the spheroids disassemble and the hepatocytes spread and dedifferentiate [131, 132]. Spheroid cultures have also been produced with support cells. A recent study produced an array of spheroids made from fetal mouse liver cells containing fetal hepatocytes and other liver cell types; hepatospecific function and differentiation induction were enhanced by co-culturing these spheroids with non-parenchymal feeder cells [133]. Other methods such as rotation have been used to make hepatocyte spheroids. Recently, a rocking method was employed to produce spheroids [134], generating spheroids faster and with fewer non-adherent hepatocytes than rotational methods, and also exhibiting preserved stable expression for many typical liver-specific genes.

Spheroids can in turn be encapsulated to control cell–cell interactions. In one method, spheroids suspended in methylated collagen are syringe-extruded into terpolymer solution to form microcapsules [135], but other methods have made use of various synthetic and natural scaffolds. Spheroid cultures have been utilized for both small-scale [91, 136, 137] and large-scale bioreactor systems [138]. While spheroid cultures can demonstrate desirable liver functions, there are several limitations including the fusion of small spheroids into larger aggregates, and death in the center of such aggregates due to limiting influx of nutrients and efflux of waste products. Thus, platforms for optimizing spheroid size and handling are under ongoing development.

Bioreactor Cultures

Though useful for many applications, the types of in vitro models described above provide a relatively homogeneous view of liver function. In vivo, there is a significant distribution of hepatic functions along the length of the sinusoid associated with translobular gradients in nutrients, oxygen, hormones, and ECM. Some bioreactor cultures attempt to capture these differences. In order to study the effects of oxygen variation across the liver lobule, a small-scale, parallel-plate bioreactor was developed that exposes hepatocyte/non-parenchymal co-cultures to a steady-state oxygen gradient [139]. These cultures were able to replicate the heterogeneous expression distribution of the drug metabolism enzymes CYP2B and CYP3A observed in vivo, and expression could be controlled with chemical inducers and growth factors. Furthermore, exposure of the culture to acetaminophen caused greatest cell death in areas of low oxygen, replicating the centrilobular death pattern observed in vivo.

Bioreactors have also been produced to culture hepatic aggregates. One device consisted of a 1 cm^2 planar polymer scaffold with 900 micro-containers that could each culture a uniformly-sized 3-D hepatic aggregate [136, 137]. The aggregates were perfused via a pore, laser-drilled within each micro-container and retained desirable morphology and liver functions for 2 weeks. Another device consists of hepatic spheroids cultured in an array of micro-channels etched into silicon wafers using deep reactive ion etching [91]. In this system, culture medium was passed across the top of the array, enabling spheroids to retain liver-specific characteristics for 2–3 weeks in culture, as assessed by gene expression profiling, protein expression, and activity of drug metabolism enzymes [91]. Recently, Domansky et al., have integrated multiple perfused bioreactors into a multi-wellplate format in which each bioreactor houses hundreds of microscale hepatic monolayers; the format of this device could be applied towards studying perfused tissue units in high-throughput [140].

Bioreactors have been developed in several other studies as well. A flat plate bioreactor was designed to study the effects of oxygenation and shear stress on hepatocyte function [141]. Additionally, alternative bioreactor configurations have been developed to minimize shear stress effects, and have included, for example, grooved substrates to protect hepatocytes from shear, or adjacent channels separated by a gas-permeable membrane to decouple oxygen exchange and volumetric flow rate [142, 143]. Such bioreactors can also be scaled for clinical applications; in one particular demonstration, grooved-culture substrates were stacked in a radial flow bioreactor [144]. To improve oxygen delivery, collagen sandwich co-cultures of hepatocytes and non-parenchymal cells from the liver were cultured in a 96-well perfused microbioreactor with a biocompatible, gas-permeable membrane [89]; hepatocytes in this system maintain liver functions such as albumin and urea expression, expression of phase I/II detoxification enzymes, and inducible expression of CYP1A1. Finally, bioreactors may be used to study more dynamic physiological processes that are not possible with conventional culture platforms; for example, a recent bioreactor device describes the ability to monitor invasion of metastatic cells into hepatic parenchyma by recreating relevant features of the liver tissue such as fluid flow and length scales [145]. Collectively, bioreactors enable complex control over hepatocyte culture parameters [146–148] and in turn hepatocyte function; as such, they will continue to be useful in studying liver biology and in applications such as drug development.

Microtechnology Tools

Microtechnology tools afford micron-scale control of tissue architecture as well as cell–cell and cell–matrix interactions, facilitating investigations of the mechanisms underlying tissue

development and function [149]. Based on methods used in the semiconductor industry, microtechnology approaches allow fine control over cell adhesion, shape, and multi-cellular interactions [150]. Consequently, they are enabling studies of biological phenomena at cellular length scales [151, 152], as well as techniques for miniaturizing and parallelizing biomedical assays (e.g. DNA microarrays, microfluidics) [92, 153].

In order to study the effects of homotypic and heterotypic cell–cell interactions between hepatocytes and non-parenchymal cells, a photolithographic-cell -patterning technique was employed to make micropatterned co-cultures in which hepatocyte islands of controlled diameters were surrounded by non-parenchymal cells [119]. Initially employed for rat hepatocyte culture, the highest levels of liver-specific functions occurred at an intermediate island diameter, implying that optimal function results from an optimal balance of homotypic/heterotypic interactions. Using soft lithography, this co-culture pattern has been recently miniaturized and adapted into a multi-well format to serve as a microscale human liver tissue model for drug development [92]. The utility of this platform for drug development has been shown through gene expression profiles, phase I/II metabolism, canalicular transport, secretion of liver-specific products, and susceptibility to hepatotoxins. Fine control over the spatial distribution of cells is also demonstrated in a recent platform developed by King et al., which uses a microfabricated device with quantitative live cell imaging to measure gene expression in real-time of individual living cells [154]; the tool is used to investigate gene-expression changes in the course of hepatic inflammation.

Another application of microtechnology is the use of microfabrication and microcontact printing techniques to develop a microarray containing hepatocyte spheroids of a uniform size [155]; this method reduces aggregate heterogeneity and minimizes cell necrosis resulting from oxygen/nutrient depletion or waste accumulation. These spheroids retain liver functions including expression of liver-enriched transcription factors, albumin secretion, and expression of urea cycle enzymes. Another application of microtechnology tools in building multicellular hepatic structures is the formation of hepatic tissue sheets by release of confluent hepatocytes from surfaces coated with the temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm) [156].

Microtechnology tools can also be used to create culture platforms in which stimuli are dynamically modified, in contrast to typical culture platforms in which stimuli are static; such tools should enable investigation and optimization of cellular responses that exhibit spatiotemporal components and thus contribute to tissue engineering applications. Microfluidic devices are typical examples of systems that permit spatiotemporal control over delivery of nutrients and other soluble mediators to cultured cells. Recently, Chao et al., described a microfluidic platform that simulates flow through the liver to predict the in vivo hepatic clearance of pharmaceutical compounds [157]. Dynamic cellular responses can also be interrogated using other nascent microfabrication approaches. In one example, a mechanically actuated "comb" device was fabricated that enabled investigation of cell–cell interactions by permitting micron-scale temporal control of cell–cell interactions [158]. When used to study hepatocyte-stromal cell interactions, this platform revealed that phenotypic stabilization of phenotypes by the non-parenchymal cells required direct contact for hours followed by a sustained short-range paracrine signal.

The fine spatial and temporal control afforded by microtechnology tools has already accelerated studies of basic liver biology and applications that were impossible without such methods.

Application of In Vitro Liver Models: Studying Liver Pathophysiology

In vitro hepatocyte cultures and co-cultures have been utilized to investigate various physiological and pathophysiological processes, including host response to sepsis, mutagenesis, xenobiotic toxicity, response to oxidative stress, lipid metabolism, and induction of the acute-phase response [119]. Among the many applications of in vitro liver tissue models is the study of the behavior of pathogens that target hepatocytes and screening for therapeutics of the associated diseases. Hepatitis C virus (HCV) and malaria are two such pathogens.

HCV is an enveloped RNA virus whose genome consists of a single positive-stranded RNA that replicates in the cytoplasm of infected hepatocytes without integrating into the host genome. The first in vitro model enabling studies of replication and screens for small molecule inhibitors of the replicative enzymes of HCV consisted of a subgenomic replicon stably-transfected into carcinoma cells [159]; however, with this system, it was not possible to study the complete viral life cycle as the structural proteins were omitted from the replicon. Collectively, at this time, researchers were unable to find a viral genotype capable of executing the full viral life cycle in vitro. In 2001, Kato et al., [160] found and sequenced a genotype-2a strain of HCV that caused fulminant hepatitis in a Japanese patient. This genotype was named JFH-1, and in 2005 it was shown that JFH-1 and a chimeric variant were able to complete the entire viral life cycle in the Huh7 carcinoma cell line and more robustly in certain Huh7 sublines [161–163]. More recently, it has become possible to study HCV infection in primary human hepatocytes [164–166]. Ploss et al., [166] demonstrate that a microscale human liver tissue model [92] is capable of recapitulating the entire viral

life cycle and can act as drug screening platform for compounds that suppress HCV replication.

Plasmodium infection is responsible for malaria disease, and distinct species, such as *P. falciparum* and *P. vivax*, are associated with different degrees of severity and patterns of pathogenesis. After transmission of the *Plasmodium* sporozoites to the human blood circulation from mosquito saliva, the sporozoites infect hepatocytes in the liver. There, they eventually proliferate and differentiate into merezoites, which then go on to infect red blood cells, where they rapidly amplify in number. In vitro liver models have the potential to enable studies of the hepatocyte stage of the malarial life cycle and presumably vaccine development and screening of small molecules that inhibit viability or proliferation of the parasite in its liver stages. Several lines of research have recently explored this possibility, for example, in a two-dimensional collagen monolayer culture model of primary human hepatocytes, investigators were able to recapitulate the complete liver development stage of *P. falciparum* [167] and *P. vivax* [168]. A similar culture model has been used to characterize the mechanistic basis of CD81-dependent invasion of hepatocytes by *Plasmodium*, with the conclusion that SR-BI enhances permissiveness to infection by increasing plasma membrane cholesterol and organizing CD81 into an entry-favorable configuration [169]. van Schaijk et al., use this model to show that disruption of the p52 gene in *P. falciparum* leads to arrest in the liver stages of development, potentially, providing a source of genetically attenuated sporozoites for vaccination purposes [170]. By reproducing key physiologic properties of liver tissue, in vitro liver models enable applications such as drug development and the study of liver pathophysiology. As discussed in this section, numerous culture configurations have been attempted, where each serve different functions. In pursuing a particular application, investigators must decide what critical aspects of the in vivo liver tissue must be replicated in their systems, informing the selection of an appropriate model.

Implantable Engineered Tissue Constructs

Transplantation of hepatocytes to perform liver functions shows great potential for the treatment of liver disease and in the development of humanized liver mouse models, but direct injection of cells is associated with variable seeding efficiency and poor long-term survival and engraftment. Hepatocyte delivery in a tissue-like structure that preserves cell attachments could increase engraftment efficiency, reduce the need for a repopulation advantage in donor cells and reduce the overall lag phase before clinical benefit is attained [171–173]. Thus, hepatic tissue engineering technologies, which seek to generate liver-like tissue in vitro prior to in vivo implantation, may provide an alternative delivery method to transplantation of suspension cells, as well as a means to implant cells and/or additional biological cues that interact with the host and ultimately serve to improve liver function.

Implantable engineered hepatic tissues have typically been created by immobilizing or encapsulating hepatocytes using biomaterial scaffolds. As such, scaffold properties and cell sourcing are both critical in the development of engineered tissue. Though great strides have been made in this field, many issues must be addressed before implantable hepatic tissue becomes a clinical reality. As work in this field advances, careful attention needs to be paid to issues that dictate the ultimate clinical translation of these therapies.

Scaffold Properties

Highly functional 3-D implantable liver tissue will likely require dense population with functional and stable hepatocytes, while also facilitating the transport of nutrients and large macromolecules. Cell seeding and nutrient transport are ultimately dictated by scaffold properties, which include material and chemical modifications, porosity, and 3-D architecture.

Material and Chemical Modifications

The choice of material determines the physicochemical and biological functions of the scaffold. For example, natural, biologically-derived materials containing binding sites for cell attachment can enhance function since hepatocytes are anchorage-dependent cells. Hepatocytes have been attached to collagen-coated dextran microcarriers and transplanted intraperitoneally into two different Gunn rat genetic models for replacement of liver-specific functions [174]. Microcarriers provide a platform for cell attachment and enhanced survival and function of the transplanted hepatocytes. Cellulose [175–177], gelatin [178], and gelatin–chitosan composite [179] microcarrier chemistries have also been explored for hepatocyte attachment. Additionally, hepatocytes have been encapsulated within natural, extracellular matrix-derived scaffolds, including the collagen gels [180, 181], hyaluronic acid [182], peptides [183], or alginate and alginate-based composites [184–187]. Microencapsulation of hepatocytes in these types of systems can facilitate hepatocyte aggregation and improve function. For instance, alginate-based encapsulation platforms have been shown to support hepatocyte spheroid culture [184, 185, 187] and thus have been proposed for use in implantable constructs.

Synthetic polymers have afforded hepatic tissue engineers an improved control over scaffold physicochemical and biological properties. The most common synthetic polymers utilized in the generation of porous tissue engineering constructs are polyesters such as poly(l-lactic acid) (PLLA) and poly(d,l-lactide-co-glycolide) (PLGA). These materials are biocompatible and biodegradable, support hepatocyte culture, and have been widely used as scaffolds for hepatocyte transplantation [93, 188–197]. A key advantage of these polyesters is the ability to finely tune its degradation time, based on the relative contribution of the PLLA versus PLGA components, each of which exhibit distinct hydrolysis kinetics. Material modifications of PLGA scaffolds have also been shown to improve hepatocyte functionality. Specifically, the addition of hydrophilic poly(vinyl alcohol) (PVA) into PLGA scaffolds enhanced hepatocyte seeding [191]. Alkali hydrolysis and ECM coating of PLGA constructs can similarly enhance hepatocyte attachment [195, 196, 198]. Importantly, a composite PLLA–PLGA scaffold coated with PVA supported long-term engraftment of hepatocytes after transplantation in the mesentery in a rodent injury model [93]. Despite these advances, the accumulation of hydrolytic degradation products upon PLLA and PLGA degradation has been shown to produce an acidic environment within the scaffold and initiate peptide degradation, stimulate inflammation, or result in poor tissue engraftment [199, 200]. As such, groups have explored methods to control peptide degradation in PLGA as well as explore alternative synthetic polymer-based systems for use in tissue engineering.

The synthetic hydrogel system based on polyethylene glycol (PEG), has also been widely utilized for various tissue engineering applications [201–219] including, recently, for hepatocellular platforms [220]. PEG-based systems are particularly useful in tissue engineering due to their high water content (which give them similar mechanical properties to tissues), hydrophilicity and resistance to protein adsorption, biocompatibility, and capacity for customization through the modification of chain length and the addition of bioactive elements [219, 221]. An additional advantage of PEG is that it can be polymerized through photo-crosslinkable diacrylate (DA) endgroups in the presence of cells, which provides for the generation of 3-D constructs with uniform cellular distribution. Studies examining PEG-DA hydrogel encapsulation of hepatic cells have utilized immortalized hepatocytes, hepatoblastoma cell lines, and primary hepatocytes, and together have shown that tailoring the design of the hydrogel network dictates hepatocyte survival and function [8, 94, 222]. Taken together, these results demonstrate that synthetic hydrogel systems represent promising platforms for implantable constructs as well as investigating in vitro hepatocellular responses in a model 3-D environment.

Although synthetic polymer scaffolds offer numerous advantages, the absence of natural cell-binding sequences in these systems often limit their capacity to promote cell adhesion. The inert nature of synthetic systems can also be used as a tool to study the basic biology of hepatocellular adhesion, by facilitating the controlled incorporation of biologically active elements aimed at regulating different aspects of cell function. Multiple approaches have been explored for modulating hepatocyte interactions with synthetic platforms by non-specific adsorption or chemical conjugation of biological molecules, including the incorporation of (1) ECM molecule coatings [195, 196], (2) various sugar residues such as lactose, heparin [223], and galactose [224, 225], (3) poly(*N*-*p*-vinylbenzyl- 4-*O* β(beta)-d-galactopyranosyl-d-glucoamide) (PVLA) in PLLA scaffolds [192, 226, 227], and (4) epidermal growth factor (EGF) in polyethylene terephthalate (PET) fabric scaffolds [227]. Each of these modifications has been implicated in improving hepatocyte adhesion within polymer scaffolds and, therefore, highlight modifications of the polymer scaffold backbone that influence hepatocyte processes. As an alternative to the incorporation of entire biomolecules, polymer scaffolds can also be conjugated with bioactive adhesive peptide sequences. Adhesive peptides, which interact with integrin receptors in the cell membrane, have been extensively utilized to promote cell attachment within polymer networks. For example, inclusion of the RGD peptide sequence within biomaterial scaffolds dramatically influences the adhesion and function of a diverse assortment of cell types [228]. In experiments using hepatocytes, grafting RGD peptides to PLLA scaffolds similarly enhanced hepatocyte attachment [197]. Notably, RGD conjugation also significantly improved the long-term stability of primary hepatocyte function in PEG hydrogels [94]. Incorporation of additional adhesive peptides that bind other integrins might further enhance hepatocyte function within synthetic polymer substrates. Additionally, the incorporation of hydrolytic or protease-sensitive peptide sequences (such as matrix metalloproteinase-sensitive peptide sequences) into hydrogel networks as degradable linkages has been shown to permit cell-mediated degradation and remodeling of the gel [210, 212, 229–232]. Although these systems have not yet been applied to hepatocellular technologies, it is interesting to note that liver regeneration proceeds in conjunction with a distinctive array of remodeling processes such as protease expression and ECM deposition [233–235]. Therefore, cell-mediated material degradation could provide a mechanism for the efficient integration of implantable constructs. The ability to modify biomaterial scaffold chemistry by introducing biologically active factors will likely allow for fine-tuned regulation of cell function and graft-host interactions.

Porosity

Many natural and synthetic implantable tissue-engineering approaches utilize porous scaffolds, which provide mechanical

support and often biological cues for growth and morphogenesis. The material's pore size can be controlled over several orders of magnitude, thus allowing materials to be tailored for purposes such as the optimization of protein exchange [94], cell–cell interaction and survival [94, 236], or the promotion of wound repair and tissue ingrowth [237]. Collagen or various alginate and chitosan composites are the most frequently used biologically-derived scaffold materials for hepatocyte tissue engineering [180, 184–187, 236, 238–251]. Collagen sponges have been utilized as scaffolds in a diverse array of cell systems and also as substrates for promoting wound repair [252–258]. In a study in which hepatocytes were seeded onto collagen scaffolds exhibiting a variety of pore sizes (10–82 μm), pore size was found to be an important factor regulating cell spreading and cell–cell interactions [236]. Additionally, alginate scaffolds with pore sizes of approximately 100 μm have been shown to encourage spheroidal aggregation of hepatocytes owing to the weakly adhesive properties of the material, and spheroid formation in turn promoted hepatocyte stabilization[239]. Variations in alginate formulation, such as an alginate–galactosylated chitosan–heparin composite system [250], can further enhance cell aggregation and viability [184, 186, 238, 249, 250]. A variety of porous synthetic materials such as PLLA and PLGA have also been used in hepatic tissue engineering, as detailed above. Porous, acellular scaffolds are normally seeded using gravity or centrifugal forces, convective flow, or through cellular recruitment with chemokines [259–261]. However, incorporation of hepatocytes into scaffolds is hindered by insufficient or non-homogeneous cellular distribution and by the relatively immotile nature and limited proliferation of hepatocytes *ex vivo*. Additionally, despite the fact that porous scaffold systems continue to be explored for use in engineered liver tissue, many of these scaffold architectures contain pores that are many times larger than individual hepatocytes, essentially making them 2-D surfaces from the hepatocyte perspective. This may limit the ability of porous scaffolds to fully recapitulate 3-D cues.

3-D Architecture

An alternative approach for tissue-engineered scaffolds strives to more closely mimic in vivo microarchitecture. The 3-D architecture of native tissues influences cellular function, mechanical properties of the tissue, and the integration of grafted engineered tissue with the host. The ability to fabricate cellular scaffolds with highly defined structure could facilitate the recapitulation of the appropriate microscale environment for cell viability, cell function, and cell–cell interactions, as well as desired macroscale properties that determine mechanical properties and nutrient delivery.

Porous scaffolds were initially produced by solvent casting or particulate leaching methods. These techniques did not permit for pre-designation of the internal scaffold structure or pore connectivity. More recently, CAD-based rapid prototyping strategies have been developed that allow for defined control by utilizing multiple assembly modes, including fabrication using heat, light, adhesives, or molding, as reviewed elsewhere [262]. Briefly, 3-D printing with adhesives combined with particulate leaching has generated porous PLGA scaffolds that were used for hepatocyte attachment [263]. Microstructured ceramic [264] and silicon scaffolds [265, 266] have additionally been proposed as systems for the culture of hepatocytes. Furthermore, molding and microsyringe deposition have been utilized to fabricate specified 3-D PLGA structures [267].

Microfabrication techniques have also been employed to pattern cells within natural and synthetic hydrogels. For example, microfluidic molding has been used to create biological gels containing patterned cells in multi-layer structures [268, 269]. In addition, syringe deposition and micropositioning were recently used to generate patterned gelatin hydrogels containing hepatocytes [270]. The ability to polymerize synthetic PEG hydrogels using ultraviolet (UV)-initiation allows for the use of photolithography to generate hydrogel networks with defined microscale patterns. In this process, patterned masks printed on transparencies localize UV exposure to selected regions of the prepolymer solution and therefore dictate the structural features of the resultant hydrogel. Photolithography-based techniques have been employed to pattern biological factors [271], produce hydrogel structures with a variety of shapes and sizes [272, 273], and build multilayer cell networks [222]. Hydrogel photopatterning is thus ideal for the regulation of scaffold architecture at the multiple size scales required for engineered hepatocellular constructs. As one example, perfusion of hepatocellular constructs is known to improve hepatocyte functions [224, 263]; therefore, photopatterning of PEG hydrogels containing hepatocyte–fibroblast co-cultures was used to create a branched 3-D network that was cultured under flow conditions for improved oxygen and nutrient transport and encapsulated hepatocyte functions [094]. At the microscale, dielectrophoretic gradients field gradients have been employed to pattern hepatocytes and fibroblasts within a pre-polymer solution of PEG prior to photoencapsulation [274]. The combined utility of photopatterning and dielectrophoresis-mediated cell patterning thus allows the construction of hepatocellular hydrogel structures with an organization defined at both the macroscale and cellular scale. Finally, the recent introduction of a new family of PEG-based photodegradable hydrogels, which allow selective degradation of hydrogels using light, will allow real-time manipulation of spatial features and mechanical properties at the microscale. Such materials create new opportunities to

study the effects of changes in scaffold microarchitecture, chemistry, and mechanics over the course of culture time [275]. In summary, the ability to dictate scaffold architecture coupled with advances in scaffold material properties, chemistries, and the incorporation of bioactive elements will serve as the foundation for the future development of improved tissue-engineered liver constructs.

Cell-Sourcing for Implantable Liver Tissues

Cell sourcing for implantable engineered liver tissue faces challenges similar to cell transplantation. Specifically, hepatocytes cultured in 3-D within biomaterial scaffolds lose phenotypic functions and the ability to proliferate in vitro in the absence of the appropriate biological cues [8]. In the effort to optimize both, phenotypic function and cell proliferation, various groups have explored the use of mature primary hepatocytes, hepatic progenitor cells, and non-parenchymal supporting cells in tissue-engineered constructs.

Mature Hepatocytes Versus Hepatic Progenitor Cells

Most tissue-engineering strategies outlined in the scaffolds section above have utilized immortalized hepatocytes, hepatoblastoma cell lines, or primary mature hepatocytes as the cell source [8, 94, 132, 201, 270, 276–278]. Of these, primary hepatocytes are the preferred cell type for clinical therapies due to safety concerns associated with cell immortalization and potential tumor formation [48]. However, similar to whole organs, the supply of primary hepatocytes is limited [8]. Additionally, the limited proliferative capacity of mature hepatocytes in vitro often results in low cell density in engineered tissues and ultimately limits the success of hepatic tissue engineering. In the attempt to identify more proliferative hepatocyte-cell sources, recent work has turned to testing the efficacy of the wide variety of hepatic progenitor cell populations (as described in detail above) in engineered tissues. As one example, bipotential mouse embryonic liver (BMEL) cells have been shown to survive and differentiate towards the hepatic lineage in PEG hydrogels [94]. Additionally, mouse embryonic stem cell-derived hepatocytes have been implanted in a tissue engineered assist device and shown to improve mouse hepatic failure [60].

While hepatic progenitor populations are an exciting alternative to primary hepatocyte-cell sources, work to further refine the unknown advantages and disadvantages of proliferative hepatic progenitors versus fully mature hepatocytes in tissue engineering is necessary. For example, it is currently unknown whether the proliferative capacity of hepatocyte progenitors in tissue engineered constructs will result in superior engraftment, tissue density, and tissue function following implantation compared to constructs containing fully functional mature hepatocytes. In fact, a major concern in the use of progenitor cell-populations for implantation therapy is that these cells may exhibit uncontrolled cell proliferation and/or differentiation towards undesired cell fates [279]. Implantation of proliferative hepatic progenitors could indeed result in "over-population" of tissue engineered constructs. In the worst situation, implantation of undifferentiated pluripotent cell types could result in teratoma formation [279]. Current work therefore speaks to address issues whether progenitor cell populations need to be pre-differentiated to cells dedicated to the hepatic lineage prior to culture in three-dimensional scaffold materials and implantation [60], whether hepatic progenitors need to be fully mature and exhibit no expression of fetal programs prior to implantation, and whether signals can be engineered strategically into the scaffold to guide appropriate differentiation within the scaffold itself [280–282]. In summary, a wealth of exciting new hepatocyte progenitor-cell sources has recently become available for use in implantable liver devices, and future studies will be needed to evaluate their therapeutic potential.

Cell–Cell Interactions

Similar to two-dimensional culture, homotypic [94, 136] and heterotypic [94, 222, 283] interactions have been found to be essential in the maintenance of hepatocyte function and survival in culture in biomaterial scaffolds. For example, pre-aggregation of primary hepatocytes or BMEL progenitor cells in PEG gels enhanced both cell survival upon encapsulation and also hepatocyte function [94], suggesting that homotypic cell interactions are important in this system. Numerous groups have also co-encapsulated non-parenchymal cell populations such as fibroblasts, endothelial cells, mesenchymal stem cells, and stellate cells with hepatocytes in biomaterial scaffolds, and some studies have shown improved hepatocyte function in the presence of non-parenchymal cell populations [94, 126, 284–290]. For example, co-encapsulation of primary hepatocytes and 3T3 fibroblasts in PEG hydrogels has been shown to improve hepatocyte survival and function [94]. Hepatocytes have also been shown to affect the morphogenesis and phenotype of non-parenchymal cells in biomaterial scaffolds. For example, when hepatocytes and microvascular endothelial cells are cultured on a collagen gel scaffold in a microfluidic device, endothelial morphogenesis is dependent on diffusion from one cell compartment to the other [291]. Additionally, 3-D culture of hepatocytes with liver sinusoidal endothelial cells allows for maintenance of the SE-1

marker in the endothelial cells, an indication of persistence of liver-specific endothelial cell phenotype [283]. Taken together, these results show that non-parenchymal cell populations have played a significant role in the development of robust engineered liver tissue to date.

Future iterations of engineered liver tissue need to further refine the non-parenchymal cell types that are necessary in implantable constructs. For example, the non-parenchymal cell types utilized in liver tissue engineering to date have typically been non-human and non-native to the liver (e.g., J2–3T3 mouse fibroblasts [94, 283]). Clinical impact of engineered liver devices will be accelerated through the use and study of human cell sources. Additionally, ease of large-scale manufacturing will likely be enhanced by minimizing the number of cell types required in any given device. In order to reduce the number of cell types in a given device, a detailed mechanistic understanding of the cues involved in cell–cell interactions, as well as liver development and regeneration is necessary. Techniques to micropattern the spatial configuration of multiple cell types with respect to one another [274], precisely regulate the spatial and temporal release of growth factors and morphogens [281], and model cell-signaling networks [61, 292, 293] should aide in determining the mechanistic roles of different cell types and biological cues. Such knowledge could be used to design engineered therapies that incorporate key signaling molecules, but limit the number of exogenous cell types needed to improve hepatocyte survival and function in engineered tissues [294]. Thus, cell–cell interactions have been shown to be important in the development of engineered liver tissue and the exact molecular mechanisms responsible for the functional benefits derived from cell–cell interactions is an area of investigation that will be critical to the design of advanced liver tissue-engineered devices.

Clinical Translation for Human Therapy

Prior to the translation of implantable device therapies to the clinic, animal models must be developed that adequately assess the safety and efficacy of these therapies. These tissues will also need to integrate with the patient's vascular and biliary systems. Finally, it will be essential that implantable engineered therapies are composed of immune-compatible cell and material components that are fit for use in humans.

Assessment in Animal Models

Prior to the translation of any cell-based liver to the clinic, the safety and therapeutic efficacy of these therapies must be demonstrated in animal models. Animal models for testing these therapies include genetic, toxic, ischemic, PHx, and total hepatectomy models. Several extensive reviews outline important criteria used in the development of animal models of fulminant hepatic failure [295–297]. These criteria include reproducibility, reversibility, liver failure-induced cell death, and presence of sufficient time interval for diagnosis and therapeutic intervention [298]. Genetic models of liver injury include the urokinase plasminogen activator overexpression (uPA++/SCID) and FAH knockout mouse models [299, 300]. Chemical induced injury models include exposure to toxic doses of carbon tetrachloride, D-galactosamine, or acetaminophen, all of which induce localized centrilobular necrosis [301, 302]. Chemical induced injury models are especially useful for testing the efficacy of cell-based liver therapies because these models most closely mimic acute liver injuries commonly found in humans (e.g. drug toxicity). Surgically induced injury models include PHx and have been widely employed because the injury stimulus is well-defined [303]. Despite the fact that the hepatectomy model is less clinically relevant (with the exception of liver resection patients), it serves as a well-controlled system to examine the importance of regenerative cues in the engraftment of hepatic constructs implanted in extrahepatic sites [303]. To this end, the optimal implantation site of implantable engineered tissue will also need to be determined. Tissue engineered constructs are frequently evaluated after implantation into subcutaneous or mesenteric spaces due to the ease of surgical access and improved imaging options. Early work in injury damage models suggested that orthotopic transplantation was necessary for hepatocyte survival due to interaction with "hepatotrophic factors" available in the portal vein. However, the effectiveness of implanted extrahepatic scaffold based systems in supporting hepatic function following hepatectomy has been demonstrated in some studies with rodent models [60, 303, 304] in which constructs were implanted subcutaneously, peritoneally, or in the fat pad, suggesting that injury cues originating in the host liver can reach extrahepatic sites. The utilization of numerous surgical and chemical animal models will be instrumental in testing the efficacy of cell-based liver therapies. Concurrently, knowledge of the mechanisms of liver injury and regeneration obtained from these experiments will influence the future design of next-generation engineered liver tissue.

Integration with Host Tissue Vasculature and Biliary System

To derive maximal therapeutic benefit from implantable engineered liver tissue, grafted tissue must integrate with the host tissue, and in particular, with the host vasculature and biliary system. Within the normal liver environment,

hepatocytes are supplied by an extensive sinusoidal vasculature [305]. This vasculature allows for the efficient transport of nutrients to the highly metabolic hepatocytes. A significant challenge in the design of implantable liver constructs is the need to sustain thick implanted tissue in the face of transport limitations prior to the establishment of functional vasculature. One strategy is to incorporate "pre-formed" vasculature into engineered constructs prior to implantation. For example, microfabricated vascular units could be created and followed by surgical anastomosis during implantation [266, 305]. Polymer molding using microetched silicon has produced channel networks with capillary dimensions [266]. Additionally, recent muscle engineering studies have demonstrated that prevascularization of engineered tissue using endothelial and mesenchymal cells (in addition to muscle cells) in vitro improves survival and vascular integration of engineered tissue with host tissue after implantation [306–308]. A second strategy is to incorporate angiogenic factors within the implanted scaffolds so that these factors can recruit the ingrowth of host vasculature immediately upon implantation. Specifically, integration of cytokines that play critical roles in angiogenesis, such as VEGF [309, 310]. bFGF [311], and VEGF in combination with PDGF [312], promotes the recruitment of host vasculature to implanted constructs. A final strategy is to "prime" the implantation site through prevascularization. For example, pre-implantation of VEGF releasing alginate scaffolds prior to hepatocyte seeding enhances capillary density and improves engraftment [313].

Finally, inclusion of excretory capabilities associated with the biliary system may be necessary in future engineered liver tissue. To date, studies have focused on the developing in vitro models that exhibit biliary morphogenesis and recapitulation of the appropriate polarization and bile canaliculi organization [314–316], as well as platforms for engineering artificial bile duct structures [317]. Future studies will determine whether the inclusion of biliary elements is necessary in implantable liver tissue.

Immune Response

Similar to whole liver or cell transplantation, an understanding of the host immune system responses following transplant of tissue-engineered constructs will be paramount to the success in translating these therapies to the clinic. To minimize the host immune response to implantable constructs, all parenchymal and non-parenchymal cells populating engineered tissue should be entirely human. Similarly, implantable tissue should be free of xenogenic materials. Towards this end, engineered tissues must be cultured under serum-free conditions and should not contain naturally-derived xenogenic biomaterials [318, 319].

The road to the ultimate immuno-compatible implantable liver therapy may be multi-tiered and will likely parallel that of cell transplantation therapies. For first-generation therapies, immunosuppressive treatments could be combined with the establishment of allogenic human primary hepatocyte or ES-derived hepatocyte cell banks that contain immunologically diverse phenotypes [320]. Next-generation therapies may be populated by autologous-cell sources such as iPS-derived hepatocytes and therefore reduce the need for rigorous immunosuppressive treatment. Furthermore, harnessing the liver's ability to induce antigen-specific tolerance [321, 322] could improve the immune acceptance of engineered grafts. Overall, immune biology challenges will be critical in the successful translation of cell-based therapies. Multiple options exist for building immune-compatible cell therapies, and careful attention to these issues during the design and development of implantable engineered liver tissue will facilitate the ease and efficiency of clinical translation of these therapies.

Conclusion

Substantial advances have been achieved in the field of liver tissue-engineering, as shown by the concurrent improvements in bio-mimetic in vitro liver systems and implantable hepatocellular constructs. This progress is enabled by integrating knowledge bases from various disciplines, including fundamental liver biology, medicine, and biomedical engineering. Although many challenges remain, our evolving understanding of key regulators of liver function and regeneration, promises to lay solid ground work for the next generation of clinically effective tissue-engineered liver systems.

Acknowledgement Funding provided by NIH R01-DK065152 and NIH R01-DK56966.

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Chapter 23
Hepatic Gene Therapy

Hiroyuki Nakai

Introduction

Around the turn of the new millennium, after its 20-year history, gene therapy experienced both an exciting success [1] and an unexpected failure [2, 3], which significantly changed our perspectives on gene therapy from "versatile therapy" that we expected would soon become available to cure difficult-to-treat diseases to "potentially effective therapy" that would surely be superior over conventional therapies, but still needs further refinement towards clinical applications. Since then, within less than a decade, exciting discoveries and the development of emerging technologies pertinent to gene therapy have occurred, re-inspiring a greater-than-ever interest to gene delivery approaches. As for hepatic gene transfer, we have already become able to deliver genes of interest to target cells in the liver at extremely high efficiency and with minimum toxicity, at least in mice. With the contemporary hepatic gene delivery methods in our hands, any disease can be effectively treated or even cured in animal models, as long as the right therapeutic targets have been identified in the liver. At the current stage of the development of new molecular therapeutics, in addition to seeking new breakthroughs, it is critical to further refine the technologies, to understand the underlying mechanisms of action, to explore the methods to minimize undesired reactions and side effects, and importantly to further the knowledge of disease pathogenesis. With this introduction, this chapter provides an overview of contemporary methods and applications of hepatic gene transfer with an emphasis on the underlying mechanisms of action of each gene delivery approach.

H. Nakai (✉)
Department of Microbiology and Molecular Genetics,
University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
e-mail: nakaih@pitt.edu

Liver in Gene Therapy

Liver: A Major Target Organ in Gene Therapy

The liver is the largest and most blood-rich solid organ in the body, with multiple important biological functions essential for maintaining good health of animals and humans. The liver consist of hepatocytes (60–65% of total liver cells), sinusoidal endothelial cells (15–20%), Kupffer cells (8–12%), hepatic stellate cells (HSCs) or Ito cells (3–8%), biliary epithelial cells (3–5%), liver dendritic cells (<1%), cells constituting blood vessels, and blood cell components passing through the hepatic circulation. Hepatocytes produce, secrete, store, and/or degrade various molecules essential for normal biological activities of organisms. Hepatic dendritic cells (DCs), Kupffer cells (KCs), sinusoidal endothelial cells, and hepatocytes in some cases, serve as antigen presenting cells (APCs). Disruption of these biological functions and metabolic homeostasis in the liver occurs due to a variety of causes including congenital genetic defects, acquired disorders, and exogenous factors including drugs, alcohol, and faulty nutrition, and leads to various hepatic and systemic diseases. Thus, the liver is involved in the development and progression of many diseases; therefore it represents the primary target organ for therapeutic interventions, especially for gene therapy. In addition, the liver is the major organ responsible for various adverse events associated with therapies.

Hepatic gene therapy approach is not limited to the treatment of diseases that affect biological functions of the liver. The liver is well equipped with cellular machinery required for production and secretion of a large quantity of proteins to be released into the blood. Therefore, hepatic gene therapy is very effective in continually producing therapeutic proteins and secreting them into the blood circulation. In this regard, skeletal muscle also serves as a protein factory in gene therapy owing to its large mass, large capacity of protein synthesis, and ability to secrete proteins. Although easier accessibility and potentially less vector spillover to remote

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_23, © Springer Science+Business Media, LLC 2011

sites make muscle-directed approach attractive, liver-directed approach could transduce a greater number of target cells and may result in more reduced immune responses against therapeutic products expressed by vectors. Potential therapeutic molecules expressed and secreted by the liver with this type of approach include blood coagulation factors, hormones, growth factors, cytokines, metabolic enzymes, and anti-proteases. In addition, gene therapy technique and the increased knowledge of developmental biology of the digestive system have made it possible to transdifferentiate hepatocytes into insulin-secreting β(beta) cells in the liver [4].

Liver also becomes a target organ to deliver therapeutic genes aiming at treating primary and metastatic liver tumors, although gene therapy for liver cancer is not generally considered as hepatic gene therapy per se. In this context, the major target cells are cancer cells and not parenchymal or non-parenchymal liver cells; however, therapeutic vectors are in general delivered to the liver through the hepatic circulation similar to the hepatic gene therapy approaches to treat non-malignant diseases.

Understanding Biological Functions of the Liver: Rare Monogenic Diseases as Targets for Hepatic Gene Therapy

The liver is a multi-tasking organ comprised of various types of professional cells as mentioned earlier, with the hepatocytes being the most versatile of them all. Hepatocytes can carry out approximately 500 biochemical reactions in a single cell and are responsible for the major metabolic processes in vertebrates. Hepatocytes are involved in the metabolism of fuels (carbohydrates, lipids, and nitrogen compounds), bilirubin, porphyrin, bile acids, steroid and thyroid hormones, vitamins and coenzymes, minerals, xenobiotics, drugs, and alcohol. All of these metabolic reactions are under the strict control of various regulatory systems. Even a single defect in one of the chains in a particular chemical reaction within a metabolic pathway can disrupt the metabolic homeostasis of the liver, leading to potentially fatal results. Although a majority of metabolic pathways and their individual chemical reactions have been biochemically well characterized, many inborn errors of metabolism are devoid of an effective therapy. Current treatment modalities include whole organ transplantation, but the global lack of donor organs requires alternative strategies for patients in need of functional liver activity. Therefore, congenital monogenic liver diseases have become ideal targets for the use of gene therapy vectors. Metabolic diseases that are potential targets for hepatic gene therapy are summarized in Table 23.1. It is important to note that hemophilia A and B, although they do not cause liver disease, are considered to be treatable by hepatic gene therapy protocols due to the exclusive production of coagulation factors in liver cells.

Understanding Molecular Pathogenesis of Diseases: More Common Diseases as Targets for Hepatic Gene Therapy

Increased knowledge regarding the cellular biology of liver cells in conjunction with the molecular pathogenesis of human diseases has allowed for the identification of new targets for molecular therapy. From the standpoint of hepatic gene therapy, an immense amount of effort has been directed toward establishing new gene therapies to treat liver fibrosis, liver ischemia-reperfusion injury, diabetes, viral hepatitis, and liver cancers. However, the development of therapeutic strategies will not likely be straightforward compared to monogenic diseases. Over the last decade, significant progress has been made in the development of new vectors and methodologies to deliver genes to the liver with high efficiency and safety. For example, genetic materials can be introduced stably and safely into a significant fraction of hepatocytes following intravenous administration of helper-dependent or gutted-adenoviral vectors and recombinant adeno-associated virus (AAV) serotype 8 vectors. Using the recently developed hydrodynamics-based method [5, 6], naked DNA or RNA could be delivered via intravenous routes to hepatocytes efficiently and globally even without a need of viral or non-viral vehicles. The newly developed gene delivery methods and the identification of new molecular targets have enabled therapeutic applications to be expanded from rare life-threatening monogenic diseases to more difficult-to-treat, but prevalent polygenic diseases.

Understanding Liver Immunology: A Key to Successful Hepatic Gene Therapy

In addition to being a protein factory and the major site of metabolic processes, the liver is considered to be a reticuloendothelial system (RES). This would constitute liver as an important and distinctive part of the immune system in the body, which would be constantly exposed to foreign antigens delivered by the portal blood flow from the digestive system. Liver sinusoidal cells, including KCs and liver sinusoidal endothelial cells (LSECs), act as a bodily defense by scavenging exogenous agents to prevent them from entering the systemic circulation (see Fig. 23.1). KCs and LSECs constitutively express major histocompatibility complex (MHC) molecules and co-stimulatory molecules allowing for the

Table 23.1 Preclinical and clinical studies of representative hepatic gene therapies

Disease	Therapeutic gene/molecule	Vector	Preclinical/clinical studies
Monogenic disease			
Hemophilia A	Factor VIII	HDAd	Human, Dog [26, 27, 29], Mouse [27, 30]
		Retro	Human [71], Dog, Mouse
		AAV	Dog [48], mouse [48, 49]
		Nonviral	Mouse [144, 150]
Hemophilia B	Factor IX	AAV	Human [8], NHP [14, 47], dog [46, 52, 53], Mouse [53, 54]
		HDAd	Dog [28], Mouse
		LV	Mouse [87, 88, 91]
		Nonviral	Mouse [148, 151, 214]
α(alpha)1-antitrypsin deficiency	α(alpha)1-antitrypsin	AAV	Mouse [294]
		HDAd	Mouse [25]
GSD type Ia	Glucose-6-phosphate-α(alpha)	AAV	Dog [295, 296], Mouse [296, 297]
		HDAd	Mouse [32]
GSD type Ib	G6PT	FGAd	Mouse
GSD type II (Pompe)	Acid α(alpha)-glucosidase	AAV	Mouse [298, 299]
		HDAd	Mouse [31]
MPS I	α(alpha)-L-iduronidase	LV	Mouse [94, 96]
		Retro	Mouse [216], Dog [73]
MPS IIIA	Sulphamidase	LV	Mouse [97]
MPS IIIB	α(alpha)-N-acetylglucosaminidase	LV	Mouse [95]
MPS VII	β(beta)-glucuronidase	AAV	Mouse [219, 300]
		Retro	Dog [74], Mouse [74, 217]
Fabry disease	α(alpha)-galactosidase A	AAV	Mouse [43]
		LV	Mouse [92]
Gaucher's disease	Glucocerebrosidase	AAV	Mouse [42]
Niemann-Pick syndrome	Acid sphingomyelinase	AAV	Mouse [44]
OTC deficiency	OTC	FGAd	Human [2, 9], NHP, Mouse
		HDAd	Mouse [33]
Crigler-Najjar syndrome	UGT1A1	LV	Rat [85, 93, 218]
		Retro	Rat [75]
Familial hypercholesterolemia	LDL receptor	Retro	Human [69], Rabbit [68]
		HDAd	Mouse [34]
Phenylketonuria	Phenylalanine hydroxylase	AAV	Mouse [45, 220]
Polygenic/acquired disease			
Liver fibrosis	MMP1/MMP8/uPA	FGAd	Rat [225, 227–229]
	TIMP antagonists[a]	FGAd/nonviral	Rat [231, 232], Mouse [230]
	Anti-HSC molecules[b]	FGAd/nonviral	Rat [233–237]
	HGF	Nonviral/FGAd	Dog [116], Rat [115, 239], Mouse [238]
Liver ischemia-reperfusion injury	Anti-ROS molecules[c]	FGAd/nonviral	Rat [242, 243, 245–247], Mouse [244]
	Others[c]	FGAd/nonviral	Rat [249, 250], Mouse [248]
Diabetes mellitus	Proinsulin precursor	FGAd/nonviral/AAV/retro	Rat [251, 253, 259, 260], Mouse [157, 257]
	Transcription factors for β(beta) cell transdifferentiation[d]	HDAd/FGAd/AAV	Mouse [262–266]
Hepatitis B	RNAi against viral RNA	AAV/FGAd/nonviral	Human, Mouse [50, 51, 155, 268–271]
Hepatitis C	RNAi against viral RNA	Nonviral	NHP [281], Mouse [120, 200, 280]
Liver cancers	p53	FGAd	Human [284, 285], Rat [283]
	None	Oncolytic Ad	Human [288–292]. Mouse

[a]TIMP (tissue inhibitors of metalloproteinases) antagonists include MMP mutants, antisense RNA and siRNA

[b]Anti-HSC (hepatic stellate cell) molecules include dominant-negative TGF-β(beta) receptor, TGF-β(beta) antisense RNA, BMP-7, CTGF shRNA, and PDGF receptor β(beta) shRNA

[c]Anti-ROS molecules include SODs, catalase, HO-1 and ferritin. Others include Bcl-2, IL-1 receptor antagonist, and CD40Ig

[d]Transcription factors for β(beta) cell transdifferentiation include Pdx-1, NeuroD, Btc, Ngn3, and MafA

Abbreviations: *FGAd* first-generation adenoviral vector, *HDAd* helper-dependent adenoviral vector, *NHP* nonhuman primate, *GSD* glycogen storage disease, *MPS* mucopolysaccharidosis, *G6PT* glucose-6-phosphate transporter, *OTC* ornithine transcarbamylase, *UGT1A1* UDP glucuronosyltransferase 1 family, polypeptide A1, *MMP* matrix metalloprotease, *uPA* urokinase-type plasminogen activator

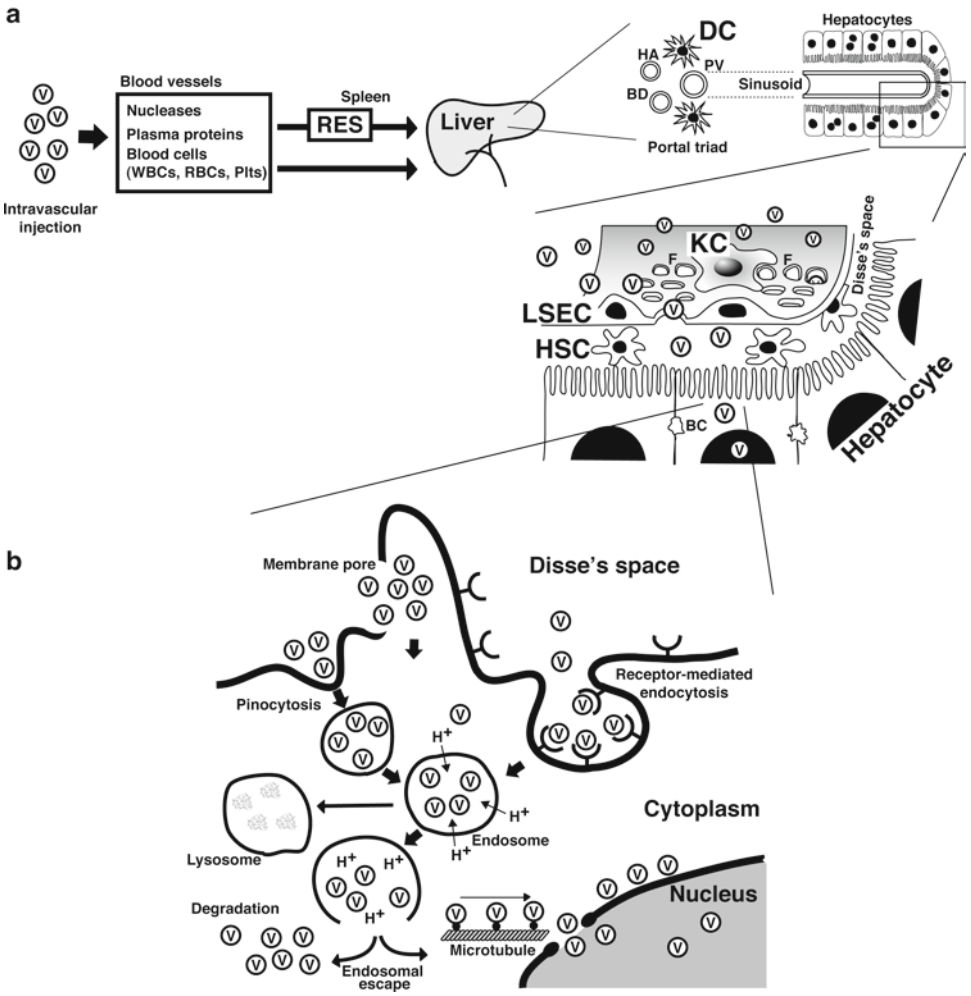

Fig. 23.1 Extra- and intra-cellular barriers in hepatic gene transfer. (**a**) *Potential extracellular barriers*. Intravascularly infused vector particles or molecules (depicted as *circled* V's) first encounter obstacles present in the blood (i.e., nucleases, plasma proteins and blood cells that degrade or sequester vectors). Before vectors reach the liver, the host's extrahepatic reticuloendothelial system (RES) such as spleen macrophages may take up vectors. Once vectors enter the hepatic circulation, vectors may be taken up by hepatic dendritic cells (DCs) that reside around the portal triads. The vector-engulfing DCs migrate to draining lymph nodes for antigen presentation, which may elicit a series of immune reactions. In liver sinusoids, Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) may trap vectors and eliminate them from the blood circulation. The LSEC barrier has small pores of 100–200 nm in diameter termed fenestrae (F) that allow small molecules to enter the space of Disse, where vectors can directly associate with hepatocytes. PEGylation of vectors (i.e., covalent attachment of polyethylene glycol to vector molecules) can overcome many of the above-mentioned extracellular obstacles. (**b**) *Potential intracellular barriers*. Vectors cross the plasma membrane by several different mechanisms that include receptor-mediated endocytosis, non-receptor-mediated endocytosis (pinocytosis), and internalization via membrane pores created by physical or mechanical methods. Endocytosed vectors need to disrupt the vesicles and escape from endosomes by taking advantage of viral machinery or endosomolytic properties of agents incorporated in nonviral vectors. Once vectors are released into the viscous cytoplasm, vectors need to be transported to the nucleus. Viruses can associate with dynein (depicted as black circles on a microtubule in the figure), the motor protein that walks along microtubules toward the nucleus, and are translocated to the perinuclear space. Nuclear import of viruses is an active process by which either viral genome or intact capsid can enter the nucleus. Unlike viral vectors, many nonviral vectors need to be equipped with artificial machinery that can overcome intracellular obstacles. Please note that the intracellular trafficking of vectors and potential barriers therein in this figure do not necessarily apply to all the vector systems. Abbreviations: *WBCs* white blood cells, *RBCs* red blood cells, *Plts* platelets, *HA* hepatic arteriole, *PV* portal vein, *BD* bile duct, *F* fenestrae, *HSC* hepatic stellate cell, *BC* bile canaliculus

presentation of internalized antigens to T cells. Surrounding the portal triads, reside a small number of DCs, the most important professional APCs, that migrate to draining lymph nodes and present engulfed antigens to T cells. Although KCs, LSECs and DCs all function as APCs, the hepatic DCs are relatively immature and are considered to be tolerogenic in comparison to their corresponding cells in the periphery [7]. This unique immunological microenvironment in the liver maintains a balance between immunity against pathogens and tolerance to harmless substances, such as food-derived foreign antigens. A disruption of the homeostatic balance between immunity and tolerance, leads to various

immune-mediated liver diseases, such as viral hepatitis, autoimmune hepatitis, and certain types of drug-induced hepatitis.

Recently, it has become evident that immune responses in gene delivery could pose a significant barrier to successful gene therapy. This is clearly the case from our experiences of the adverse events observed in adenoviral and AAV vector-mediated, liver-directed gene therapy trials in humans [2, 8]. Intrahepatic arterial infusion of a replication-defective first generation adenoviral vector led to the tragic death of Jesse Gelsinger, an 18-year-old boy who voluntarily participated in a dose escalation safety study of adenoviral vector-mediated gene therapy to treat ornithine transcarbamylase (OTC) deficiency [2, 9]. The infusion of a relatively high dose of adenoviral vector into the liver of Mr. Gelsinger induced an unexpectedly strong innate immune response against the adenovirus capsid proteins. This resulted in an overwhelming systemic inflammatory reaction leading to disseminated intravascular coagulation (DIC) and multiple organ failure (MOF) [2]. Since adenoviral vectors are selectively taken up by APCs in the liver and spleen in humans, it is presumed that APCs engulfing pathogenic adenovirus immediately secreted proinflammatory cytokines including IL-6, initiating systemic inflammatory reactions [2].

Similar immune-related effects were observed in a clinical trial investigating the therapeutic potential of recombinant AAV serotype 2 (AAV2) vectors for the treatment of severe hemophilia B. Intrahepatic arterial infusion of AAV2 vector induced AAV2 capsid-specific cytotoxic T lymphocytes (CTLs), eventually destroying vector-transduced hepatocytes in combination with mild liver toxicity [8]. This was unexpected since recombinant AAV was long considered to have little to no immunogenicity. These immune-related observations that were not predictable from all of the preclinical animal studies have prompted researchers to start devoting a substantial amount of effort to better understand the immunology of the liver so that more effective hepatic gene therapy applications can be established. Towards addressing these issues, significant advances have been made over the past several years in understanding the adenovirus-mediated innate immune responses [10]. Recent studies have elucidated the molecular sensors of adenovirus and subsequent signal transduction pathways that trigger the cascade of immune responses, which include the Toll-like receptor (TLR)-9/myeloid differentiating factor 88 (MyD88)-dependent and independent pathways that are activated by adenoviral genomic DNA [11] and the interleukin-1α(alpha) (IL-1α(alpha))-mediated proinflammatory pathway that is triggered by β(beta)3 integrin activation through binding to the arginine-glycine-aspartic acid (RGD) motifs of the viral capsid [12]. In addition, recent studies have underscored the roles of CD4+ CD25+ regulatory T cells (Treg) in AAV-mediated adaptive immune responses [13] and provided clues on how to avoid undesirable immune responses effectively either through the use of immunosuppressive regimens [14] or even by hepatic gene transfer itself [15]. Intriguingly, hepatic gene transfer can be utilized as a therapeutic strategy to suppress autoimmunity by taking advantage of the tolerogenic properties of the liver [16]. Although our knowledge in this new area is in its infancy, autoimmune diseases may become a common therapeutic target for hepatic gene therapy.

Vectors and Methods for Hepatic Gene Transfer and Their Mechanism of Action

Barriers in Hepatic Gene Therapy

For therapeutic molecules to function in target cells within a particular organ, gene therapy vectors and the *de novo* expressed therapeutic molecules need to overcome a number of extracellular and intracellular obstacles (Fig. 23.1). It is important to note that many viral vectors have evolved machinery to overcome intracellular barriers with high efficiency, but they are quite vulnerable to extracellular host defense systems when infused into the blood circulation. Entry of a significant amount of wild type virus particles into the blood does not occur in the course of a natural infection. For this reason, nature has never provided viruses the opportunity to evolve mechanisms to overcome extracellular barriers posed in such an artificial infection process, which is important aspect to consider for gene therapy processes. Unlike viruses, non-viral vectors require mechanisms to surmount not only extracellular hurdles but also intracellular ones that viral vectors can generally get over.

For the in vivo hepatic gene transfer approaches, vectors are first infused into blood circulation in most of the cases. Immediately after vectors are mixed with the blood, their journey to the site of action in the target cells is full of hardships as shown in Fig. 23.1a. Serum components represent the first obstacles for many vectors. Naked DNA and RNA are rapidly degraded with nucleases in the blood. Cationic liposomes and polymers become associated with negatively charged serum proteins and aggregate, forming particles too large to pass through capillary beds and become trapped in the first pass organ, mostly the lung. Adenoviral vectors become trapped by platelets, decreasing their bioavailability [17]. Vectors trapped by the blood components are then captured by the RES and quickly cleared from the blood circulation. Therefore, gene delivery vectors exhibit pharmacokinetic profiles unfavorable for systemic administration in most of the cases. Upon successful arrival to the liver, vectors face the next barriers, KCs and LSECs. KCs are macrophages

residing in the liver sinusoids and efficiently take up foreign particles. LSECs also contribute to the removal of vectors to some extent. LSECs are interspersed with fenestrae of approximately 100–200 nm in diameter and form a physical barrier to Disse's space into which microvilli of hepatocytes extend. It is difficult for particles bigger than the size of fenestrae to reach the hepatocytes [18]. Upon entry to Disse's space, vectors need to cross the negatively charged hydrophobic plasma membrane of hepatocytes, which represents the biggest barrier in gene delivery (Fig. 23.1b). Receptor-mediated endocytosis is the most desirable mechanism for vector uptake; however, in most of the cases, vectors enter cells in a nonspecific manner by mechanisms that remain largely unknown. Cytoplasmic degradation and nuclear transport represent the next obstacles. Endocytosed vectors are subject to degradation and need to escape from endosomes for nuclear transport before they are transported to lysosomes. Adenovirus capsid proteins can catalyze the lysis of endosomal membranes under acidic conditions [19], while many nonviral vectors need to be equipped with endosomolytic agents. It should be noted that cationic polymers such as polyethylenimine (PEI) have some intrinsic endosomolytic properties by the proton sponge mechanism [20]. Nonetheless, viral vectors, in general are very good at surmounting the above mentioned intracellular hurdles. The barriers in nucleus include uncoating vector particles [21], genome processing [22], chromatinization, and gene silencing [23, 24].

Adenoviral Vectors

Recombinant adenovirus (Ad) vectors are the most frequently used viral vectors for in vitro and in vivo experiments, in large part due to their high transduction efficiency in a wide range of cell types in a cell cycle-independent manner, including quiescent hepatocytes in the liver. The most commonly used Ad vectors are those derived from human Ad type 5 (Ad5). In nature, wild type Ad5 is pathogenic and causes self-limited upper respiratory tract infection (i.e., common cold); however, intravascularly infused recombinant Ad5 vectors exhibit strong tropism to the liver, which makes them highly attractive for hepatic gene therapy. For the purpose of hepatic gene transfer, first generation Ad (FGAd) vectors have been extensively studied since the early 1990s to examine their ability to treat monogenic diseases, including hemophilia, OTC deficiency, familial hypercholesterolemia, and α(alpha)1-antitrypsin deficiency. Although these pre-clinical studies showed some encouraging results, the increasing body of evidence from other pre-clinical studies and the unexpected tragic death of Jesse Gelsinger in 1999 demonstrated that early generation Ad vectors have significant drawbacks that limit their further clinical use for the treatment of monogenic diseases due to a number of immune-related problems. They are currently being superseded by helper-dependent Ad (HDAd) vectors, which can direct persistent transduction and exhibit less toxicity [25] as described later. Better safety profiles and persistent therapeutic efficacy of HDAd vectors have been shown in animal models for hemophilia A and B [26–30], glycogen storage diseases (GSDs) [31, 32], OTC deficiency [33], and familial hypercholesterolemia [34].

Adenoviruses are nonenveloped, icosahedral viruses of approximately 100 nm in diameter and their virions contain a single copy of double-stranded linear DNA genome. Wild type Ad5 has a 36-kilobase pair (kb) DNA genome containing a set of the early genes (E1, E2A, E2B, E3, and E4) and a set of the late genes (L1–L5). The tropism of the adenovirus was initially believed to be attributed to the 12 protruding fiber proteins bound to the penton base on the viral capsid. However, there are more recent studies demonstrating the importance of another part of the capsid, which is predominantly comprised of hexons. All of these three major Ad capsid proteins (fiber, penton base, and hexon) appear to play crucial roles in Ad5 infection. In most of the cases, Ad5 infection is mediated by the two-step infection mechanism. First, Ad5 fiber knob positioned at the end of each spike first binds to the coxsackievirus-adenovirus receptor (CAR). Second, the RGD motif in Ad5 penton base protein interacts with α(alpha)vβ(beta)3 or α(alpha)vβ(beta)5 integrins on cell surface, mediating virus internalization. However, infection of intravascularly infused Ad5 to hepatocytes occurs in a CAR independent manner [35], and the most recent studies support a model in which hepatocytes transduction with Ad5 in vivo is mainly mediated by coagulation factor X which bridges Ad5 hexons and an alternative receptor on the surface of hepatocytes [36].

Traditional FGAd vectors are replication-defective viruses with the E1 gene or often both E1 and E3 genes being deleted and replaced with an exogenous gene to be expressed. The E1 gene expression is essential for expression of other viral genes; therefore, E1-deleted Ad vectors do not replicate or produce progenies in infected cells and provide a level of safety with this vector system. Ad vectors with further modifications or deletions that abolish the functions of the E2 and/or E4 genes are referred to second or third generation Ad vectors; however, their advantages over FGAd vectors have remained unclear. Although they are still used for animal studies, early generation of Ad vectors are not appropriate for human use except for vaccination purposes or cancer gene therapy due to the following reasons.

First, to attain therapeutic benefits for hepatic gene therapy, early generation Ad vectors required high vector doses, which inevitably induced significant activation of innate immune responses, which, in some cases, were fatal. Mechanistically, intravascularly infused Ad vectors are first

taken up exclusively by phagocytic cells in the RES, particularly KCs, resulting in sequestration of infused Ad vector particles. The interaction between Ad vectors and KCs is, in part, mediated by blood components, especially platelets, forming virus-platelet aggregates that are eventually entrapped by KCs, leading to thrombocytopenia [2]. The sequestration of the Ad vector by the RES substantially reduces the number of vector particles that can reach hepatocytes and thereby necessitates the use of higher vector doses to achieve efficient hepatocyte transduction. This phenomenon is known as the "threshold effect" [37]. This effect makes it difficult to safely administer therapeutic doses of Ad vectors in humans. In addition, soon after vector administration, Ad vector-activated immune cells, particularly KCs and splenic macrophages initiate the production of various proinflammatory cytokines and chemokines by yet-to-be determined mechanisms. However, there is evidence that the activation of both intracellular sensors such as Toll-like receptor 9 (TLR9) and cell surface receptors such as integrin β(beta)3 are involved [11, 12]. Ultimately, this results in the induction of dose-dependent innate immune responses leading to significant systemic toxicity.

Second, therapeutic gene expression from early generation Ad vectors is transient and generally lasts for only a few weeks following hepatocyte transduction. This is primarily due to the induction of adaptive immune responses against viral proteins and therapeutic gene products, which generate cytotoxic T lymphocytes (CTLs) that specifically eliminate vector-transduced hepatocytes. It has been determined that Ad viral proteins, which in theory should not be expressed from E1-deleted Ad vectors, can be expressed at low levels from the adenoviral backbone genome sequence and serve as their own adjuvant in adaptive immune responses.

To overcome these drawbacks of early generation Ad vectors, HDAd vectors have been developed and become more widely examined as a viable gene transfer vehicle for the liver. HDAd vectors are devoid of all the viral coding sequences with only the viral inverted terminal repeats (ITRs) and packaging signal remaining in the vector genomes, which theoretically makes them safer than the early generation Ad vectors. The nomenclature "helper-dependent" comes from the fact that propagation of HDAd vectors in cells requires the co-infection of a helper virus to produce viral proteins in trans. HDAd vectors can accommodate therapeutic genes of up to 35 kb, but are often stuffed with noncoding mammalian genome-derived sequences, such as an intron from the human hypoxanthineguanine phosphoribosyltransferase (HPRT) gene containing matrix attachment regions (MAR). Care must be taken not to include heterologous DNA with negative effects on the vector, such as lambda phage DNA [38]. Although it is not possible to completely avoid the vector sequestration by the RES and the induction of innate immune responses, HDAd vectors can, in general, direct persistent transgene expression from the liver even though the adaptive immune responses against the viral capsid proteins are elicited. Encouraging results in early preclinical studies led to the advancement to the use of HDAd vectors in a clinical trial to treat a patient with severe hemophilia A in the early 2000s [30], but it lacked therapeutic efficacy while promoting acute hepatotoxicity and thrombocytopenia. More recent studies have greatly advanced HDAd-mediated hepatic gene transfer technologies toward clinical applications. Vector doses and liver transduction efficiency can be significantly decreased and increased, respectively, by delivering HDAd from the hepatic artery with the hepatic vein outflow temporarily blocked by a balloon occlusion catheter percutaneously inserted into the inferior vena cava [39]. In all, HDAd vectors are a significant improvement in the rational design of adenoviruses as gene therapy vehicles, but it is clear that further study is necessary to fully understand their potential as a consistently safe gene therapy modality.

Adeno-associated Virus Vectors

Recombinant adeno-associated virus (AAV) vectors are the most robust in vivo gene delivery vehicle for hepatic gene transfer among all of the currently available viral and nonviral vectors. Although the classical recombinant AAV vectors derived from AAV2 can transduce only 5–10% of hepatocytes in the liver at maximum, contemporary recombinant AAV vectors derived from newly identified serotype such as serotype 8 (AAV8) can achieve almost 100% hepatocyte transduction without any noticeable acute and chronic toxicity in animal models [40, 41]. And importantly, transduction or therapeutic gene expression can stably persist lifelong, as long as hepatocytes do not undergo substantial regeneration due to liver injury. Therefore, recombinant AAV vectors are particularly suitable for gene therapy for diseases that require persistent therapeutic gene expression in hepatocytes such as inborn errors of metabolism and hemophilia, and for diseases that require nearly complete hepatocytes transduction such as viral hepatitis. In fact, their therapeutic efficacy has been shown in the corresponding animal models [42–54].

AAV is a non-enveloped icosahedral replication-defective virus of approximately 20 nm in diameter. Unlike other viral vectors created from pathogenic viruses, AAV has never been associated with any human and animal diseases and elicits minimal or no innate immune responses, making recombinant AAV vectors attractive for clinical use. Wild type AAV has a single-stranded DNA genome of approximately 4.7 kb. Recombinant AAV vectors are devoid of all the viral genome sequence except for the 145-nucleotide

inverted terminal repeat (ITR) at each genome terminus. Recombinant AAV vectors derived from serotypes other than AAV2 have become widely available and are referred to recombinant AAV1, AAV5, and AAV8 vectors and so on. Except for recombinant AAV2 vectors, new serotype vectors are, in general, those that have recombinant AAV2 vector genome encapsidated with a viral coat derived from a serotype other than AAV2 (i.e., pseudoserotyped); therefore, they are often referred to AAV2/1, AAV2/5, and AAV2/8 and so on (genotype/serotype). For in vivo hepatic gene transfer, AAV2 vectors were widely used in preclinical and clinical studies; however, they are being superseded by more robust vectors such as AAV8 vectors [40] and surface-exposed tyrosine mutated AAV vectors [55]. AAV2 vectors become uncoated quite inefficiently in hepatocytes while uncoating of AAV8 vectors is a very rapid process, which might explain the robustness of AAV8 in hepatocytes [21]. Laminin receptor has been identified as one of the potential receptors for AAV8 [56]; however, the exact viral entry pathways of robust serotype vectors remain elusive at present. Capsid ubiquitination, which triggers AAV degradation by the ubiquitin proteasome system, requires phosphorylation of several key tyrosine residues by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK). Therefore, tyrosine mutated AAV capsids are resistant to ubiquitination and hence they can escape from degradation resulting in enhanced transduction [55]. Double-stranded (ds) or self-complementary (sc) AAV vectors have also become increasingly popular as a means to substantially increase transduction efficiency, although their packaging capacity is decreased to half [57]. DsAAV vectors can skip one of the rate-limiting steps in AAV vector transduction (i.e., conversion from single-stranded genomes to double-stranded DNA) and show 1–2 log higher transduction efficiency in the liver and other tissues compared to the conventional single-stranded vectors [58, 59].

The mechanisms of the persistent expression from AAV vector-transduced hepatocytes are yet to be fully understood. Unlike integrating retroviral or lentiviral vectors, the persistent nature of AAV vector-mediated hepatocytes transduction does not result from vector genome integration into the host genome [60]. In hepatocytes, vector genomes predominantly reside as episomal double-stranded circular genomes comprised of only therapeutic gene sequences and AAV-ITRs. The advantage of this genome configuration is that, as demonstrated with minicircles vectors [61], the episomal circular monomer AAV vector genomes have the ability to escape from gene silencing and allow long-term transgene expression presumably due to a lack of negatively-acting cis elements. Induction of immune tolerance to transgene products expressed by AAV vectors also contribute to the persistence in some instances. Studies have shown that recombinant AAV vector-mediated hepatic gene transfer quite often induces immune tolerance to immunogenic transgene products (heterologous proteins or neoantigens), particularly when the transgene expression is restricted to hepatocytes by the use of liver specific promoters [62]. AAV vector-mediated transgene expression in hepatocytes efficiently induces transgene product-specific CD4+ CD25+ regulatory T cells (Tregs) that suppress production of transgene-specific antibody and induction of cytotoxic T lymphocytes against vector-transduced hepatocytes, leading to immune tolerance to the transgene products and transgene-expressing hepatocytes [13].

Although vector genome integration is not the mechanisms for AAV vector-mediated persistent transgene expression in most cases, integration does occur in hepatocytes at nonrandom genomic sites with the ribosomal RNA gene, transcriptionally active genes, and DNA palindromes being significantly preferred [63, 64]. The frequency of integration in hepatocytes is low, but not negligible and is reported to be approximately 0.1% hepatocytes when vector is injected into newborn mice [65]. One study has shown that vector genome integration could cause insertional mutagenesis leading to hepatocarcinogenesis in wild type and mucopolysaccharidosis (MPS) type VII mice when newborn mice received AAV vector [66]. Others failed to show the link between AAV vectors and liver cancer development [67]. Although a question remains as to whether or at what frequency insertional mutagenesis could occur in larger animal models and humans, recombinant AAV vector-mediated liver gene transfer, in general, is considered to be safe.

Retroviral Vectors

Recombinant retroviruses are most commonly derived from gammaretroviruses, which are best exemplified by the murine leukemia virus (MLV). Retroviral vectors were first explored for hepatic gene transfer prior to any other type of viral vectors becoming available. Because of the intrinsic ability of retroviruses to integrate into the host genome, these viruses were initially touted as an ideal gene therapy system, particularly for the treatment of monogenic liver diseases that required life-long therapeutic gene expression. In fact, retroviral vector-mediated ex vivo hepatic gene therapy for familial hypercholesterolemia was shown to be effective in a rabbit disease model [68] and safe in a human clinical trial [69]. However, retroviral vectors are currently limited in use for hepatic gene transfer due to their inefficient ability to transduce hepatocytes in vivo. Induction of liver regeneration by administration of hepatocyte growth factor (HGF) is required for transduction in adult animals [70]. In addition, intravascular injection of retroviral vectors into adult animals induces CTLs against transduced hepatocytes [70]. A phase I trial of intravenous infusion of a retrovirus vector expressing human

coagulation factor VIII into adult severe hemophilia A patients did not show any efficacious outcomes [71], which discouraged further pursuit of this type of in vivo approaches to treat genetic diseases. At present, preclinical and clinical applications of retroviral vector-mediated in vivo hepatic gene therapy are considered mainly in the context of neonatal gene transfer. Neonatal hepatic gene therapy for mucopolysaccharidosis (MPS) type I, MPS type VII, and Crigler-Najjar syndrome by in vivo retroviral vector approaches has been studied in small and large animal models and has been shown to be effective and safe [72–75]. Taken together, retroviral vector-mediated approaches do not appear to be suitable for hepatic gene therapy for adult patients, and they are effective only in ex vivo or neonatal approaches. In addition, there remain ethical issues that need to be addressed for this type of approach to be utilized in fetuses or neonates.

Retroviruses are enveloped, spherical, positive-sense strand RNA viruses of 80–100 nm in diameter with glycoprotein surface projections. Their nucleocapsid contains two copies of polyadenylated single-stranded linear RNA genome of 7–11 kb. MLV has 8.3-kb genome containing the gag, pol and env genes. Recombinant retroviral vectors are made replication defective by replacing the most of the viral genome with a transgene, leaving only the long terminal repeats (LTRs) and short cis-acting elements including the packaging signal and primer binding site. Gammaretroviral vectors integrate into the host genome and express their transgenes only after the host cells undergo a cell division because viral genome integration requires nuclear membrane breakdown. The liver is a quiescent organ with very slowly dividing hepatocytes, the life span of which is several hundred days or more; therefore, it is difficult to transduce hepatocytes with retroviral vectors without any pharmaceutical or surgical intervention. In vivo administration of retroviral vectors are only effective in developing babies whose hepatocytes are still actively dividing, or when liver regeneration is induced physically (i.e., partial hepatectomy, blockage of hepatic circulation, etc.) or chemically (i.e., administration of liver damaging agents such as carbon tetrachloride, growth factors such as HGF and keratinocyte growth factor [KGF or fibroblast growth factor 7, FGF7], and hormones [e.g., thyroid hormone, T3]) [76, 77]. Although retroviral vector-mediated gene therapies were considered to be safe until recently, leukemia caused by gene therapy developed in five patients with X-linked severe combined immune deficiency (SCID) who were treated with retroviral vector-transduced hematopoietic stem cells [3, 78, 79]. This has raised a significant concern about retroviral vector-mediated insertional mutagenesis. It turned out that MLV vectors preferentially integrate near transcription start sites of the host genome [80], which decreases the chance of disrupting open reading frames of cellular genes while increasing the chance of activating a particular set of growth promoting genes/oncogenes (e.g., Lmo2) by inserting the strong viral LTR promoter near the 5′ end of the genes. In hematopoietic stem cell gene transfer, continuous cell division might help accumulate harmful genetic alterations and facilitate the selection for cells with growth advantage; however, this is likely not the case in hepatic gene transfer. Although the potential risk of retroviral vector-mediated hepatocarcinogenesis is considered to be low and no MLV vector-related hepatocellular carcinoma has been reported in animal studies [81], this issue may need further investigation. The use of self-inactivating (SIN) retroviral vectors with a deletion within U3 region of the LTR may overcome this issue to some extent and reduce the risk of insertional mutagenesis and vector mobilization. The U3 deletion abolishes the viral LTR's strong enhancer-promoter activity, and hence it is expected to avoid the activation of neighboring genes and transcription of viral genomic RNA upon integration. However, unfortunately, retroviral vectors with SIN LTRs are hampered by low titers. In addition, it remains to be addressed whether SIN retroviral vectors can indeed substantially decrease their oncogenic potential.

Lentiviral Vectors

Recombinant lentiviral vectors have recently gained much attention and have become increasingly popular in many gene delivery applications due to their ability to integrate into the host genome of not only dividing, but also non-dividing cells in vitro and in vivo and hence, stably transduce quiescent cells, including hepatocytes in the liver [82, 83]. The most widely used lentiviral vectors are those derived human immunodeficiency virus type 1 (HIV-1) coated with an envelope containing the glycoprotein (G) of vesicular stomatitis virus (VSV) (i.e., VSV-G pseudotyped HIV vectors). In hepatic gene transfer, the ability to integrate in both dividing and non-dividing cells has made lentiviral vectors particularly suitable for permanent genetic modifications of hepatocytes by both ex vivo and in vivo approaches. Primary hepatocyte transduction with MLV vectors in vitro is highly inefficient and requires induction of DNA synthesis or cell cycling by growth factors such as HGF. On the other hand, lentiviral vectors can achieve high or even nearly complete in vitro hepatocyte transduction without supplementation of a growth factor using a cell suspension of either freshly isolated hepatocytes or those thawed from frozen stocks [84]. Therapeutic efficacy of the lentiviral vector-mediated ex vivo approach has been shown in Gunn rats [85], an animal model for Crigler-Najjar syndrome, and its feasibility and safety has been demonstrated in non-human primates [86]. In vivo approaches, i.e., intravascular injections of lentiviral vectors, have been studied in animal models of hemophilia A and B [87–91] and several types of inborn errors of metabolism

including Fabry disease [92], Crigler-Najjar syndrome, [93] and mucopolysaccharidoses (MPSs) [94–97]. These studies have shown partial to full correction of the disease in prenatal, neonatal, and adult animals. Hepatocytes in prenatal and neonatal livers are more susceptible to transduction with lentiviral vectors than those in adult livers; therefore, in vivo intervention in the earlier stages of life is more effective than vector administration into adult animals [92, 96]. One drawback in lentiviral vector-mediated hepatic gene transfer is the potential induction of cytotoxic T lymphocyte (CTL)-mediated immune responses against the expressed transgene products, limiting the duration of transgene expression [88, 89, 93, 98]. However, this issue has recently been overcome in mouse models by using a combination of a liver-specific promoter and a microRNA (miRNA)-regulated system that maximally abolishes transgene expression in cells in hematopoietic lineages including APCs [99].

Lentiviruses are in the family, Retroviridae, and their vial genomes contain accessory genes in addition to the gag, pol, and env genes. HIV-1 has six accessory genes, the vif, vpr, vpu, tat, rev, and nef genes, which are involved in the pathogenesis of AIDS. Among these accessory genes, only the rev gene product is important for recombinant HIV vector production, and it can be supplied in trans. The third generation VSV-G pseudotyped HIV vectors, the most widely used lentiviral vectors at present, are devoid of all the accessory genes as well as the gal, pol, and env genes, are in a self-inactivating (SIN) configuration, carry only minimal cis elements, and are produced from at least four split components. This multi-component system minimizes vector genome recombination in packaging cells that may cause inadvertent production of replication competent viruses; therefore, it offers maximal biosafety. The VSV-G envelope facilitates fusion with cellular membranes of a variety of cell types and significantly increases the host range. When VSV-G pseudotyped HIV vectors are infused intravascularly in mice, the vectors mainly transduce the liver and spleen. Specifically, liver sinusoid endothelial cells (LSECs), KCs, and splenic macrophages, dendritic cells, and B cells are more efficiently transduced than hepatocytes [100, 101]. This not only poses the immune-related problem as described earlier, but also causes sequestration of vectors by non-hepatic cells, which makes gene transfer to hepatocytes inefficient and necessitates administration of relatively high vector doses for hepatocyte transduction [100, 101].

Despite the ability of lentiviral vectors to integrate non-dividing cells and transduce hepatocytes better than MLV vectors, the quiescent nature of hepatocytes makes hepatocyte transduction relatively inefficient as some studies have shown that efficient hepatocyte transduction in vitro and in vivo requires cell cycling [102, 103]. However, liver transduction could be enhanced by the use of newer versions of lentiviral vectors that carry additional cis-acting elements including the sequence responsible for forming a central DNA flap found in the HIV pol gene (i.e., the central polypurine tract (cPPT) and central termination sequence [CTS]) and woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) [87, 100, 101, 104]. The inclusion of the central DNA flap markedly enhances nuclear transport of vector genomes in non-dividing cells [105, 106] and increases transduction efficiency in hepatocytes in vivo.

Insertional mutagenesis is always a concern in any integrating vectors and so is it to lentiviral vectors. Unlike MLV vectors, lentiviral vectors preferentially integrate in transcriptionally active genes with no preference for near transcription start sites [107]. This represents a safer spectrum of integration sites than that of MLV vectors [108]. In mouse models, SIN HIV-1 vectors are less oncogenic than MLV vectors [109], and in humans, there has been no evidence of oncogenesis attributed to HIV integration in patients with active HIV infection. For these reasons, the risk of insertional mutagenesis by lentiviral vectors has been considered to be significantly lower than MLV vectors. However, in May 2009, the French Medicine Agency reported that clonal dominance emerged in one of two β(beta) thalassemia patients infused with hematopoietic stem cells modified by a SIN HIV-1 vector. Clonal dominance is a consequence of insertional mutagenesis potentially leading to carcinogenesis, and has been observed in patients treated with retroviral vector-transduced hematopoietic stem cells [3, 78, 79]. Although leukemogenesis and hepatocarcinogenesis involve different oncogenic processes, the risk of insertional mutagenesis leading to liver cancer will need to be carefully addressed. One study has reported a frequent occurrence of murine liver cancers in lentiviral vector-mediated in utero and neonatal gene transfer [110]. The lentiviral vectors used in this study was equine infectious anemia virus (EIAV)-based vectors in SIN configuration and not HIV-1 vectors. To date, the cause of the tumorigenesis in this study has yet to be determined. In all, lentiviral vectors remain the most commonly used integrating vectors for hepatic gene transfer. Because integration is not an absolute requirement for persistent transgene expression in hepatocyte as demonstrated by other viral and nonviral vectors, and because there is a potential problem with insertional mutagenesis, non-integrating lentiviral vectors are being pursued as alternative methods to mediate persistent transgene expression in hepatocytes [111, 112].

Non-viral Vectors

Commonly used non-viral vectors can be categorized into the three main groups, according to the type of vector particle formulations; lipoplex, polyplex, and naked nucleic acids

in which cargos are complexed and protected with liposome, linear or spherical (dendritic) polymers, and nothing, respectively. In general, nonviral vectors have three major advantages over viral vectors; ease to manufacture on a large scale, absence of products derived from potentially infectious agents, and potentially low immunogenicity. Disadvantages include low in vivo transfection efficiency and short-lived transgene expression. In addition, certain types of vectors such as lipoplexes exhibit significant dose-dependent acute toxicities. Over the last two decades, there have been significant advances in nonviral vectors in the following three aspects: the development of new and improved nonviral carriers; the development of new technologies to physically or mechanically deliver genes to target cells; and the improved design of cargos (e.g., plasmid DNA) to avoid cellular innate responses and transgene silencing. In the context of hepatic gene transfer, particularly important are liver-targeting nonviral vehicles and naked DNA vectors in conjunction with hydrodynamics-based gene delivery [5, 6]. These new vectors and methods are currently under intensive investigation toward the establishment of gene therapy for hemophilia, liver fibrosis, diabetes, liver ischemia-perfusion injury, hepatitis and liver cancers, demonstrating proof-of-principle of the approaches in various animal models (Table 23.1).

Naked DNA vectors represent the simplest type of nonviral vectors. The proof-of-concept that naked DNA can be delivered and function in animal tissue by direct in vivo injection was first established in the muscle [113] and subsequently in other organs including the liver [114]. However, unless the hydrodynamics-based strategy is applied, simple intravascular injection of naked DNA vectors can achieve only limited transfection efficiency in the liver, which is too low to attain therapeutic efficacy to treat genetic diseases. Although nonparenchymal liver cells (i. e., KCs, LSECs, and HSCs) can be transfected to some extent, it is very difficult to transfect hepatocytes. Notwithstanding such inherent inefficiencies, intravascular infusion of naked DNA has still remained as one of the appealing approaches to treat acquired diseases that do not require high levels of transgene expression in the liver, such as liver fibrosis [115, 116].

In nonviral hepatic gene delivery, development of liver-targeting nonviral vectors is key to success and has been the major focus in the research. In most of the settings in hepatic gene therapy, vectors should be delivered intravascularly to the liver because local or regional transfection in liver cells should not be sufficient for therapeutic purposes. Since nonviral vehicles by themselves are not normally equipped with tissue or organ-specific targeting machinery, dissemination of vectors to non-target tissues is a significant concern in systemic administration of nonviral vectors. In this regard, it has been shown that liver targeting can be achieved by the incorporation of a liver-homing device into chemical formulations of the vehicles. Such a modification not only helps vectors home in the liver, but also has a potential to facilitate vector uptake by target cells by hijacking a receptor-mediated endocytosis pathway(s). Liver-targeting devices include: asialoglycoprotein receptor ligands such as asialoorosomucoid [117–119], apolipoprotein A-I [120] and hepatitis B virus (HBV) L antigen [121] for hepatocyte targeting; hyaluronan, which is the ligand for hyaluronan receptor for endocytosis (HARE), for targeting to LSECs [118]; vitamin-A for hepatic stellate cell targeting [122]; and liver-targeting synthetic peptides indentified from random peptide libraries by selection [123]. Using these targeting nonviral vehicles, therapeutic nucleic acids (plasmid DNAs, siRNAs, and oligonucleotides) can be delivered to hepatocytes or LSECs following intravascular vector injection. Therapeutic levels of blood coagulation factors VIII or IX could be expressed in mouse livers by intravenous infusion of a hepatocyte-targeting polyplex or LSEC-targeting nanocapsule encapsulating each corresponding therapeutic plasmid DNA [117, 118], or by intravenous injection of a human hepatoma cell-targeting HBV L particle-based vector in a mouse xenograft model of hepatocellular carcinoma [121]. A recent study has shown that efficient in vivo delivery of siRNA specifically to hepatocytes can be achieved by intravenous injection of siRNA conjugated with a polyethylene glycol (PEG)-shielded asialoglycoprotein receptor-targeting polymer that only disassembles in acidified endosomes and therefore mediates dynamic activation of siRNA specifically in hepatocytes [124]. Relatively efficient albeit no or less-specific delivery of cargos to hepatocytes via intravascular routes can be achieved by polyethylenimine (PEI) polyplexes, cationic liposomes including HVJ liposome [125], polycationic lipid-cholesterol liposome [126], and cationic-lipid protamine DNA complex [127]. However, vectors unequipped with a liver-targeting or shielding device may need to be infused directly into the hepatic circulation for efficient liver transfection. This is because cationic polyplexes and liposomes tend to associate with negatively charged blood components due to electrostatic attraction, resulting in aggregation and entrapment by capillary beds in the first pass organ, which is the lung, when infused intravenously. siRNAs conjugated with cholesterol or those conjugated with a liposomal formulation termed SNALP (stable nucleic acid lipid particles) can deliver siRNAs efficiently, although not specifically, to hepatocytes to knockdown target gene expression (e.g., the apolipoprotein B gene) [128–130]. Cholesterol-conjugated antagomir (i.e., chemically modified single-stranded RNA oligonucleotides complementary to microRNAs) can be delivered to hepatocytes in mice efficiently but not specifically by intravenous injection and mediate a marked reduction of endogenous microRNAs including liver-enriched miR-122 [131], which is a potential target for the therapy of hepatitis C [132] and hyperlipidemia [131].

Another important key to success is the design of cargos, which are most of the time plasmid DNA. Transgene expression cassettes constitute an integral part of plasmid DNA vectors; therefore, it is critical to design expression cassettes that can direct efficient and persistent liver-specific transgene expression. Hepatocyte-specific, strong enhancer-promoter elements that are currently widely used are chimeric elements comprising a promoter derived from either the α(alpha)1-antytripsin gene or the thyroid hormone-binding globulin (TBG) gene and an enhancer or a regulatory sequence derived from either the apolipoprotein E gene, the α(alpha)1-microglobulin/bikunin precursor gene or other types of liver specific elements [54, 133–136]. However, even with such strong liver-specific promoters, transgene expression from conventional plasmid DNA vectors persist only for a short period of time in hepatocytes, declining to baseline levels in days to weeks. Initially, the short-lived hepatic expression from plasmid DNA vectors was presumed to be mainly due to their non-integrative nature causing gradual loss of vector genomes overtime. However, it has turned out that plasmid DNA vector genomes can be stably maintained and do not necessarily disintegrate in hepatocytes even if transgene expression has disappeared. Now it has become clear that plasmid backbone sequences covalently attached to a transgene expression cassette can cause heterochromatinization of the neighboring transgene, leading to transgene silencing in hepatocytes for a not-yet-defined mechanism [23, 137, 138]. In addition, the presence of unmethylated CpG motifs derived from bacterial sequence in DNA cargos can have significant detrimental effects on transgene expression as described below [139–141]. Therefore, attention needs to be paid not only to transgene expression cassettes, but also to plasmid DNA backbones composed of bacteria-derived sequences.

An important issue in systemic intravenous injection of plasmid DNA vectors is that, when complexed with cationic liposomes (i.e., injected as lipoplex), vectors induce production of proinflammatory cytokines including interferon γ(gamma) (IFN-γ(gamma)) and tumor necrosis factor α(alpha) (TNF-α(alpha)) leading to acute inflammatory responses and hepatocyte damage [139–141]. These adverse effects are usually self-limited, but can be fatal. Studies have identified unmethylated CpG motifs present in plasmid DNA vectors as the main culprit of this toxicity [139]. CpG motifs in mammalian genomes occur at a much less frequency than those in bacteria-derived sequences, and cytosine residues in the motifs are frequently methylated. On the other hand, CpG motifs in bacteria-derived sequences are all unmethylated. This makes plasmid DNA vector genomes unusually unmethylated CpG-rich molecules serving as pathogen-associated molecular patterns (PAMPs) that are recognized by toll-like receptor 9 (TLR9) [142]. TLR9 mediates activation of the NF-κ(kappa)B pathway resulting in production of pro-inflammatory cytokines, although alternative pathways seem to be involved [142]. Interestingly, plasmid DNA or liposome alone does not cause the above toxicities, indicating complexity of the reaction [141].

Nevertheless, recent advances in understanding the mechanisms of action of nonviral vectors have led to clues on how to overcome the obstacles. CpG-methylated or CpG-depleted plasmid DNA have been created and proved to reduce acute toxicity [143]. It has been shown that plasmid DNA vectors linearized with a restriction enzyme(s) in such a way that plasmid backbone sequence is completely separated from its neighboring transgene expression cassette can avoid gene silencing and mediate life-long transgene expression at high levels in hepatocytes in rodents [23, 138]. In this approach, the DNA fragments containing only the transgene cassette and devoid of all bacterial sequences are self-circularized in hepatocytes, forming supercoiled double-stranded monomeric molecules termed "minicircles," which have the ability to escape from gene silencing [61, 137]. Minicircles can be produced in E. coli on a large scale using a bacteriophage Φ(phi)C31 integrase-based system and can mediate stable and long-term transgene expression in hepatocytes in vivo [61]. Certain types of liver-specific enhancer-promoters have been shown to escape the plasmid backbone silencing effects to some extent even in the presence of covalently attached bacterial sequences. Such liver-specific elements consist of a combination of one or more of the following elements: the human apolipoprotein E hepatic locus control region, the human albumin enhancer and/or promoter, the human α(alpha)1-antitrypsin promoter, and human coagulation factor IX intron A [136, 144, 145].

Hydrodynamics-Based In Vivo Transfection Method

Naked DNA vectors have recently gained significant attention as simple and potentially more efficient and safer nonviral vectors for hepatic gene transfer. This is mainly due to the advent of the hydrodynamics-based hepatic gene delivery, which was developed in late 1990s by two independent research groups [5, 6]. In mouse models, this method involves a rapid (5–7 s) injection of naked DNA in a volume of 8–10% body weight saline through the tail vein. With this rapid and high volume injection, up to 40% of hepatocytes can be transfected in mouse liver. The application of this approach is not limited to gene delivery with naked plasmid DNA vectors and can be extended to delivery of siRNAs, oligonucleotides, and other types of nucleic acids [146], and argumentation of transduction efficiency with viral vectors [147]. In fact, the therapeutic efficacy of this approach has been demonstrated in small animal models for various human diseases including

hemophilia [144, 148–151], inborn errors of metabolism [152–154], viral hepatitis [155], acute liver failure [156], diabetes [157] and cancers [158–162]. This method also provides an important tool to create new mouse models of human diseases [155, 163], to study biological functions of various gene products in vivo, and to optimize transgene expression cassettes in gene therapy vectors [134, 145]. One drawback is that the hydrodynamic method, even though effective in rodents, cannot readily be translated into the clinic due to the requirement of a large-volume intravenous infusion in a very short period of time, which would not be tolerated in humans. To overcome this obstacle, a computer-controlled hydrodynamic gene delivery system has been developed in which a computer connected to the infusion device monitors real-time intravenous pressure and controls the rate of infusion to follow the most appropriate venous pressure-time curve for hepatic gene transfer [164]. This system has been shown to direct plasmid DNA transfection in hepatocytes in pig liver at 2–3 logs higher efficiencies than those in previous pig studies by a minimally invasive procedure [165]. This approach will become increasingly feasible by further studies on efficacy and safety in large animal models.

The mechanism of action of hydrodynamic gene delivery has been partially elucidated. Rapid injection of a large volume of solution containing DNA significantly increases venous return to the heart, thereby increases cardiac preload which is far in excess of cardiac output, causing transient cardiac congestion. Rapidly elevated central venous pressure produces a backflow to the liver, and a significant volume of injected solution, which is minimally contaminated with the nuclease-rich blood, enters the liver sinusoids in retrograde. The increased fluid volume in the liver and the resulting force enlarge the fenestrae of LSECs, partially disrupt the structure of LESC layers, and create small pores in the plasma membranes of hepatocytes [166, 167], the process of which is called hydroporation [166]. This allows easier extravasation of DNA-containing solution, and enables DNA uptake via the membrane pores. Alternative mechanisms have been proposed that involve massive formation of endocytic vesicles in hepatocytes [168] or a receptor for DNA [169]. For more details, the reader is referred to the two review papers by the groups who have done pioneering research in this field [170, 171].

Transposon and Phage Integrase-Based Approaches: Sleeping Beauty Transposon and Phic31 Integrase Systems

Permanent insertion of transgenes into the host genome leading to stable expression of therapeutic gene products is an attractive approach for the treatment of many genetic diseases and some of acquired diseases. Until late 1990s, efficient in vivo vector genome integration could be attained only by the use of viral vectors. With the awakening of Sleeping Beauty (SB) transposons that can transpose (jump from one chromosomal location to another) in mammalian cells [172] and with the discovery that bacteriophage site-specific integrase phiC31 mediates integration of donor DNA sequences in specific genomic sites in mammalian cells [173], it has become possible to establish systems that allow insertion of foreign genetic materials into the host genome of mammalian cells with high efficiency and in a predicted manner. In both SB transposon and phiC31 integrase-based systems, donor DNA (vector) carrying a transgene is delivered to target cells together with transposase or integrase-expressing elements in a form of either DNA or mRNA. This results in integration of a portion of donor DNA containing the transgene (in the SB transposon system) or the whole donor DNA sequence (in the phiC31 system) into the host genome at relatively random locations (in SB transposon system) or at a limited number of locations (in phiC31 system). These two systems are currently widely used as efficient integrating vector systems in various in vitro and in vivo applications. Transposition or integration occurs in adult quiescent hepatocytes in mouse liver and human primary T cells without prior stimulation, making it most likely that the systems have the ability to modify the host genome in nondividing cells [148, 151, 174]. In the context of hepatic gene therapy, naked plasmid DNA vectors equipped with this integration machinery have been shown to mediate stable marker gene expression and have curative potential for hemophilia [118, 148, 151, 175] and metabolic diseases [152, 153, 176]. Although the systems have been utilized mostly in the context of naked DNA vectors, the integral elements can be incorporated in helper-dependent adenoviral vectors [177, 178] or nonintegrating lentiviral vectors [179, 180]. The advantage of the system is the ability to direct persistent and stable transgene expression upon hepatocyte division or liver regeneration, which may occur in association with normal growth in young children, hepatitis, or drug-induced liver injury, and experimentally in association with partial hepatectomy or administration of hepatotoxic agents such as carbon tetrachloride [60, 148, 175, 177]. Another potential advantage could be the ability to escape transgene silencing, which is an issue of episomal plasmid DNA vectors, although a study has shown that a significant fraction of integrated SB transposon vector genomes become silenced in vitro [24].

SB transposon is a synthetic DNA transposon that belongs to the Tc1/mariner family of mobile elements. This member of DNA transposons have been found in a wide range of lower and higher eukaryotes. Although nematodes and flies contain active elements, Tc1/mariner transposons in vertebrates are defective due to multiple inactivating mutations; therefore they are viewed as fossils that were once active at their birth. Phylogenic analysis of dead copies of the salmonid

subfamily of fish elements, identified consensus DNA and protein sequences, which led to successful reconstitution of a fully active transposase, SB10, by directed mutagenesis [172]. The SB transposon vector system involves co-transfection of a plasmid DNA containing a transgene placed between the two terminal inverted repeats of approximately 0.2 kb derived from a Tanichthys albonubes Tc1-like transposon and the second plasmid DNA or RNA expressing the SB transposase. In transfected cells, SB transposase binds on the two inverted repeats, precisely excises the transgene, cuts the target sequence at a TA dinucleotide, and mediates insertion of the excised piece of DNA into the host genome, the process of which is called a "cut and paste" mechanism [172]. Unlike HIV-1, MLV, and AAV vectors, which show significant integration site preference for particular genomic regions [63, 80, 107], SB transposons integrate relatively randomly although there are minor preferences for AT-rich regions and microsatellites [181]. Since the original SB10 was constructed, several new versions of hyperactive SB transposases have been identified by site-directed mutagenesis or using directed evolution techniques. Such newly evolved transposases can mediate transposition reactions at up to 100-fold higher efficiency in vitro; however, their in vivo activities have yet to be better characterized [182].

PhiC31 integrase is an enzyme encoded by Streptomyces bacteriophage phiC31 and mediates site-specific recombination between the attP site (attachment site Phage) in the phiC31 phage genome and attB site (attachment site Bacteria) in the host Streptomyces genome. In the recombination process, a crossover occurs between the attB and attP sites and allows insertion of the whole circular genetic material containing the attP site (donor) into the attB site in the host genome (recipient). The crossover creates two attB and attP hybrid sequences (i.e., the attR and attL sites) between which the donor sequence is inserted. An excision reaction between the attR and attL sites can occur, but it requires the phage-encoded excisionase. In mammalian cells, phiC31 integrase can recognize somewhat degenerate attachment sites, and mediate integration of attB site-carrying episomal circular donor DNA into degenerate attP sites in the host genome. Such degenerate attP sites are called pseudo attP sites. In mammalian genome, there are only a limited number of pseudo attP sites that are recognized by phiC31 integrase, which confers site specificity of the system [151, 173, 183]. Recent studies using in vitro culture cells have shown that at least 80% of integration events are likely a consequence of phiC31 integrase-mediated attB-pseudo attP recombination with 7.5–8.7% of integrations occurring at a specific pseudo attP sites in the human chromosome 19q13.31 [183, 184]. Other pseudo attP sites that have been identified as hot spots are the one at human chromosome 8p22 [173] and the mpsL1 site in the mouse genome [151]. The mpsL1 was originally identified as a hot spot of vector genome integration in the liver of mice that received a hydrodynamic injection of naked DNA vectors with phiC31 integration machinery; however, it did not represent a hot spot when the phiC31 integrase system was used in mouse liver in the context of helper-dependent adenoviral vector [178]. Although this system offers an attractive tool to restrict vector genome integration sites, recent studies have revealed that the system induces various types of genomic aberrations including deletions, rearrangements, and chromosomal translocations in vitro [183–185]. Such genomic aberrations are rarely found in the SB transposon vector system. Whether phiC31-induced genomic aberrations occur in vivo is yet to be determined. Studies are ongoing to further improve the system, which include the identification or creation of integrase variants with improved properties [186].

Gene Repair and Gene-Targeting-Based Approaches

Targeted repair of defective genes in somatic cells in vivo, if successful, would be the perfect approach for gene therapy for genetic diseases. The approach would result in no insertion of genetic materials that may contain elements detrimental to the host. In addition, it would not disrupt functional sequences in the host genome. Furthermore, repaired genes would be regulated in a physiological manner by their own autologous promoters. On the other hand, gene targeting approaches can insert therapeutic genes into a specific safe locus in the host genome or specifically disrupt dominant-negative disease genes. Different types of methods for targeted gene repair have been developed and are currently available. Among them, gene targeting by homologous recombination using zinc finger nucleases [187, 188] and gene targeting adeno-associated virus (AAV) vectors [189] represent the systems that are most efficient and have become increasingly popular. Studies have demonstrated efficient gene targeting or gene correction in vitro with the above-mentioned techniques using clinically relevant experimental models [190, 191], showing promise of these approaches for ex vivo gene and cell therapies. However, in vivo gene modification would be a significant challenge because efficiencies achievable with currently available methods are too low to be therapeutic. In the in vivo context, gene targeting AAV vectors could correct defective genes in hepatocytes only at frequencies of 10^{-4} to 10^{-5} of hepatocytes in mouse liver [192]. Other gene targeting strategies include single-stranded oligonucleotides, triplex-forming oligonucleotides, small fragment homologous replacement (SFHR), and chimeraplasts (RNA/DNA oligonucleotides) [193]. With these methods, successful targeted gene modifications can be achieved but at low frequencies, and improving targeting efficiency remains

as a significant challenge. Chimeraplasts-mediated in vivo hepatic gene correction once gained significant attention due to its impressively high gene modification frequencies with up to ~50% alleles being modified in rats [194, 195]. However, the efficacy of chimeraplasts has remained controversial because many laboratories have failed to reproduce such high efficiencies [196].

RNA Interference-Based Approach

RNA interference (RNAi) was discovered a decade ago and rapidly emerged as powerful tool to inhibit gene expression in a gene-specific manner [197]. Since then, introduction of RNAi into cells has quickly evolved into a novel approach with enormous potential for the treatment of various diseases [198, 199]. In general, RNAi-mediated targeted gene knockdown can be achieved by delivering either of the following elements into cells: small interfering RNAi (siRNAs), which are double-stranded linear RNA molecules of 19–25 bp with 2-nucleotide overhangs at each terminus; or nonviral or viral RNAi vectors that express short hairpin RNA (shRNA) transcribed by either RNA polymerase pol II or pol III promoter. In 2002, in vivo RNAi in mammals was first demonstrated in mouse liver by delivering naked siRNA to hepatocytes using a hydrodynamics-based in vivo transfection method [200]. Since then, many studies have investigated therapeutic potential of RNAi delivery as well as its mechanism of action using various animal models. In the context of hepatic gene therapy, we have now come to the point that one can deliver siRNA or shRNA to a substantial fraction or virtually all of hepatocytes and attain dramatic and persistent reduction of target gene expression in the liver, at least in animal models, by a single intravenous injection of AAV8 vectors [51] or repeat intravenous injections of newly-developed polyplexes [129]. Therapeutic efficacy or phenotypic changes due to targeted gene knockdown in the liver have been demonstrated in animal models of chronic hepatitis B virus (HBV) infection by HBV RNA shRNA expression [50, 51, 201, 202], acute liver failure by delivering siRNA against Fas or caspase 8 [156, 203], non-alcoholic fatty liver disease by fatty acid transport protein 5 (FATP5) shRNA expression [202], and arterial atherosclerosis by introduction of cholesterol-conjugated siRNA against apolipoprotein B (ApoB) [128].

However, accumulated experience of RNAi in mammalian cells has revealed new obstacles that impose significant challenges particularly in the in vivo use of RNAi for therapeutic purposes. First, although siRNAs had been thought to be short enough to evade the recognition of RNA sensors (e.g., protein kinase R (PKR), TLR3 and retinoic acid inducible gene-1 (RIG-1)) that trigger type I interferon responses, recent studies have revealed that siRNAs also serve as potential triggers of innate immune responses [204, 205], particularly when delivered with chemically synthesized vehicles [130]. Second, it has become evident that it is not easy to specifically knockdown only the target gene due to the difficult-to-control, widespread, off-target gene silencing effects [206]. This unintended gene silencing is mainly due to the microRNA-like off-target effects, the result of gene silencing mediated by a short ~7-nucleotide region analogous to the "seed region" found in microRNA [206]. The microRNA-like silencing of many unintended target transcripts has been demonstrated in the liver of mice receiving siRNA, adding greatly to the challenge of identifying siRNAs with perfect specificity in vivo [207]. Third, exogenously introduced siRNAs or shRNAs can be toxic in vivo and even lethal when they are highly expressed. AAV8 vector-mediated overexpression of shRNA in mouse hepatocytes could result in functional saturation of exportin 5, the endogenous microRNA nuclear export machinery, leading to fatal damage of hepatocytes [51]. Thus, although RNAi-mediated targeted gene knockdown represents a promising approach, the method is still in its infancy for clinical translation. Nevertheless, the research in this field is progressing very rapidly, and some of the obstacles have been overcome to some extent by the use of noninflammatory 2′ O-methyl-modified siRNAs [208], asymmetric interfering RNAs (aiRNAs) which reduce off-target silencing [209], or shRNAs expressed by a Pol II promoter [201], or by expressing interfering RNAs in the context of microRNA [210, 211].

Preclinical and Clincal Applications of Hepatic Gene Therapy

A summary of preclinical and clinical applications of representative hepatic gene therapies is provided in Table 23.1. The reader is referred to the references cited therein for details.

Hemophilia

Hemophilia is a group of X-linked inherited bleeding disorders caused by deficiency of either blood coagulation factor VIII (hemophilia A) or IX (hemophilia B). Hemophilia, particularly hemophilia B, is the genetic disease that has been most extensively studied in the context of hepatic gene therapy. This is attributed to the following features that make hemophilia the most attractive target disease for gene therapy. Only >1% levels of coagulation factors in the blood can significantly improve the symptoms of severe hemophilia (patients with <1% levels of coagulations factors).

In addition, the therapeutic window of transgene expression levels is pretty wide from 1% to supraphysiological levels; therefore, tight control of transgene expression by a regulated promoter is not necessary. Furthermore, both murine and canine hemophilia A and B models are available that faithfully recapitulate the human disease, and therapeutic efficacy in these animal models can be easily and reliable assessed.

In the 1990s and early 2000s, many preclinical studies using hemophilia animal models demonstrated safety and potential therapeutic efficacy of liver-directed approaches using retroviral, adenoviral, and AAV vectors. However, the studies have also revealed some limitations. The representative limitations in these earlier studies include very low transduction efficiency with retroviral vectors, short duration of transgene expression with adenoviral vectors, and occasional production of inhibitors against factor VIII or IX with any types of vector. Nonetheless, the strong safety profiles with many encouraging results in the earlier preclinical studies led to the initiation of the three liver-directed gene therapy phase I clinical trials in the United States between 1999 and 2001. These liver-directed trials were the single-treatment, dose-escalation study of gene therapy for severe hemophilia A by intravenous infusion of an MLV vector expressing B-domain deleted factor VIII [71], the trial of factor VIII gene transfer for severe hemophilia A by intravenous infusion of a minimal adenovirus (mini-Ad) vector carrying the full-length factor VIII gene [30], and the safety study in subjects with severe hemophilia B by hepatic arterial infusion of AAV2 vector expressing factor IX [8]. Although >1% levels were detected in all the trials with the highest level being 11% in the AAV2 trial [8], none of the clinical studies demonstrated persistent and stable expression of circulating blood coagulation factors in the blood, and the maximum duration of expression at therapeutic levels was limited to 8 weeks [8]. It is important to note that mini-Ad and AAV2 trials experienced significant adverse events; thrombocytopenia and hepatotoxicity in the mini-Ad trial resulting in early termination of this trial, and hepatotoxicity in the AAV2 trial [8]. A body of evidence has indicated that AAV2-capsid specific cytotoxic T lymphocytes were activated upon AAV2 vector infusion and destroyed AAV2-transduced hepatocytes in humans, which caused hepatotoxicity and limited the duration of transgene expression [8, 212]. Facing such immunological impediments, the current main research focus of hepatic gene therapy for hemophilia has been directed toward understanding immunology in gene therapy and establishing the strategy to avoid inhibitor formation and immune responses against viral capsids. An appropriate regimen that suppresses unwanted adaptive immune responses has been identified as a combination of mycophenolate mofetil (MMF) and sirolimus (rapamycin), an immunosuppressive regimen that does not eliminate Tregs [14]. This regimen is currently under investigation in a new clinical trial for AAV2-mediated liver-directed gene therapy for severe hemophilia B [90]. Self-complementary AAV8-factor IX vector [47] will also be tested in an upcoming clinical trial [90]. Although the following approaches are still in preclinical stages, safety and therapeutic efficacy has been demonstrated with hepatic gene delivery by AAV factor VIII vectors [48, 213], helper-dependent adenoviral vectors [26, 28, 29], and nonviral vectors [118, 148, 150, 214].

Inborn Errors of Metabolism

Inborn errors of metabolism constitute a wide range of categories of genetic diseases caused by defects of a gene involved in various metabolic pathways. Among them, GSDs and lysosomal storage diseases (LSDs) including MPSs are the representative target diseases for hepatic gene therapy. These storage diseases result from inability to catalyze a biochemical reaction(s) in a particular metabolic pathway(s) due to deficiency of an enzyme or an enzyme-associated protein (e.g., enzyme activator protein). The diseases also could be attributed to impairment of other types of biological activities such as deficiency in a transport protein. As a consequence, upstream or incompletely processed metabolites accumulate in multiple organs including the liver, spleen, brain, muscle, heart, bones, joints, eyes, vessels, bone marrow, and lymph nodes, showing complex and progressive clinical symptoms. The liver is commonly affected and often exhibits massive enlargement. Although many of GSDs and LSDs are monogenic diseases, not all of them are good targets for gene therapy. Some enzyme defects in GSDs affect only liver or skeletal muscle; however, many LSDs including GSD type II (Pompe's disease), affect various types of cells in multiple organs, making it in theory difficult to genetically correct all the affected cells by gene delivery. What makes hepatic gene therapy approaches quite feasible for the treatment of such systemic lysosomal diseases is "cross-correction" [215], the mechanism by which affected cells, remote from the liver, endocytose soluble lysosomal enzymes that are secreted into the bloodstream from genetically corrected cells. This endocytosis process involves mannose 6-phosphate receptors on the surface of the plasma membrane of cells to be corrected. In cells, lysosomal enzymes receive mannose-6-phosphate tags in the Golgi apparatus and are directed to and fused with lysosomes through binding to mannose-6-phosphate receptors on the inner surface of the Golgi membrane. A majority of lysosomal enzymes traffic to lysosomes in this manner under normal conditions; however, they can traffic to the plasma membrane and can be secreted when overexpressed in hepatocytes by gene transfer.

Gene therapy approaches to treat GSDs and LSDs favor intervention at earlier ages (i.e., neonatal gene transfer) rather than adult ages [72, 215]. Although vector injection at adult ages has been shown to be effective in animal models, it generally shows less therapeutic efficacy than neonatal injection and is often hindered by inhibitor formation [72, 96, 98, 215]. Neonatal gene therapy approaches have advantages, in that they can prevent many clinical manifestations before they become overt and potentially prevent unwanted immune responses against therapeutic proteins due to the immaturity of the immune system. In many applications of the neonatal gene therapy, the main target organ to be transduced is the liver, where hepatocytes and nonparenchymal cells undergo many cycles of cell division associated with growth of the host. Therefore, MLV and lentiviral vectors appear to be preferable because liver transduction with these integrating vectors in theory should not decline with growth once transduction has been established. In fact, neonatal gene therapy with MLV and lentiviral vectors has been shown to be effective in small and large animal models of MPS I [73, 96, 216], MPSVII [74, 217], Fabry disease [92] and Crigler-Najjar syndrome [75, 218]. In contrast, AAV vector-mediated hepatocyte transduction dramatically drops by >100-fold in weeks due to the episomal nature of AAV vectors when vector is injected into neonates [65]. However, AAV vector-mediated neonatal gene therapy is also effective in MPS VII mice [219]. Neonatal injection of AAV vectors can mediate transient albeit early elevation of transgene expression at high levels in the liver [65], which appears to be critical for successful prevention of clinical manifestations of the disease [72]. AAV8 vectors are outstanding in treating animal models of symptomatic inborn errors of metabolism due to their ability to transduce hepatocytes stably and at extremely high efficiencies when vectors are injected at older ages [42–45, 220].

Liver Fibrosis

Liver fibrosis is the consequence of chronic liver injury caused by a variety of causes, most commonly alcohol abuse, viral hepatitis, and non-alcoholic steatohepatitis (NASH). The process of fibrosis involves continuing hepatocyte damage and loss, continuing hepatocyte regeneration, and a progressive increase in extracellular matrix (ECM), which eventually leads to disruption of the architecture of the liver and severe hepatic dysfunctions. Historically, advanced liver fibrosis or cirrhosis was viewed as an irreversible disease stage. However, in the last two decades, our understanding of molecular pathogenesis of liver fibrosis has been greatly advanced, and now it is viewed as a dynamic process that involves a perturbed balance between profibrotic and antifibrotic mechanisms, and therefore liver fibrosis is potentially reversible by augmenting the antifibrotic mechanisms [221, 222]. Such contemporary concepts of the disease have opened a new avenue to the treatment of liver fibrosis by intervening the profibrotic and antifibrotic processes using gene delivery techniques. The potential gene therapy approaches include, inhibition of collagen production and enhancement of collagen degradation, the blockage of HSCs activation and induction of HSC apoptosis, protection from hepatocyte apoptosis and augmentation of hepatocyte regeneration, and modulation of inflammatory responses by cytokines.

The key players in liver fibrosis are hepatocytes, KCs, HSCs, a series of chemical mediators and enzymes derived thereof, and collagen, the major constituent of ECM in fibrotic livers. Hepatocyte injuries caused by various insults induce inflammation and release a variety of chemical mediates (cytokines, chemokines, and reactive oxygen spices (ROS)) from dying hepatocytes and their neighboring cells. These mediators activate HSCs directly and indirectly through the mediators released from KCs and T cells that become activated in the process of the innate immune responses. Upon activation, quiescent HSCs proliferate and undergo substantial phenotypic changes and transform into myofibroblast-like cells. The HSC-derived myofibroblasts are the major source of the increased collagen [223] and tissue inhibitors of metalloproteinases (TIMPs) in ECM. Myofibroblasts are also recruited from bone marrow [224]. HSCs produce metalloproteinases (MMPs) for tissue remodeling; however, their collagenolytic activity has been inhibited by increased levels of TIMPs secreted from activated HSCs. Based on such current knowledge about molecular pathogenesis of liver fibrosis, the following gene therapy approaches have been investigated and proven to be effective in liver fibrosis animal models such as carbon tetrachloride and bile duct-ligation models. Adenoviral vector-mediated overexpression of MMPs [225–228] or urokinase-type plasminogen activator (uPA) [229] in hepatocytes have been shown to enhance ECM degradation and hence regress liver fibrosis. Similar effects have been observed when the function of TIMPs is inhibited by TIMP antagonists delivered by adenoviral or nonviral vectors. Such antagonists include inactive MMP mutants [230], TIMP antisense RNA [231], and TIMP siRNA [232]. Transforming growth factor-β(beta) (TGF-β(beta)), platelet-derived growth factor (PDGF), tumor necrosis factor-α(alpha) (TNF-α(alpha)) and connective tissue growth factor (CTGF) have been identified as cytokines that activate HSCs and promote fibrogensis. Therefore, antagonizing these cytokines using gene delivery techniques represents potential approaches to inhibit HSC transformation and reduce the production of ECM. These types of approaches have been shown to be effective in the context of adenoviral vector-mediated hepatic expression

of dominant-negative TGF-β(beta) receptor [233], TGF-β(beta) antisense RNA [234] and bone morphogenetic protein-7 (BMP-7) that counteracts the TGF-β(beta)'s fibrogenic signaling [235], and plasmid DNA vector-mediated expression of shRNA against CTGF or PDGF receptor β(beta) subunit [236, 237]. Forced expression of hepatocyte growth factor (HGF) in the liver by viral or nonviral gene delivery [115, 116, 238, 239] has been shown not only to protect hepatocytes from apoptosis and augment regeneration, but also to possess antifibrotic activity, in small and large animals, by the mechanisms that include the suppression of the profibrotic TGF-β(beta) pathway and induction of MMPs [115]. A recent report demonstrates that intravenous injection of a vitamin A-coupled liposome can target HSCs via receptors for retinol binding protein and successfully ameliorate fibrosis in rat livers when this vehicle carries siRNA against the rat homology of human heat shock protein 47 (HSP47), a chaperone that facilitates collagen secretion [122]. Improvement of current vector systems and further elucidation of the molecular mechanisms of liver fibrosis will make gene therapy for liver fibrosis a more efficient and feasible approach for clinical application.

Ischemia-Reperfusion Injury

Hepatic ischemia-reperfusion injury is one of the serious complications in liver transplantation and could cause graft dysfunctions in the early phase after transplantation. It also occurs in many other pathologic conditions including trauma, shock, and surgical hepatectomy. Studies have identified key players and various important molecular mediators and signal transduction pathways responsible for the onset and progression of cellular damage in the dynamic process of hepatic ischemia-reperfusion injury. They include KCs, T cells, neutrophils, reactive oxygen species (ROS), damage associated molecular pattern molecules (DAMPs), proinflammatory cytokines such as TNF-α(alpha) and IL-1, nitric oxide (NO), and signal transduction pathways involving TLRs and NF-κ(kappa)B. Briefly, hepatic ischemia-reperfusion triggers release of an excess of ROS from activated KCs and induces oxidative stress in hepatocytes and sinusoidal endothelial cells. These liver cells also become exposed to increased levels of endogenous ROS released from mitochondria. This oxidative environment damages hepatocytes releasing DAMPs. ROS and DAMPs activate signal transduction pathways involving NF-κ(kappa)B and activator protein 1 (AP-1) in KCs, releasing proinflammatory cytokines and chemokines, which subsequently activate T cells and recruit neutrophils. Recruited neutrophils also release ROS and damage hepatocytes. Although it remains difficult to establish effective preventive or curative treatment of ischemia-reperfusion injury that is readily translatable into the clinic, several targets for molecular therapy have been identified and tested in animal models [240, 241].

The most common approach is to express antioxidant genes to counteract ROS and prevent tissue damage. In this context, over expression of superoxide dismutase 1 (SOD1 or Cu/Zn SOD) or SOD2 (Mn SOD) [242, 243], catalase [244], heme oxygenase 1 (HO-1) [245, 246], or ferritin [247] by hepatic gene delivery has been shown to attenuate liver tissue damage in animal models of liver ischemia-reperfusion injury. SODs and catalase scavenges ROS; HO-1 has cytoprotective effects; and ferritin sequesters ferrous ions (Fe^{2+}), the source of ROS. Other hepatic gene delivery approaches include expression of anti-apoptotic genes such as Bcl-2 in hepatocytes [248], expression of IL-1 receptor antagonist to suppress proinflammatory signals [249], and blocking the CD40-CD154 co-stimulatory pathway by CD40Ig expression, which suppresses T cell activation by APCs expressing CD40 on the surface membrane [250]. The majority of reported preclinical studies have utilized first generation adenoviral vectors to transduce donor livers in situ, which in some studies was followed by orthotopic liver transplantation in recipient animals. Adenoviral vectors have been preferred for this purpose due to the ease of production of high titer stocks, the high liver transduction efficiency, and the rapid onset of transgene expression; however, clinical application of this approach may require the use of more advanced and safer viral or nonviral vector systems.

Diabetes

Despite remarkable progress in the management of diabetes mellitus by oral drugs, insulin replacement therapy, and pancreatic islet transplantation with improved immunosuppressive regimens, no curative therapies are available and the current treatment still remains unsatisfactory. In this regard, gene delivery-based approaches hold promise as a long-term curative therapy for diabetes, in particular for type I diabetes. The approaches include delivery of cytoprotective and/or growth promoting genes to pancreatic β(beta) cells, genetic manipulation of immunomodulatory systems to prevent autoreactive T cell-mediated destruction of β(beta) cells, and gene delivery to hepatocytes to make insulin-producing cells in the liver.

A straightforward approach in liver-directed diabetes gene therapy is to deliver the preproinsulin (proinsulin precursor) gene to hepatocytes by means of viral or nonviral vectors [251]. This approach is often referred to hepatic insulin gene therapy (HIGT) [252, 253]. Since non-β(beta) cells including hepatocytes do not possess the specific endoproteases (prohormone convertases) that cleave proinsulin into mature insulin, the insulin gene used for HIGT has

generally been engineered in such a way that proinsulin can be efficiently processed into active and mature insulin by a ubiquitously-expressed endoprotease such as furin [254]. Physiological control of insulin secretion from engineered hepatocytes is possible to some extent by incorporating hormone and glucose-responsive elements in transgenes. Such positive and negative regulatory elements include those derived from the genes for insulin-like growth factor binding protein (IGFBP), L-type pyruvate kinase, glucose-6-phosphatase (G6Pase), glucose transporter-2 (GLUT2) and insulin [255–258]. In preclinical animal studies, various types of the insulin gene constructs with or without regulatory machinery have been delivered to hepatocytes in the contexts of adenoviral, retroviral, AAV, and naked DNA vectors, demonstrating sustained insulin secretion and phenotypic improvement of diabetic animal models [157, 251, 253, 259–261].

Although it is still in its infancy, an emerging and more compelling hepatic gene transfer approach is the induction of transdifferentiation of hepatic cells (hepatocytes and hepatic progenitor cells) to insulin-secreting β(beta) cells. The proof-of-concept that this approach can reprogram liver cells into β(beta) cells and indeed ameliorate the disease was first demonstrated in streptozotocin-induced diabetic mice injected with an adenovirus vector expressing the pancreatic and duodenal homeobox gene 1 (Pdx-1) [4]. NeuroD, betacellulin (Btc), neurogenin 3 (Ngn3), and RIPE3b1 (MafA), which are downstream factors of Pdx-1 in the course of pancreatic differentiation, also have the ability to drive the transdifferentiation process when they are delivered to mouse livers by adenoviral or Ad/AAV hybrid vectors [262–264]. When Pdx-1 or Ngn3 was expressed in the liver in the context of AAV or naked DNA vectors, their effect was substantially diminished [265, 266], but could be restored by irrelevant adenoviral vector coinfection [266]. This suggests potential roles of host proinflammatory responses in the transdifferentiation process triggered by overexpression of the key transcription factors. Nonetheless, generation of self surrogate β(beta) cells in the liver would provide a promising and effective approach and potentially solve many issues inherent to other therapies.

Viral Hepatits

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are prevalent diseases, and approximately 400 and 200 million people are affected worldwide, respectively. They pose a significant health threat eventually leading to liver cirrhosis and hepatocellular carcinoma in a substantial portion of affected individuals. Currently available antiviral therapies using interferon α(alpha) (IFN-α(alpha)) and nucleoside/nucleotide analogs are effective only in a minority of the patients. Facing the limitations of current therapeutics, there has been an increasing enthusiasm for developing novel effective therapies by taking advantage of the emerging RNAi technology. HBV is an enveloped spherical virus containing a partially double-stranded DNA genome of approximately 3.2 kb. The genome is in a relaxed circular form maintained by overlapping 5′ ends of the two DNA strands. HBV life cycle is unique among DNA viruses, in that the viral DNA genome replicates via reverse transcription of viral mRNA. The negative-sense DNA strand is transcribed into four overlapping mRNAs with 3′ regions being shared. The longest is the 3.5-kb pregenomic RNA from which negative sense DNA strand is reverse-transcribed during viral replication. HCV is an enveloped spherical virus containing a positive-sense single-stranded RNA genome of approximately 9.6 kb. The 5′ untranslated region (UTR) of the viral genomic RNA contains an internal ribosome entry site (IRES) essential for translation of viral polyproteins, and in the 3′ UTR resides an element responsible for replication. HCV genome replication occurs through negative sense strands and is catalyzed by the virally-coded RNA-dependent RNA polymerase. Although the viral life cycles of HBV and HCV are significantly different, both involve RNA intermediates in viral genome replication. This makes RNAi-based approaches particularly effective for both viruses because RNAi not only eliminates viral proteins but also directly inhibits virus replication by destroying viral pre-genomic or genomic RNAs. Successful suppression of viral protein expression and viral replication by over 90% has been demonstrated in vitro and in vivo experiments in which RNAi was delivered into cells in a form of shRNA or siRNA using viral or nonviral vectors [267]. To introduce persistent RNAi to hepatocytes, adenoviral and AAV8 vectors expressing pol II or pol III promoter-driven shRNAs have been used [50, 51, 268, 269]. However, highly efficient nonviral methods for in vivo delivery of siRNA to hepatocytes have begun to emerge [124, 199, 270, 271] and they are expected to become increasingly common in the near future although repeated injections will be required for sustained effects.

In vivo efficacy of RNAi-based hepatitis B therapies have been investigated in mouse models of chronic HBV infection. The first model is HBV transgenic strains, the hepatocytes of which carry episomally replicating HBV genomes and secrete high titers of HBsAg, HBeAg, and HBV DNA into the blood circulation [50, 51, 268]. The other commonly used model is the murine hydrodynamic injection model of HBV replication, in which HBV DNA genome is delivered to the liver as plasmid using hydrodynamic tail vein injection [155]. This model also recapitulates HBV replication in human hepatocytes chronically infected with HBV. Target genomic sites for effective HBV RNAi are the sites that are contained in multiple HBV RNA species, such as the surface

antigen coding region [50, 51, 155, 268, 272]. Long-term inhibition of HBV replication in hepatocytes that lasts for at least 5 months has been demonstrated with AAV8 vectors [50, 51], while adenoviral vector-mediated shRNA delivery results in limited persistence up to several weeks [268, 269] and could trigger interferon responses even in the context of helper-dependent adenoviral vectors [273]. One shortcoming of the above animal models is that they do not develop chronic hepatitis; therefore, a question remains as to whether or not RNAi therapies can ameliorate chronic hepatitis in humans. Nonetheless, the first clinical trial for liver-directed RNAi-based therapy was initiated in United States in 2008. In this trial, plasmid DNA expressing four shRNAs that target different sites of the HBV genome are conjugated with liposome and delivered to patients' liver to treat chronic hepatitis B, according to the sponsoring company's press release.

As for hepatitis C gene therapy, although in vitro HCV replicon systems has been established [274], and significantly accelerated research on HCV virology and anti-HCV drug discovery, preclinical studies of hepatitis C gene therapy have been hampered by the lack of convenient animals models. The best mouse model for hepatitis C at present is SCID-Alb-uPA mice with humanized liver [275]. In this model, human hepatocytes transplanted in SCID-Alb-uPA mouse livers repopulate and replace mouse hepatocytes by selective destruction of mouse hepatocytes expressing urokinase-type plasminogen activator (uPA) [275]. However, this model is not readily available and has several issues including complexity of the system. Nonetheless, with the in vitro replicon system, RNAi-mediated suppression of viral protein expression and inhibition of viral genome replication by at least 80–90% has been demonstrated [276–279]. Targets for effective HCV RNAi include the NS5B region coding RNA-dependent RNA polymerase and 5′UTR [276–278]. Preclinical studies using rodents have shown that intravascularly delivered siRNAs or shRNAs by means of nonviral or viral vectors could substantially eliminate RNA molecules containing HCV-derived target sequences and suppress protein expression in hepatocytes [120, 200, 280]. However, whether RNAi-based approaches can inhibit HCV replication in vivo has yet to be addressed. In this regard, inhibition of viral replication by RNAi therapy has been demonstrated in the GB virus B/monkey system, a nonhuman primate model for hepatitis C [281].

Although RNAi-based therapies for chronic hepatitis B and C hold promise, there are issues as described in above (RNA Interference-Based Approach). Another important issue is that viruses would eventually become resistant to RNAi making the therapies ineffective. Because HBV's reverse transcriptase and HCV's RNA-dependent RNA polymerases are not proofreading enzymes and prone to introduce mutations, viruses can rapidly evolve in vivo and make a large pool of quasispecies, a population of viruses exhibiting genetic diversity. Antiviral therapies provide the power of selection that leads to the emergence of resistant clones from the pool of quasispecies. It is the Achilles' heel of RNAi that a single mismatch generated during virus evolution is enough to abolish the effect. Targeting multiple sites or alternative strategies will be required to overcome this issue.

Liver Cancers

Hepatocellular carcinoma (HCC) and metastatic colon cancer in the liver are difficult to treat with currently available therapeutic modalities due to their multifocal and chemoresistant nature. Therefore, gene therapy approaches for these two cancers have been enthusiastically investigated in preclinical animal studies and human clinical trials. The strategies can be classified into three categories: delivery of therapeutic molecules (genes or other types of nucleic acids) to cancer cells, delivery of therapeutic molecules to nonmalignant cells surrounding or associated with liver tumors, such as hepatocytes or tumor vessels, and virotherapy using oncolytic viruses. The first category includes p53 gene therapy, suicide gene therapy based on herpes simplex virus thymidine kinase or cytosine deaminase, TRAIL (TNF-related apoptosis inducing ligand) gene therapy, and immunomodulation by introducing cytokine genes. The second strategy creates the milieu suppressive to tumor growth by expressing cytokines, antiangiogenic factors, and antiproteolytic factors. In the context of hepatic gene delivery for liver cancers, adenoviral vectors have been most commonly used. Representative approaches that have been studied in human clinical trials are intravascular or intratumoral administrations of adenovirus p53 (Ad-p53) or oncolytic adenoviruses.

Ad-p53 gene therapy has been extensively studied in patients with advanced lung cancers, showing excellent safety and proof-of-principle of the approach [282]. This approach has also been shown to be effective against HCC in a rat liver tumor model, in which restoration of wild type p53 in p53 deficient liver tumors could suppress tumor growth and induce apoptosis following intrahepatic arterial infusion of Ad-p53 [283]. In the Ad-p53 human clinical trials, Ad-p53 was delivered to the liver via hepatic artery in combination with hepatic transcatheter arterial chemoembolization (TACE) in patients with advanced HCC [284, 285]. Although Ad-p53-mediated gene therapy for HCC in the current form did not appear to provide significant additional benefits over conventional therapies [284], the clinical studies demonstrated safety profiles of the therapy [284, 285].

Oncolytic adenovirus or conditionally-replicating adenovirus (CRAd) is an Ad that is designed to kill cancer cells by

taking advantage of its lytic life cycle. The principle of this strategy is to allow adenovirus to progress into the lytic life cycle only in cancer cells, leading to cancer-specific cell lysis. This can be achieved by either of the following: deletion or mutation of the Ad E1A or E1B gene, the gene product of which binds to Rb and p53, respectively, and inhibits their function; or driving the Ad E1 gene by a tumor-specific promoter such as the α(alpha)-fetoprotein (AFP) gene promoter. An oncolytic adenovirus in the former type, named *dl*1520 (ONYX) [286], was originally predicted to replicate and lyse cells only in cancer cells that do not express p53; however, it has turned out that cancer selectivity is not dependent on the status of p53 and rather it is mediated by other mechanisms including the loss of E1B55K-mediated late viral RNA transport that limits viral replication in normal cells [287]. Nonetheless, the excellent tumor specificity has led to dl1520-mediated clinical trials for metastatic liver cancers [288, 289], HCC [290], and hepatobiliary carcinomas [291]. The studies demonstrated safety, but revealed no significant responses. It should be noted that one patient who received dl1520 via the intrahepatic artery developed transient albeit grade 4 systemic inflammatory response [288], which is a reminiscent of the fatal adverse event in the Ad-OTC clinical trial [2]. A subsequent clinical study of dl1520 for the treatment of metastatic colon cancer in patients who failed prior chemotherapy has shown potential clinical benefits [292].

These earlier clinical studies with Ad-p53 and dl1520 showed only limited responses. However, various novel and potentially more effective approaches are currently under investigation in culture cells and experimental animals [293]. With the advanced technologies of in vivo gene delivery and novel therapeutic modalities such as RNAi, increasing knowledge about molecular pathogenesis of liver cancers will provide new opportunities for gene therapy to substantially improve therapeutic outcomes in difficult-to-treat liver cancers.

Conclusions

The liver is the most studied organ for in vivo gene transfer because the liver is involved in a variety of diseases, many vectors have inherent preference for the liver, and the liver provides an excellent platform to study various types of biologicals and drugs. Gene delivery technologies are rapidly progressing and it appears that we have started overcoming the biggest hurdle in hepatic gene transfer, i.e., inefficient delivery of therapeutic cargos to target cells in the liver. We now have modalities to deliver cargos to the liver with high efficiency and safety, at least in animal models. Immunological barriers, nonspecificity of vectors, requirement of high doses that may be toxic to humans, and uncertainty of long-term safety including the risk of hepatocarcinogenesis in humans, still remain as issues to be addressed and solved. With the solution of these issues, hepatic gene delivery would become an indispensable and common modality to treat various hereditary and acquired, and benign and malignant human diseases.

Acknowledgement Preparation of this chapter is in part supported by the National Institution of Health (R01 DK078388) and Cystic Fibrosis Foundation (R883-CR02). The author is most grateful to Nicole Kotchey, Frank Park and Christopher Naitza for their invaluable assistance in preparation of the manuscript.

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Part IV
Basic Principles of Hepatobiliary Pathology

Chapter 24
Liver Cell Death

Harmeet Malhi and Gregory J. Gores

Introduction

Hepatocyte cell death is an indispensible and cardinal element that precipitates and perpetuates liver injury [1]. Accordingly, liver injury can result in acute or chronic liver disease. This distinction between acute and chronic liver disease is arbitrary; a reflection of the etiology and time course of liver injury. Hepatocytes are the predominant parenchymal cell type in the liver. Commonly encountered injurious stimuli, such as viruses, alcohol, fatty acids, and bile acids, primarily target hepatocytes [2–9]. Cholangiocytes and sinusoidal endothelial cells are targeted in a stimulus-specific and disease-specific manner, such as, in biliary disease, allograft rejection, and vascular insults [10–14]. These insults result in cell death which leads to secondary pathologic processes such as the occurrence of inflammatory infiltrates which further accentuate liver injury. The inflammatory cells mediate and facilitate cell death via secretion of cytokines [15]. Homeostatic renewal in the liver also hinges on cell death. In this instance, senescent or damaged cells are removed by apoptosis, as liver mass and function are renewed. This process ensues continuously and seamlessly from birth to death and is not associated with inflammation or with liver injury.

Several modes of cell death are described in liver diseases as in other organ systems [16, 17]. Apoptosis and necrosis in the liver can occur in any cell type and in response to various stimuli [2, 6, 7, 9, 13, 18–20]. Knowledge of these modes of cell death and their recognition in the liver is based on specific morphologic features and detection of specific signaling mechanisms in human disease as well as experimental models. One or both modes of cell death can be detected in the liver depending on the nature of the injurious stimulus. While features of both apoptosis and necrosis are observed at the organ level and activated at a cellular level, in the death of an individual cell only one mode of cell death is exclusively operative, i.e., either apoptosis or necrosis. When both pathways are activated, an intrinsic failure of one pathway to progress or exogenous blockage of one pathway leads to cell death via the alternative pathway [21, 22]. Necroptosis, a relatively newly named mode of cell death, refers to necrotic cell death that is manifest under very specific and well-defined conditions and is "programmed" similar to apoptosis. Necroptosis occurs in cells with a genetic, e.g., lack of caspase 8, or chemical, e.g., caspase inhibitors, blockade of apoptosis, upon stimulation with death receptors (DRs), Fas ligand, or tumor necrosis factor α(alpha) [23–25]. Thus, apoptosis and necrosis share many similarities. For example, they can be activated by the same stimulus and both are dependent on mitochondrial dysfunction. On the other hand, the cellular signaling pathways and resultant morphology are indeed distinct. In either case, understanding the mechanisms that lead to death is more important than classifying the morphology of the dead cell. In this chapter the authors first provide an overview of each mode of cell death; the role of membrane-bound intracellular organelles is also discussed in the context of each mode of cell death. The later portion of this chapter is focused on select molecular mediators of apoptosis that have a well-recognized role in liver cell death.

Overview of Apoptosis

Apoptosis is morphologically characterized by condensation of nuclear chromatin, membrane blebbing, nuclear fragmentation, and breakdown of the dying cell into membrane-bound apoptotic bodies [26]. This morphology is affected by a series of well-defined, active, energy-requiring, biochemical pathways that are conserved across cell types and intrinsic to the dying cell, which culminate in the activation of caspases (cysteine proteases which cleave at aspartate residues) [27] and endonucleases. Mechanistically, apoptosis can be triggered by extracellular or intracellular pathways (Fig. 24.1). Cell surface DRs and their cognate ligands activate the extracellular pathway of apoptosis. The intracellular (mitochondrial) pathway of apoptosis can be activated by a

G.J. Gores (✉)
Division of Gastroenterology and Hepatology,
Mayo Clinic College of Medicine, Rochester, MN, USA
e-mail: gores.gregory@mayo.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_24, © Springer Science+Business Media, LLC 2011

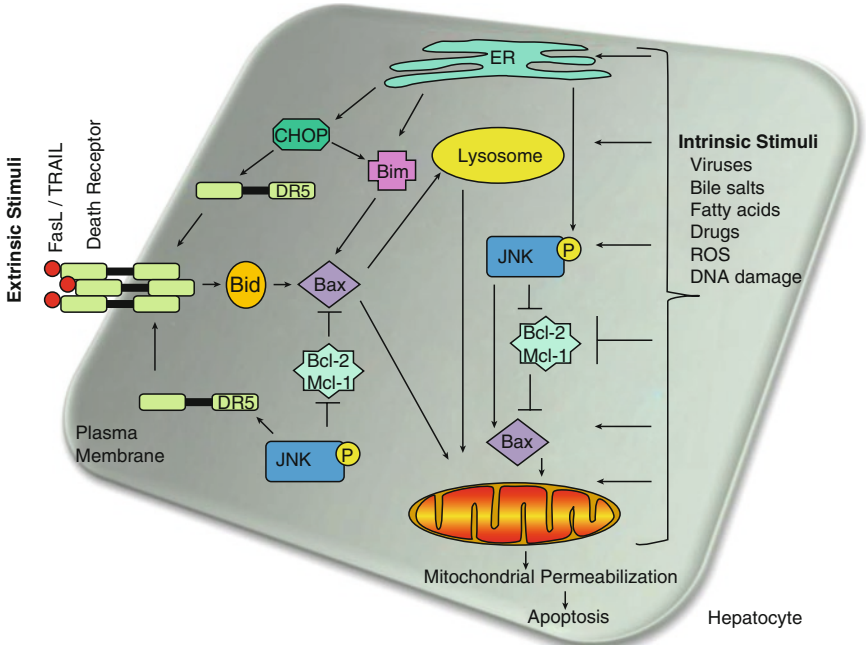

Fig. 24.1 Shared cellular apoptotic machinery. The extrinsic and intrinsic apoptotic pathways converge on mitochondria in hepatocytes. Extrinsic death stimuli, Fas Ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), ligate with their cognate receptors, leading to receptor oligomerization and intracellular signal transduction. Tumor necrosis factor alpha (TNFα(alpha)), not shown here, activates a different signaling pathway. The success of death ligands in inducing hepatocyte apoptosis is further governed by the proapoptotic (Bid, Bax, Bim) and antiapoptotic (Bcl-2, Mcl-1) Bcl-2 family proteins. These can be activated by transcriptional or posttranslational events. Bid is cleaved by activated caspase 8 (not shown here), further activating Bax, which can permeabilize lysosomes and mitochondria. This process is inhibited by Bcl-2 and Mcl-1. Intrinsic apoptotic stimuli cause intracellular perturbations that activate the apoptotic machinery. Endoplasmic reticulum (ER) stress results in transcriptional upregulation of C/EBP homologous protein (CHOP), which further transcriptionally upregulated DR5 and Bim expression. C-jun N-terminal kinases (JNK) can activate Bax and inactivate Bcl-2 via phosphorylation. JNK can also transcriptionally enhance the expression of DR5. All these events culminate in mitochondrial permeabilization and postmitochondrial events, resulting in the activation of effector caspases and apoptosis

myriad of cellular stressors, e.g., metabolic stress including oxidative stress, DNA damage, chemotherapeutic agents, toxins, and intracellular calcium [1]. In type II cells, defined exclusively in the context of Fas signaling, hepatocytes being a prime example, mitochondrial involvement is an obligate requirement for the execution of apoptotic cell death, therefore apoptotic signals from the extracellular pathway also converge on mitochondria [28]. In isolated hepatocytes, attached to collagen or matrigel, Fas-induced apoptosis occurs in the absence of mitochondrial activation, converting the cells to type I; however, this phenomenon has only been observed in hepatocytes isolated from C57/Black 6 mice upon attaching the cells to collagen or matrigel matrix [29].

Homeostatic apoptosis in the liver, similar to developmental apoptosis, is nonimmunogenic, and apoptotic bodies are presumably effectively removed by hepatic macrophages, i.e., Kupffer cells within the liver. This process is difficult to detect in vivo; however, in experimental models, Kupffer cells effectively phagocytose hepatocyte apoptotic bodies [30]. This is based on recognition of specific cell surface signals on apoptotic bodies; the best recognized is phosphatidylserine. In health, a balanced activation of both proinflammatory (TNF-α(alpha)) and anti-inflammatory (TGF-β(beta) and IL-10) signals is observed in Kupffer cells upon engulfment of apoptotic bodies. On the other hand, apoptosis of infected or injured hepatocytes results in the activation of immune response and inflammatory pathways. The exact underlying mechanisms for immune activation by apoptosis of infected or pathologically injured hepatocytes are not fully understood. One possible, albeit simplistic, explanation is that this is a purely quantitative phenomenon. Homeostatic apoptosis and resultant immune activation are well regulated and of low-grade and occur without tissue injury. Pathologic apoptosis, on the other hand, either overwhelms the regenerative capacity of the liver as in fulminant hepatitis, or activates inflammatory cascades as in chronic hepatitis, leading to liver injury. The more complex and probable explanation is that injured and infected cells display distinct cell surface signals that communicate this "diseased death" to macrophages, leading to the activation of inflammation and immune cells. It is also probable that the actual contents of the apoptotic body contribute to the ultimate tissue response, inflammatory vs. noninflammatory. Activated lymphocytes and other immune cells are also removed by apoptosis; derangements in this process could also amplify

liver injury. Ongoing liver apoptosis activates regenerative pathways in the liver, and indeed, in an experimental model of Fas-induced apoptosis, hepatocytes resistant to apoptosis expand and repopulate the liver [31]. Furthermore, stellate cell (cells that line liver sinusoids and function as pericytes in health and myofibroblasts in disease) activation occurs upon engulfment of apoptotic bodies leading to hepatic fibrogenesis [32]. Stellate cell apoptosis, in turn, terminates the fibrogenic response activated upon engulfment of hepatocyte apoptotic bodies [19, 33, 34]. In animals resistant to Fas-induced hepatocyte apoptosis, abrogation of apoptosis is associated with decreased inflammation, injury, and stellate cell activation, thus mechanistically linking apoptosis, inflammation, and fibrosis in the liver [35].

The Extracellular Pathway

DR signaling forms the basis of the extracellular pathway of apoptosis. DRs are members of the tumor necrosis factor (TNF)/nerve growth factor superfamily [36, 37]. They are type I transmembrane proteins with an intracellular C-terminal tail and an extracellular N-terminal domain. These receptors share sequence homology in their intracellular or cytoplasmic portion that is designated the death domain (DD) and functions in the propagation of apoptotic signaling by permitting homotypic interactions with adaptor proteins that also contain the conserved DD. DRs are activated upon ligation by their cognate ligands. The receptor ligand pairs of significance in liver injury and disease are: tumor necrosis factor alpha (TNF-α(alpha)) and its receptor tumor necrosis factor receptor 1 (TNF-R1); Fas (CD95/Apo-1) and Fas Ligand (FasL); tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptors TRAIL receptor 1 (TRAIL-R1/DR4) and TRAIL receptor 2 (TRAIL-R2/DR5/KILLER/TRICK).

Oligomerized, ligand-bound receptors undergo conformational change bringing together the functional DDs and forming the death inducing signaling complex (DISC), which recruits adaptor proteins Fas-associated death domain (FADD) to form Fas and TRAIL-induced death inducing signaling complexes or TNF receptor-associated death domain (TRADD) in the case of TNF-α(alpha) via their DD (Figs. 24.2 and 24.3) [36, 38]. FADD and TRADD also possess death effector domains (DED) that share homology with the DED of procaspase 8 and 10. Procaspase 8 and 10 bound to the DISC undergo activation by presumably an "induced proximity" model, according to which there is autoproteolytic cleavage and activation of caspase 8 and 10 [39]. Caspase 8 cleaves the cytoplasmic protein proapoptotic Bcl-2 family protein Bid into two fragments; one of these two fragments designated tbid (retains the BH3 domain) translocates to the mitochondria and leads to mitochondrial permeabilization via activation of proapoptotic proteins Bax and Bak (discussed further in the following text). This is the so called Type II signaling paradigm in hepatocytes [28, 40]. In contrast, lymphocytes produce sufficient active caspase 8 at the DISC to directly activate the effector caspase 3, bypassing the need for mitochondrial dysfunction, in Type I signaling.

Cellular FLICE like inhibitory protein (cFLIP) is an important regulator of the extrinsic pathway of apoptosis at the level of the DISC [41]. cFLIP shares sequence homology with procaspase 8, and therefore, binds to FADD interfering with binding and activation of procaspase 8. The expression of cFLIP is upregulated by nuclear factor κ(kappa)-B (NF κ(kappa)B), such as in response to TNF-α(alpha) signaling or dexamethasone signaling [42]. Degradation of cFLIP via ubiquitination and the proteasome is a regulated process and sensitizes cells to apoptosis [43].

The BCL-2 Family of Apoptosis Regulators

Proteins of the Bcl-2 family are important intrinsic regulators of the mitochondrial apoptosis pathway [44]. Bcl-2 proteins share 1–4 structural domains designated Bcl-2 homology (BH) domains, which impart different functions to these proteins. The antiapoptotic members share all four BH domains and include Bcl-2, Bcl-XL, Mcl-1, A1, Bcl-W, and Boo. BH123 proteins and BH3-only proteins form the two subfamilies within the proapoptotic Bcl-2 proteins. Bax and Bak are the BH123 proteins of importance in hepatocyte apoptosis as they are essential for mitochondrial permeabilization [45]. The BH3-only proteins are the most numerous Bcl-2 proteins, including Bad, Bim, Puma, Noxa, Bid, Bik, Hrk, and Bmf [46]. The Bcl-2 antiapoptotic proteins and Bax and Bak, in addition to their well-recognized role in mitochondrial permeabilization, also are associated with other membrane-bound organelles, including the endoplasmic reticulum (ER) and nuclear membranes [47]. The BH3-only proteins vary in subcellular localization and abundance. They are either activated or expressed in response to specific intrinsic apoptotic stimuli and with respect to Bid, extrinsic DR signaling. The variety is not an expression of redundancy, but provides the ability to respond to different intrinsic apoptotic stressors by activating distinct BH3-only proteins, e.g., JNK activates Bim, and DNA damage leads to p53-dependent expression of Noxa and Puma [48, 49].

Activation of Bax and Bak by intrinsic apoptotic stimuli is proposed to occur by several different mechanisms. Some BH3-only proteins are direct activators of Bax and Bak, e.g., tBid, Bim, and Puma [50]. Some other BH3-only proteins act indirectly as "activators" of Bax and Bak by facilitating their release. In this model, the antiapoptotic proteins bind and sequester Bax and Bak, and these "activators" displace Bax

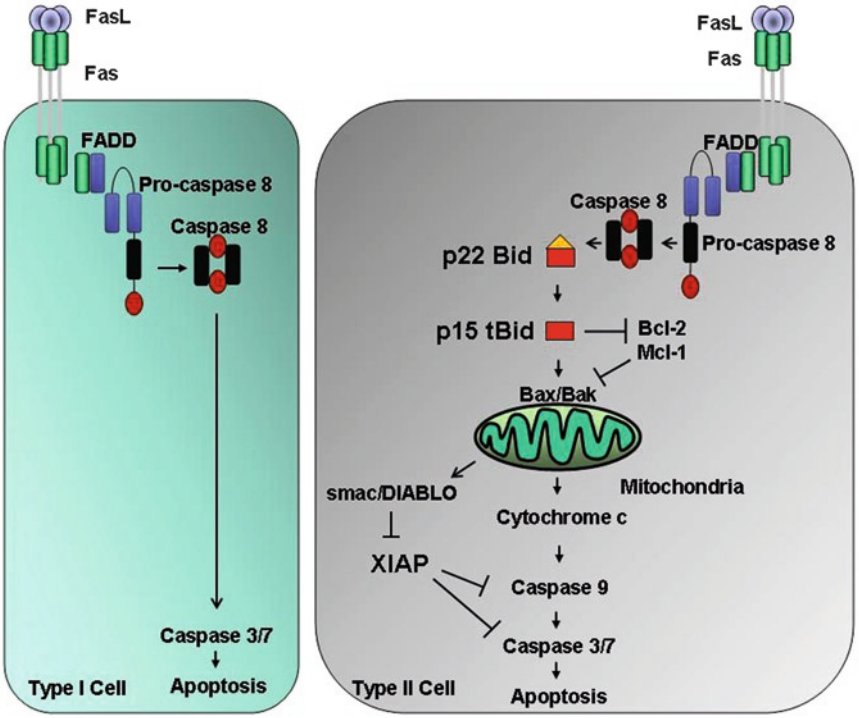

Fig. 24.2 Fas signaling. The death receptor Fas is activated upon ligation with its cognate receptor, Fas Ligand (FasL), on its extracellular domain. This results in receptor oligomerization and intracellular signal transduction based on homotypic interaction between the intracellular death domains (shown in *green*) of Fas and Fas-associated death domain (FADD), an adaptor protein. FADD recruits procaspase 8 via conserved death effector domains (shown in *blue*), resulting in autoproteolytic cleavage and formation of active caspase 8. In a type I cell, such as a lymphocyte, active caspase 8 is sufficient to activate caspase 3 and 7, leading to apoptosis. In a type II cells, such as a hepatocyte, Bid is cleaved by caspase 8 to truncated Bid (tBid), followed by tBid-induced activation of Bax/Bak and mitochondrial permeabilization. Mitochondrial permeabilization releases proapoptotic effectors, including second mitochondrial activator of caspases/direct IAP binding protein with low pI (smac/DIABLO), which inhibits X-chromosome linked inhibitor of apoptosis (XIAP), releasing active caspase 3 from inhibition. Bid and XIAP expression are critical determinants of type II signaling

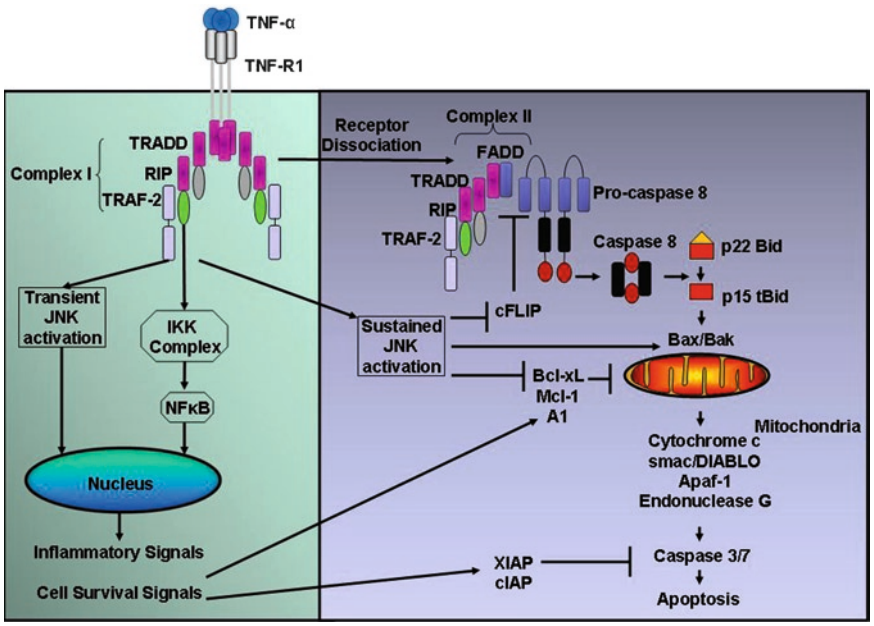

Fig. 24.3 Tumor necrosis factor alpha signaling. Tumor necrosis factor alpha receptor 1 (TNF-R1), upon activation by binding its ligand, tumor necrosis factor alpha (TNFα(alpha)), initiates two sets of signals, designated complex I and complex II. The adaptor proteins tumor necrosis factor receptor-associated protein with death domain (TRADD), RIP, and TNF-associated factor 1 (TRAF-2) form the adaptor protein complex that activates nuclear factor κ(kappa)B (NFκ(kappa)B) and c-jun N-terminal kinase (JNK). Following this, the receptor complex dissociates from TNFα(alpha), undergoes a conformational change, and is internalized. With recruitment of the adaptor protein Fas-associated death domain (FADD), complex II is formed with processing and activation of caspase 8, Bid, and mitochondrial permeabilization

and Bak from antiapoptotic Bcl-2 protein binding and inhibition, so that they can execute mitochondrial permeabilization. Other BH3-only proteins can directly neutralize antiapoptotic Bcl-2 proteins; however, this would not be sufficient to trigger cell death without the activation of Bax and Bak [51].

The Mitochondrial/Intracellular Pathway

Mitochondria are organelles consisting of two specialized membranes, an inner mitochondrial membrane and an outer mitochondrial membrane, and two compartments. This dual membrane structure forms the intermembrane space which contains proapoptotic proteins. The inner mitochondrial membrane is folded into cristae and the compartment it forms contains the mitochondrial matrix. The proapoptotic proteins contained in the intermembrane space are cytochrome c, SMAC/DIABLO (second mitochondrial activator of caspase/direct IAP binding protein with low pI), HtrA2/Omi, AIF (apoptosis-inducing factor), and endonuclease G [52, 53]. The outer mitochondrial membrane is impermeable to these proapoptotic proteins, thus sequestering them till mitochondrial permeabilization signals are received. Bax translocates to mitochondria from the cytoplasm upon activation, and Bak, which is located on the mitochondrial outer membrane, undergoes a conformational change upon activation, form homooligomers that lead to mitochondrial outer membrane permeabilization (MOMP), and permit the release of cytochrome c [54]. Cytochrome c release occurs well before mitochondrial swelling or rupture. Cytochrome c associates with apoptotic protease activating factor 1 (Apaf 1) and adenosine triphosphate (ATP) to form the apoptosome that cleaves and activates procaspase 9. Caspase 9 then leads to the activation of terminal, effector caspases 3 and 7 [39]. These effector caspases and endonucleases released from mitochondria target cellular and nuclear proteins and result in the well-recognized apoptotic morphology. The magnitude of caspase 3 activation and its interaction with XIAP also determine cellular response to Fas. Robust caspase 3 activation leads to Type I cells, whereas in Type II cells, XIAP binds to and inhibits active caspase 3 [40]. The alternative theory of mitochondrial permeabilization is based on the mitochondrial permeability transition pore complex (MTP) and mitochondrial permeability transition (mPT) and does play a role in other modes of cell death such as ischemic cell death and it is discussed further in the section on necrotic cell death.

Lysosomes

Lysosomes are membrane-bound organelles that contain hydrolytic enzymes that function at an acidic pH and perform the vital role of breakdown and degradation of endocytosed macromolecules and obsolete and senescent subcellular components; the later process is known as autophagy. Lysosomes contain a variety of hydrolytic enzymes including proteases, known as cathepsins, and therefore possess the tools necessary for participation in cell death [55]. Total lysosomal rupture with release of all their content leads to necrotic cell death, whereas selective permeabilization, such as by the proapoptotic protein Bax, participates in apoptosis. In hepatocytes, lysosomal permeabilization leads to selective release of cathepsins and occurs upstream of mitochondrial permeabilization in the apoptotic cascade [56]. It is well accepted that cathepsins B, D, and C, of the known 11 mammalian cathepsins, participate in apoptosis. Furthermore, lysosomal involvement in hepatocyte apoptosis is not universal and occurs only in response to specific stimuli. It can be secondary to extrinsic or DR-mediated signals or be activated by the intrinsic pathway [57, 58]. Lysosomal participation has been observed in apoptosis secondary to Fas, TNF-α(alpha), bile salts, free fatty acids, ceramide, photodamage, and reactive oxygen species.

The availability and utilization of cathepsin B deficient mice in various models of liver injury has demonstrated a role for the lysosomal pathway in hepatocyte apoptosis. Isolated hepatocytes treated with TNF-α(alpha)/Actinomycin D to induce apoptosis demonstrate cathepsin B release into the cytosol [56]. Furthermore, purified lysosomes can be permeabilized by caspase 8, and cathepsin B can in turn permeabilize mitochondria, a process enhanced in the presence of cytosol. Cathepsin B deficient mice are protected from the apoptotic effects of TNF-α(alpha) administration. In this model Bid is required for selective lysosomal permeabilization; however, Bax activation is independent of cathepsin B release [59]. In the bile duct-ligated mouse model of cholestatic liver injury, cathepsin B deficiency protects against hepatocyte apoptosis and its consequences, liver injury, and fibrosis [60]. Free fatty acids can activate Bax leading to lysosomal permeabilization and release of cathepsin B [57]. Furthermore, Bax inhibition prevents free fatty acid-induced Cathepsin B release [61]. Animals fed a high fat diet develop liver steatosis and increased hepatic cathepsin B activity. Glycyrrhizin stabilizes lysosomal membranes and prevents free fatty acid-induced and high fat diet-induced increase in cathepsin B activity [62]. Lysosomal enzyme activity in serum is elevated in chronic liver diseases [63, 64]. Nonselective or total lysosomal permeabilization likely plays a role in necrotic cell death as elevated lysosomal enzymes are also observed in fulminant hepatic failure [65].

Endoplasmic Reticulum

The ER forms a network of specialized and dynamic membrane-bound organelles that hepatocytes and other secretory cells are enriched in. The unfolded protein response

is activated upon accumulation of misfolded or unfolded proteins in the ER, a state of ER stress due to excess "client proteins." [66] Alterations in calcium homeostasis, glucose deprivation, tumicamycin, UV irradiation, oxidative stress, and obesity can also lead to ER stress (Fig. 24.4). A series of ER stress-specific responses are activated that are aimed at minimizing misfolded proteins from accumulating [67]. The ER-associated degradation of proteins (ERAD) facilitates removal of defective proteins. Global translation attenuation decreases new protein synthesis, with selection upregulation of specific proteins, such as ER chaperones to allow the ER to adapt to the misfolded protein load. Lastly, apoptosis mediators are activated as well, especially during unresolved or protracted ER stress. The UPR is characterized by the activation of three resident ER transmembrane stress sensors, inositol requiring enzyme 1 alpha (IRE-1 alpha), activating transcription factor 6 alpha (ATF-6 alpha), and RNA-activated protein kinase (PKR)-like ER kinase (PERK). Each of these is temporally regulated and has distinct downstream effects. IRE-1 alpha functions as a protein kinase as well as endoribonuclease. It cleaves the transcription factor XBP1 to form spliced XBP1 (sXBP1) that activates many genes involved in the UPR and ERAD. IRE-1 alpha can also bind the TNF receptor-associated protein 2 (TRAF2) leading to JNK activation and can interact with Bax and Bak, thus effecting apoptosis. PERK is a Ser/Thr protein kinase, activated by autophosphorylation, which phosphorylates and inactivates eukaryotic translation initiation factor 2 alpha (eIF2α(alpha)); however, activating transcription factor 4 (ATF4) remains active and induces C/EBP homologous protein (CHOP) expression. ATF6 is cleaved and released into the cytosol, and then translocates to the nucleus. The transcription factor CHOP has binding sites for ATF4, ATF6, and XBP1 in its promoter, and its expression is partially regulated by all three ER stress sensors.

Sustained or severe ER stress can activate apoptotic pathways in several ways. Calcium homeostasis is sensitive to the Bcl-2 family proteins [68]. Bcl-2 and Bcl-xL can reduce

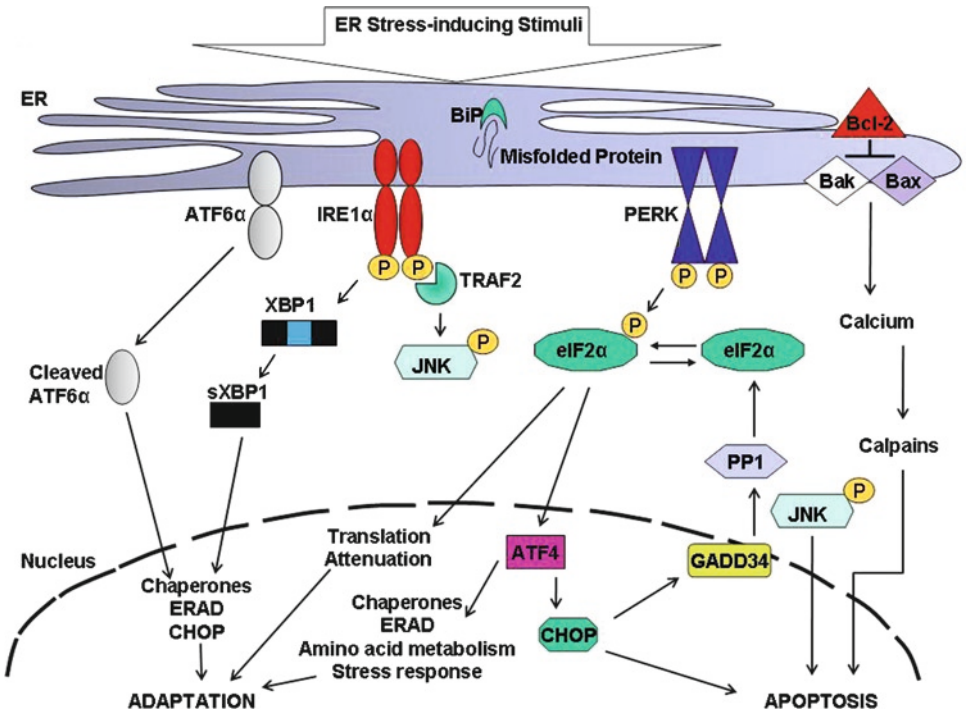

Fig. 24.4 Endoplasmic Reticulum stress signaling. The accumulation of misfolded client proteins in the endoplasmic reticulum (ER) creates ER stress and activates the unfolded protein response. Some other ER stress inducing stimuli include Thapsigargin, Tunicamycin, lipid excess, and increased polypeptide synthesis. The chaperone BiP/GRP78 (heavy chain binding protein/glucose-regulated protein 78) binds to client proteins. Three known ER stress sensors, each possessing a lumenal domain, a transmembrane domain, and a cytoplasmic domain, are activated upon dissociation of BiP from their lumenal domains. Activating transcription factor 6 (ATF6) is cleaved and the cleaved N-terminal fragment translocates to the nucleus, increasing the expression of adaptive genes. Inositol requiring kinase (IRE) 1α(alpha) is activated by autophosphorylation. It also has endoribonucleolytic activity leading to the splicing of the transcription factor X-box binding protein 1 (XBP1) to its spliced form (sXBP1), which selectively upregulates expression of adaptive genes as well. IRE1α(alpha) also activates JNK via the adaptor protein TRAF2. PKR like ER protein kinase (PERK) phosphorylates eukaryotic initiation factor 2 alpha (eIF2α(alpha)), leading to global attenuation of transcription to decrease the client load in the ER. It also selectively upregulates activating transcription factor 4 (ATF4) and CHOP. Sustained ER stress is associated with a failure of adaptation and apoptosis. Apoptosis can occur due to the transcription factor CHOP-induced upregulation of the proapoptotic protein Bim, or TRAIL receptor, DR5. Bax and Bak-regulated calcium release from the ER as well as IRE1α(alpha)-dependent JNK activation can also determine apoptotic cell fate

basal calcium concentration in the ER, and Bax can increase basal calcium concentration in the ER. In cells lacking Bax and Bak, basal calcium levels are lowered. Alterations in calcium can affect many intrinsic apoptotic pathways, including calpains (calcium dependent proteases), and direct effects on mitochondria. CHOP is a transcription factor, activated by the ER stress response and known to regulate the proapoptotic protein Bim and TRAIL-R2 expression, sensitizing cells to apoptosis [69, 70]. ER stress kinases can activate c-Jun N-terminal kinase [71], which can then activate the mitochondrial pathway of apoptosis [72]. Bax inhibitor-1 (BI-1), an inhibitor of Bax-induced apoptosis, has recently been shown to suppress the IRE1-α(alpha) branch of the UPR resulting in decreased sensitivity to ER stress-induced cell death [73]. The role of ER stress pathways in liver injury has been studied in several experimental models. Cycloheximide induces acute ER stress followed by liver cell death and injury [74]. GCDC can activate ER stress pathways in isolated hepatocytes leading to increased apoptosis; and in the bile duct-ligated mouse model of cholestasis, in CHOP-deficient animals, abrogation of hepatocyte apoptosis and fibrosis are observed [75, 76]. Recently, it has been demonstrated that the transcription factor Foxa 2 is essential for bile acid metabolism in hepatocytes; its deletion leads to intrahepatic cholestasis and activation of ER stress pathways [77]. Nutritional obesity is associated with ER stress and JNK activation [78]. In free fatty acid-induced lipoapoptosis, saturated fatty acids activate ER stress pathways [79]. Cholesterol accumulation also activates the UPR, expression of CHOP, calcium depletion, and activation of apoptosis [80]. In a model of alcohol-induced hepatocyte apoptosis, CHOP-deficient mice are protected against hepatocyte apoptosis, in spite of developing hepatic steatosis and activating the ER stress response [81]. On the other hand, the acute and severe induction of ER stress results in hepatic steatosis mediated via the transcriptional control of de novo lipogenesis and VLDL secretion [82].

Overview of Necrosis

Necrotic cell death is recognized in the liver under conditions of massive metabolic disturbance, mitochondrial dysfunction, and ATP depletion leading to swelling of mitochondria and other organelles, rupture and release of intraorganelle contents, and cellular swelling, cell membrane bleb formation with eventual bleb rupture and release of intracellular contents [1]. Spillage of intracellular contents stimulates an inflammatory response. Necrotic cell death is seen with ischemic reperfusion injury, uncoupling of mitochondrial oxidative phosphorylation, and drug-induced liver injury. The same stimulus, differing only in magnitude, can activate apoptotic or necrotic cell death within hepatocytes. As in apoptosis, mitochondrial dysfunction plays a significant and central role in necrotic cell death. Historically, necrotic cell death was defined morphologically, and cellular signaling pathways involved in necrotic cell death were not well defined [16]. Mechanistically, necrosis was a definition of exclusion, based on the absence of apoptotic or autophagic cell death. Emerging data have highlighted some of the signaling molecules that mediate necrotic cell death. Some of this information has been obtained from studies based on the inhibition of apoptotic signaling, either by utilizing molecular inhibitors, or cells genetically deficient in certain molecules and hence incapable of apoptotic signaling [25, 83]. Nevertheless, signaling pathways that mediate necrotic cell death are being defined by such experiments, though liver-specific information may not be available.

Receptor-interacting proteins (RIP) are intracellular adaptor kinases with homologous N-terminal kinase domains; the kinase domains are essential for induction of necrosis, which has primarily been described upon TNFα(alpha) treatment of cells [84–86]. RIP1 and RIP2 interact with DRs and caspases through shared DD and caspase recruitment domain (CARD). RIP3 interacts with RIP1 via a RIP homotypic interaction motif (RHIM). RIP1 is recruited to the TNFα(alpha) signaling complex, Complex I, formed upon ligation of TNFR1. Signals emanating from this Complex I lead to prosurvival signals, e.g., activation of NFκ(kappa)B and mitogen-activated protein kinases (MAPK), in a RIP1-dependent manner [87, 88]. Inactivation of RIP1 signaling, by deubiquitination, or the genetic absence of RIP1 sensitizes cells to TNFα(alpha)-induced cell death. However, RIP1 also mediates TNFα(alpha)-induced necrotic cell death and the production of reactive oxygen species (ROS). RIP1-induced necrotic cell death requires the participation of RIP3. RIP3 phosphorylates and stabilizes RIP1 in a heterodimeric complex that leads to necrotic cell death. RIP3 was also identified as a molecular switch between apoptotic and necrotic cell death in NIH 3T3 cells upon treatment with TNFα(alpha). RIP3 activation was linked to the metabolic state of the cell. RIP3 associated with seven metabolic enzymes under conditions of TNFα(alpha) + caspase inhibitor treatment. Modulation of expression of some of these metabolic enzymes, which play a key role in breakdown of glycogen or the utilization of amino acids as an energy source, correlated with the production of ROS and necrotic cell death [89].

The Mitochondria in Necrosis

The MTP is formed by the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane and adenine nucleotide transporter (ANT) on the inner mitochondrial

membrane along with cyclophilin D and other proteins in the mitochondrial matrix [90]. The inner mitochondrial membrane contains the respiratory chain that performs oxidative phosphorylation and ATP generation for the cell. As electrons are transported through the respiratory chain, protons are pumped out of the matrix creating a transmembrane electrochemical gradient, the inner mitochondrial membrane potential (Δ(delta) Ψ(psi) m). The mPT refers to a rapid increase in the permeability of the inner mitochondrial membrane that results in rapid membrane depolarization, uncoupling of oxidative phosphorylation, release of the contents of the mitochondrial matrix, eventual rupture of the outer membrane, and release of apoptotic mediators. ATP depletion can also occur from lack of blood flow as occurs in ischemic injury leading to necrotic cell death. The role of this mPT in cell death regulated by Bcl-2 family proteins is unclear, as in mice lacking cyclophilin D, hepatocyte mitochondria are efficiently permeabilized by Bax and tBid, but are resistant to calcium-induced permeabilization [91]. Mitochondria isolated from hepatocytes from mice lacking ANT or VDAC are susceptible to cell death induced by various stimuli [92, 93], which contradicts data that Bax and Bak-induced MOMP is followed by the mPT [90, 94]. Furthermore, neither Bax and Bid, nor calcium-induced or oxidative stress-induced mitochondrial permeabilization was dependent on VDAC. This observed difference may be a function of the experimental models used or the existence of discrete mutually exclusive pathways of mitochondrial permeabilization. These studies further highlight the involvement of mitochondria in hepatocyte cell death, whether the stimulus is apoptotic or necrotic.

Molecular Mediators of Apoptosis

Fas

Fas is a DR protein expressed on every cell type in the liver. Upon ligation with its cognate ligand, Fas Ligand (Fas L), on the extracellular domain of the receptor, the Fas receptor oligomerizes and activates caspase 8 and caspase 10 [28]. Caspase 8 cleaves Bid to tbid which leads to mitochondrial permeabilization and activation of effector caspases. In health, Fas is expressed at low levels on the plasma membrane; a majority of Fas localizes to the Golgi complex and trans-Golgi network in hepatocytes. This prevents spontaneous unwanted apoptosis and provides a reservoir for the rapid translocation of Fas to the cell surface by injurious stimuli as is seen with bile salts [95]. FasL in the liver is derived from activated cytotoxic T cells and natural killer cells. Proteolytic cleavage of FasL from the cell membrane of T cells results in a soluble form (sFasL), the toxicity of which is unclear, though elevated circulating levels are found in many liver diseases. The ligated Fas receptor signals through the DISC (vide supra), leading to cleavage of Bid to tBid, mitochondrial permeabilization, and apoptosis (Fig. 24.2). The importance of Fas in hepatocyte apoptosis is underscored by the observation that exogenously administered anti-Fas antibody leads to massive hepatocyte apoptosis and fulminant hepatic failure in mice [96]. Mice deficient in Fas are protected. As hepatocytes are Type II cells, the antiapoptotic Bcl-2 proteins can inhibit Fas-induced apoptosis. Indeed, transgenic mice overexpressing human Bcl-2 are protected against the lethal apoptosis induced by Fas [97]. Bid is required for Fas-induced apoptotic signaling, as mice deficient in Bid do not develop the characteristic hepatocyte apoptosis and fulminant hepatic failure induced by exogenous administration of Fas [98]. X-chromosome linked inhibitor of apoptosis (XIAP), an antiapoptotic protein, governs sensitivity to Fas-induced cell death downstream of Bid [40]. In the combined absence of XIAP and Bid, hepatocytes that were resistant to Fas-induced apoptosis in the absence of Bid alone were sensitized to Fas-induced cell death. Mice deficient in XIAP alone were more sensitive to the fatal effects of anti-Fas agonistic antibody. Thus, XIAP functions as a negative regulator or Fas signaling.

Following the observation of fulminant hepatic failure induced by anti-Fas antibody, there has been an explosion of research on the role of Fas in liver pathobiology and hepatocyte apoptosis. Though not essential for organogenesis, as Fas-deficient animals develop normal livers, Fas is important in maintaining hepatic homeostasis and renewal. In Fas-deficient mice, with age, hepatic hypertrophy develops [99], more hepatocytes are seen per liver lobe, and within hepatocytes nuclear abnormalities are seen. The cell surface expression and availability of Fas control a cell's susceptibility to Fas-induced apoptosis. Most Fas in located within the cell, thus limiting its availability to bind to its ligand. Cell surface Fas is bound to the hepatocyte growth factor receptor, Met [100]. This interaction effectively sequesters Fas and inhibits spontaneous death signaling. Met and FasL are competitive in their binding to Fas, and Met prevents FasL binding to Fas at low concentrations of FasL. On the other hand, on the addition of exogenous HGF, Met dissociates from Fas and sensitizes hepatocyte cell lines to apoptosis. High concentration of FasL can overcome the Met–Fas interaction. Thus, the complex interaction between Met and Fas results in either life or death for a stressed hepatocyte. Indeed, this paradigm has been advanced further by the observation that in fatty liver Fas is dissociated from Met, both in human fatty liver samples and in animal models [101]. Mice with diet-induced hepatic steatosis are sensitized to Fas-induced apoptosis [102]. Human biopsy samples from patients with nonalcoholic steatohepatitis demonstrate enhanced Fas expression as

well as hepatocyte apoptosis [103]. In cell culture models of free fatty acid-induced steatosis, sensitization to Fas-induced apoptosis is also observed [5].

Chronic viral hepatitis, both due to hepatitis B virus (HBV) (see Chap. 37) and hepatitis C virus (HCV) (see Chap. 38), demonstrates activation of the Fas-FasL death signaling cascade [104]. Indeed, in both forms of viral hepatitis, hepatocyte Fas expression is upregulated. FasL expressing cytotoxic T lymphocytes infiltrate the liver in chronic viral hepatitis. Fas and FasL expression is geographically localized to areas of inflammatory activity within hepatic lobules [105]. Circulating levels of soluble Fas (sFas) and sFasL are elevated in patients with chronic hepatitis B, more so than in inactive carriers [106]. Fas-induced apoptosis is also seen in patients with fulminant hepatitis B infection [107]. Similarly, in chronic hepatitis C elevated sFasL levels are observed [108].

The accumulation of toxic, hydrophobic bile acids within the liver is a key feature of cholestatic liver injury. Toxic bile acids, e.g., glycochenodeoxycholate (GCDC), induce Fas-dependent and Fas-independent apoptosis in hepatocytes [95, 109]; detoxification of bile acids is discussed in Chap. 12. In cultured hepatocytes, GCDC treatment leads to Fas oligomerization and activation of caspase 8 [109]. Cellular trafficking of Fas is also regulated by GCDC, which enhances translocation of Fas from the cytosol to the cell surface [95]. Spontaneous receptor oligomerization and activation of downstream death signals results from increased cell surface expression of Fas. Bile salts can also activate the Src family kinase Yes, resulting in activation of the epidermal growth factor receptor and phosphorylation-induced activation of Fas [110]. Further proof of the importance of Fas is established on the basis of studies in mice lacking Fas receptor (*lpr*). Following bile duct ligation, hepatocyte apoptosis, liver injury, and fibrosis are abrogated in the Fas receptor-deficient mouse [35].

TRAIL

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a death ligand, related to FasL and TNF-α(alpha) [111]. TRAIL is of particular interest in liver disease, as normal hepatocytes are resistant to TRAIL-induced apoptosis [112]. TRAIL receptors, especially DR5 and DR4, can, however, mediate apoptosis in sensitized hepatocytes. Under normal conditions, the expression of TRAIL and its receptors in human livers is low. TRAIL has been shown to mediate hepatic steatosis in animal models of alcoholic hepatitis and acute adenoviral hepatitis [113]. Metabolic or viral stress may sensitize hepatocytes to TRAIL toxicity. Susceptibility to TRAIL-induced cell death can be regulated by cell surface expression of DR5 and DR4 as well as the production of TRAIL ligand for paracrine activation of its receptors. Indeed, in models of acute viral hepatitis, adenoviral infection sensitizes hepatocytes to TRAIL-induced apoptosis [112]. TRAIL also mediates *Listeria monocytogenes*-induced acute hepatitis and concanavalin A-induced acute liver injury. Further support for the role of inflammation in preferentially sensitizing the liver to TRAIL is demonstrated in an experimental model of constitutive hepatocyte-specific NF-κ(kappa) B activation. These mice demonstrate upregulation of DR5 expression on hepatocytes and constitutive activation of NK cells within the liver [114].

In chronic liver disease, such as in nonalcoholic fatty liver disease (see Chap. 34) and chronic viral hepatitis, upregulation of TRAIL receptors with concomitant sensitization to TRAIL-induced cell death is observed [115]. In patients with chronic hepatitis B infection, cytokine-induced enhancement of TRAIL expression on intrahepatic NK cells and DR5 expression on hepatocytes is observed [116]. This correlates with serum ALT levels as well as flares of inflammation. In cell culture models, hepatitis C viral proteins as well as hepatitis B viral proteins can selectively modulate the expression of cell surface TRAIL receptors [117, 118]. This may have a proapoptotic or antiapoptotic effect. For example, hepatitis B core antigen levels correlate inversely with sensitivity to TRAIL-induced cell death via modulation of DR5 expression [119]. The emerging model in viral hepatitis suggests an important role for TRAIL-mediated apoptosis in removal of virus infected cells. Failure of this or viral trickery to subvert this process may play a role in persistence of chronic viral infection. Nutritional stress, such as free fatty acids and intracellular ER stress, can lead to enhanced DR5 expression, the later via the transcription factor CHOP [70, 120]. The stress kinase, JNK, can also enhance DR5 expression [120]. Thus, the potential for hepatic toxicity of TRAIL agonistic antibodies, which are in clinical trials for cancer chemotherapy, exists especially in patients with acute or chronic hepatitis, justifying careful and necessary monitoring.

TNF-Alpha

Tumor necrosis factor alpha (TNF-α(alpha)) is a pleiotropic cytokine that plays important roles in liver regeneration, inflammation, injury, cell survival, and cell death [37]. It is produced predominantly by activated macrophages, but can be expressed by other cell types as well. Circulating TNF-α(alpha) is a ligand for two characterized receptors, tumor necrosis factor receptor 1 (TNF-R1) and tumor necrosis factor receptor 2 (TNF-R2). TNF-R1 is widely expressed in tissues, and TNF-R2 expression is generally restricted to immune cells; however, both are expressed on hepatocytes.

TNF-R1-mediated apoptosis is better described. The opposed effects of TNF-α(alpha) emanate from the same receptor complex (Fig. 24.3) [121]. Both receptors lack intracellular catalytic domains and rely on adaptor proteins to propagate their signals. Upon receptor ligation, the trimerized TNF-R1 recruits intracellular adaptors via a conserved DD. Downstream signals from the receptor–ligand complex are arbitrarily designated as complex I and complex II.

Complex I is formed by tumor necrosis factor receptor-associated protein with death domain (TRADD), TNF receptor-associated factor-2 (TRAF-2), and receptor-interacting protein1 (RIP). Via a kinase cascade involving RIP1, the transcription factor, nuclear factor-κ(kappa) B (NF- κ(kappa) B) is activated and translocates to the nucleus. NF- κ(kappa) B target genes are subsequently activated and fall broadly into two categories, inflammatory genes, such as interleukin 6, iNOS, and antiapoptotic genes, such as, cFLIP, XIAP. TNF-α(alpha)-induced JNK activation also occurs via complex I and the adaptor protein TRAF2. Thus, complex I mediates the cell survival and inflammatory signals of TNF-α(alpha). Following this, the adaptor complex undergoes a conformational change, dissociates from trimerized TNF-R1, and recruits the adaptor protein FADD to form complex II. Caspase 8 activation occurs in this secondary complex. Apoptosis can occur downstream of this, both via Bid cleavage and in a Bid-independent manner. In the later scenario, JNK-dependent Bim phosphorylation occurs downstream of TNF-α(alpha) signaling [122]. Due to the earlier formation of complex I, the predominant consequences of TNF-α(alpha) signaling are cell survival and the activation of inflammatory pathways. Furthermore, due to the activation of opposing forces, in order to induce apoptosis, TNF-α(alpha) survival signals have to be overcome or inactivated. Experimentally, in cultured hepatocytes or liver cell lines, this can be achieved by inhibition of NF-κ(kappa)B, inhibition of transcription (actinomycin D), or translation (cycloheximide). The role of protracted JNK activation as an in vivo switch in TNF-α(alpha) signaling has been recognized [43]. It has been demonstrated that prolonged JNK activation results in cFLIP degradation which shifts the balance of TNF-α(alpha) signaling toward cell death.

In spite of and due to its varied potential effects, there is no doubt that TNF-α(alpha) plays an important role in hepatic homeostasis and disease. The predominant consequences of in vivo activation of TNF-α(alpha) signaling are promotion of hepatic regeneration and inflammation. In many chronic liver diseases TNF-α(alpha) signaling is upregulated and plays a role in liver injury, though this is most likely not due to hepatocyte apoptosis. Mice deficient in TNF-R1 demonstrate impaired liver regeneration and increased mortality following partial hepatectomy [123]. Further dissection of this pathway has demonstrated the requirement for interleukin 6 for liver regeneration [124]. Constitutive increase in TNF-α(alpha) expression and activity in the liver leads to spontaneous hepatocyte cell death and inflammation and failure of liver regeneration following partial hepatectomy due to apoptosis secondary to sustained activation of TNF-α(alpha) [125].

TNF receptor expression is upregulated on hepatocytes in acute and chronic liver diseases. Indeed, experimental models of fulminant hepatitis are based on massive activation of TNF-α(alpha) signaling, and in patients with fulminant hepatic failure elevated circulating levels of soluble TNF-α(alpha), TNR-R1, and TNF-R2 can be detected. Soluble TNF-R1 levels also correlate inversely with survival [126, 127]. Similarly, in alcoholic hepatitis circulating TNF-α(alpha) and TNF-R1 are elevated and correlate with mortality [128, 129]. In a murine model of alcoholic hepatitis, utilizing mice deficient in TNF-R1 or TNF-R2, it was demonstrated that TNF-R1 is essential for alcohol-induced liver injury [130]. Analysis of a TNF-α(alpha) gene promoter polymorphism associated with increased TNF-α(alpha) expression has demonstrated that this polymorphism is associated with increased susceptibility to steatohepatitis [131]. Similarly in patients with chronic viral hepatitis, secondary to hepatitis C viral infection or hepatitis B viral infection, TNF-α(alpha) signaling is activated [132, 133].

JNK

C-jun N-terminal kinases (JNKs) are members of the MAPK family [134, 135]. They are ubiquitously expressed and mediate cellular responses to myriad extracellular and intracellular stresses. The three known JNK genes encode ten JNK isoforms by alternative splicing, of which JNK1 and JNK2 isoforms are expressed in the liver. JNKs are activated via a kinase signaling cascade that links the extracellular or intracellular stress to JNK phosphorylation. Upon activation, JNKs phosphorylate and regulate mediators of cell survival, inflammation or cell death in a stimulus-specific and time-dependent manner. MAP kinase phosphatases (MAP) dephosphorylate JNK and thus serve as negative regulators of JNK signaling. Chemical inhibition and genetic deletion studies have demonstrated that depending on the model of injury, there is either a redundancy in the role of JNK 1 and JNK 2 or each can modulate specific aspects of liver injury. Temporal kinetics of JNK activation also influence the outcome; the early phase of JNK signaling favors cell survival, and therefore if JNK activation is transient, survival is the likely outcome. However, sustained JNK activation promotes apoptotic signaling.

JNK can regulate cell death by modulation of many pro- and antiapoptotic proteins, either transcriptionally or via phosphorylation (Fig. 24.1). The cytokine TNF-α(alpha) leads to JNK activation, and JNK in turn is a positive regulator of TNF-α(alpha) expression. In TNF-α(alpha)-induced cell

death, the sustained activation of JNK leads to phosphorylation and activation of the E3 ubiquitin ligase Itch. Itch activation leads to ubiquitination and degradation of cFLIP, releasing caspase 8 from cFLIP-inhibition promoting TNF-α(alpha)-induced apoptosis [43]. Utilizing models of fulminant hepatitis based on Concanavalin A or lipopolysaccharide injection, others have demonstrated that JNK 1/2 in bone marrow-derived cells is required for TNF-α(alpha) production, but not for TNF-α(alpha)-mediated cell death, and hepatocytes that lack JNK 1/2 are equally susceptible to TNF-α(alpha)-induced cell death [136]. In another model of TNF-α(alpha)-induced hepatocyte apoptosis, JNK 1-mediated Mcl-1 phosphorylation increases the half life of Mcl-1 lending an antiapoptotic role to JNK 1. Acetaminophen (APAP)-induced liver injury is also mediated by JNK. In mouse models, APAP treatment leads to robust JNK activation as well as Bax translocation [137]. Pharmacologic inhibition of JNK activation attenuates liver injury, as does absence of apoptosis signal-regulating kinase 1 (ASK 1). The toxic bile acid, deoxycholic acid, also activates JNK and increases hepatocyte apoptosis by JNK-dependent TRAIL-R2 expression [138]. In this model, JNK1 mediated cell death, whereas JNK2 was protective.

JNK is chronically activated in the liver in obesity-associated fatty liver; this mediates cellular insulin resistance [78]. Free fatty acids, which are elevated in patients with nonalcoholic fatty liver disease, can also induce JNK-dependent lipoapoptosis by enhancing the expression of Bim, a proapoptotic BH3-only protein, as well as sensitize to DR toxicity by enhanced expression of Fas and TRAIL-R2 [5, 120, 139]. In high fat diet (HFD)-induced steatohepatitis in mice, JNK 1-deficient mice are resistant to weight gain, whereas JNK 2-deficient mice are significantly more obese than their wild type controls [140]. JNK 1-deficient mice also demonstrate less injury and inflammation in the liver, which may be a consequence of less adiposity, whereas JNK 2 is a negative regulator of Bim expression and sensitivity to apoptosis in spite of greater liver steatosis. JNK activation is also linked to the redox state of the cell, and oxidative stress-induced apoptotic signaling [141]. In TNF-α(alpha)-induced cell death, oxidative inactivation of MAP kinase phosphatases promotes prolonged JNK activation and cell death [142]. JNK activation occurs in ischemia–reperfusion injury of the liver, and pharmacologic inhibition of JNK activation decreased hepatocyte cell death in experimental orthotopic liver transplantation [143–145].

Conclusion

Hepatocyte cell death is a central event in liver injury, regardless of etiology, even though the susceptibility of an individual hepatocyte to intracellular or extracellular stressors is variable. The effects of TNFα(alpha) are pleiotropic, and in the absence of NF-kappaB inhibition favor cell proliferation, survival, and inflammation. Fas signaling results in apoptotic cell death with characteristic morphologic changes; and diseased hepatocytes are sensitive to TRAIL-induced apoptosis as well. Recent studies have highlighted the "program" of necrotic signaling, specifically the role of RIP kinases in mediating necrotic cell death. Intracellular perturbations, such as protracted ER stress, sustained JNK activation, or lysosomal permeabilization can also trigger hepatocyte cell death. In cell death induced by any of these stimuli, mitochondrial dysfunction is mandatory for the cell to die. Hepatocyte cell death promotes inflammation and fibrosis in the liver, perpetuating the injury cascade. Advancements in the exact molecular pathways of hepatocyte cell death present several areas, such as caspase inhibitors and JNK inhibitors, which have therapeutic potential.

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Chapter 25
Macroautophagy

Ying-Hong Shi, Jia Fan, Chih-Wen Lin, Wen-Xing Ding, and Xiao-Ming Yin

Introduction

Two major intracellular degradation systems have been defined: the ubiquitin–proteasome system and the autophagy–lysosome system. Degradation of ubiquitin-conjugated proteins is mediated by proteolysis in the proteasome. However, autophagic degradation of intracellular components is mediated by the lysosome. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy, which differ in their physiological functions and in the way the cytoplasmic materials are delivered to the lysosomes [1]. Macroautophagy is an evolutionarily conserved and perhaps quantitatively most important autophagy process, in which macromolecules and subcellular organelles are delivered to the lysosomes via a vesicular structure, called autophagosome. This chapter will focus on the role of macroautophagy (hereafter referred to as autophagy) in the pathobiology of the liver.

The autophagic process was first described by Clark in 1957 using electron microscopy, but the term "autophagy" was first introduced by de Duve and Wattiaux in 1963 [2, 3]. In the mammals, autophagy has been extensively studied in the liver. Much of the pioneering work in this field was conducted in the context of liver pathobiology, such as the determination of the morphology and the membrane protein composition of the autophagosomes, and the characterization of regulatory molecules such as amino acids, insulin, and the mammalian target of rapamycin (mTOR) [4].

It is now well defined that autophagy is important for multiple physiological and pathophysiological processes in cell growth, development, and homeostasis by maintaining balance between the synthesis, degradation, and subsequent recycling of cellular products [5]. Deregulation of autophagy in hepatocytes can be observed in liver injury and hepatic carcinogenesis. Involvement of autophagy has been investigated in a variety of liver diseases, such as α-1-antitrypsin deficiency, alcoholic liver disease, ischemia reperfusion liver injury and hepatocellular carcinoma (HCC) [1].

Y.-H. Shi (✉)
Department of Liver Surgery, Liver Cancer Institute, Shanghai, P.R. China
e-mail: shi.yinghong@zs-hospital.sh.cn

Basic Biology of Autophagy

Morphological Studies of Macroautophagy

In macroautophagy, cytoplasmic components including long-lived proteins and organelles are sequestered within double-membrane vesicles, which are known as autophagosomes. The origin of the sequestering membrane is still unknown, but in mammalian cells it has been long suspected that the endoplasmic reticulum (ER) is one of the likely membrane sources [6]. Autophagosomes then fuse with the lysosomes to form autolysosomes, and the sequestered contents are degraded by lysosomal hydrolases and are recycled. In hepatocytes, it has been reported that small-sized autophagosomes (<0.5 μm in diameter) near the bile canaliculi can be detected by electron microscopy following starvation in 24 h. The main contents of the autophagosomes are cytoplasm with mitochondria and peroxisomes rarely detected. However, after 24 h, relatively large-sized autophagosomes (approximately 1–1.5 μm in diameter) that contain mitochondria and rough ER appear [7]. In general, multiple vacuoles at different maturation stages in the cytosol can be found in hepatocytes under starvation.

Autophagosomes and autolysosomes, which are the fused vesicles of autophagosomes with the lysosome, thus containing more electron dense materials and demonstrating wider morphology heterogeneity, can be visualized clearly by electron microscopy in steady state. Electron microscopy is a valid and important method both for the qualitative and quantitative analysis of changes in autophagy status (Fig. 25.1a) [8]. Although definitive, electron microscopy could be technically challenging and tedious and may not provide dynamic data in a timely fashion. Additional tools for detection of autophagy such as immunofluorescent localization of LC-3

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_25, © Springer Science+Business Media, LLC 2011

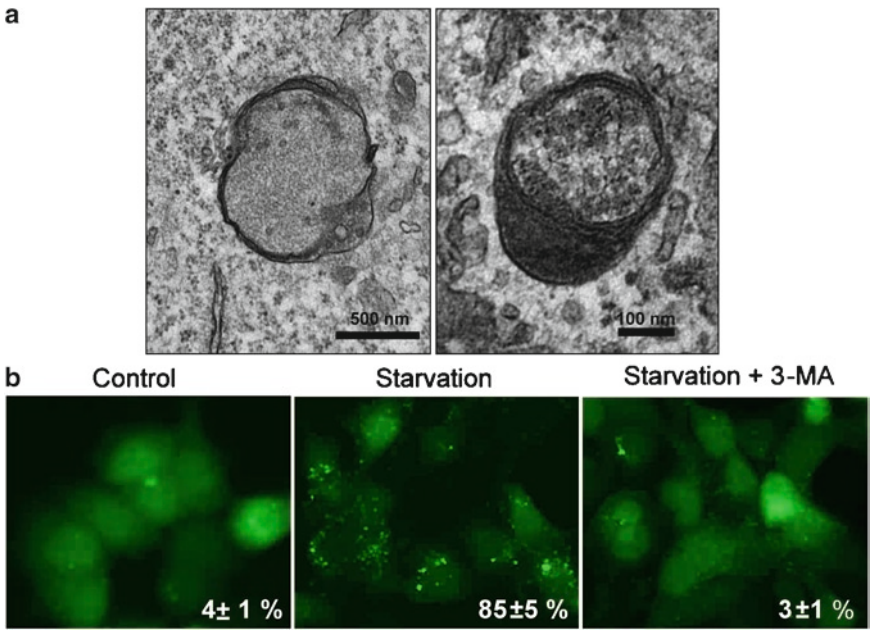

Fig. 25.1 Detection of macroautophagy. (**a**) Classically, autophagy is analyzed by electron microscopy, which detects autophagosomes (*left* panel) and the fused form with the lysosome i.e., the autolysosomes (*right* panel). These two examples are from 293 cells treated with 10% calcium phosphate precipitates for 4 h. (**b**) Fluorescence microscopic methods have been developed recently with the discovery of molecules involved in the autophagosome biogenesis. By far the most commonly used in the mammalian cells is LC3, which translocates from the cytosol to the autophagosomal structure upon autophagy stimulation. The example shown here is HCT116 cells that stably express GFP-LC3 in the complete medium with 10% serum (control) where GFP-LC3 is diffusively distributed in the cytosol with only about 4% of cells exhibiting punctated GFP-LC3 distribution. However, when the cells were cultured in medium deprived of amino acids and serum (starvation) for 6 h, about 85% of cells demonstrated punctated location of GFP-LC3, suggesting that this molecule was translocated to the autophagosomal structure, which could be suppressed by 3-methyladenine (3-MA), an inhibitor of the class III PI-3 kinase (VPS34), which is required for autophagy activation

to autophagosomes in the cytoplasm are being developed currently (Fig. 25.1b). The number of autophagosomes, autolysosomes, and total autophagic vesicles can be determined in a given cell section, or area. Because autophagy is a dynamic and continuous process, quantification of vesicles only reflects the turnover status of the autophagosomes at a given time. Flux analysis is necessary to differentiate whether the accumulated autophagosomes are due to increased production or reduced degradation [9]. Thus, it may be possible to determine whether a positive flux is present by examining whether there is an increase in autolysosome in the presence of lysosome inhibitors, such as chloroquine. They are increasingly being supported by biochemical studies of autophagy molecules.

Molecular Machinery of Macroautophagy

Although autophagy was first defined in mammalian cells, the understanding of molecular machinery comes largely from the discovery of autophagy related genes (ATGs) in the yeast system. More than 30 ATGs have been identified [10] and many have corresponding mammalian homologues, including Atg1 to Atg10, Atg12 to Atg14, and Atg16 to Atg18 [10, 11]. The Atg1 complex is required for the induction of autophagy downstream of Tor signaling in response to nutrient deprivation [12]. In mammalian cells, the Atg1 complex is comprised of ULK1 (a mammalian Atg1 homolog), Atg13, and FIP200 (the mammalian homology of Atg17) [13].

Formation of a class III phosphatidylinositol 3-kinase (PtdIns3K) complex is also necessary for the induction of autophagy. In the mammals, this complex is comprised of the Ptdins3K complex: Vps34 and p150 (the yeast Vps15 homologue), and their regulators, Beclin 1 (the yeast Atg6 homologue) and Barkor/Atg14 [14–17]. Autophagy signals, such as amino acid deprivation, will activate Beclin 1/Atg6 and Atg14, which in turn enhance the lipid kinase activity of Vps34, leading to an increased generation of phosphatidylinositol 3-phosphate (PtdIns3P) (Fig. 25.2). PtdIns3P will facilitate other Atg proteins, such as Atg16 (see below), to be recruited to the precursor membranes of autophagosomes, also known as phagophore.

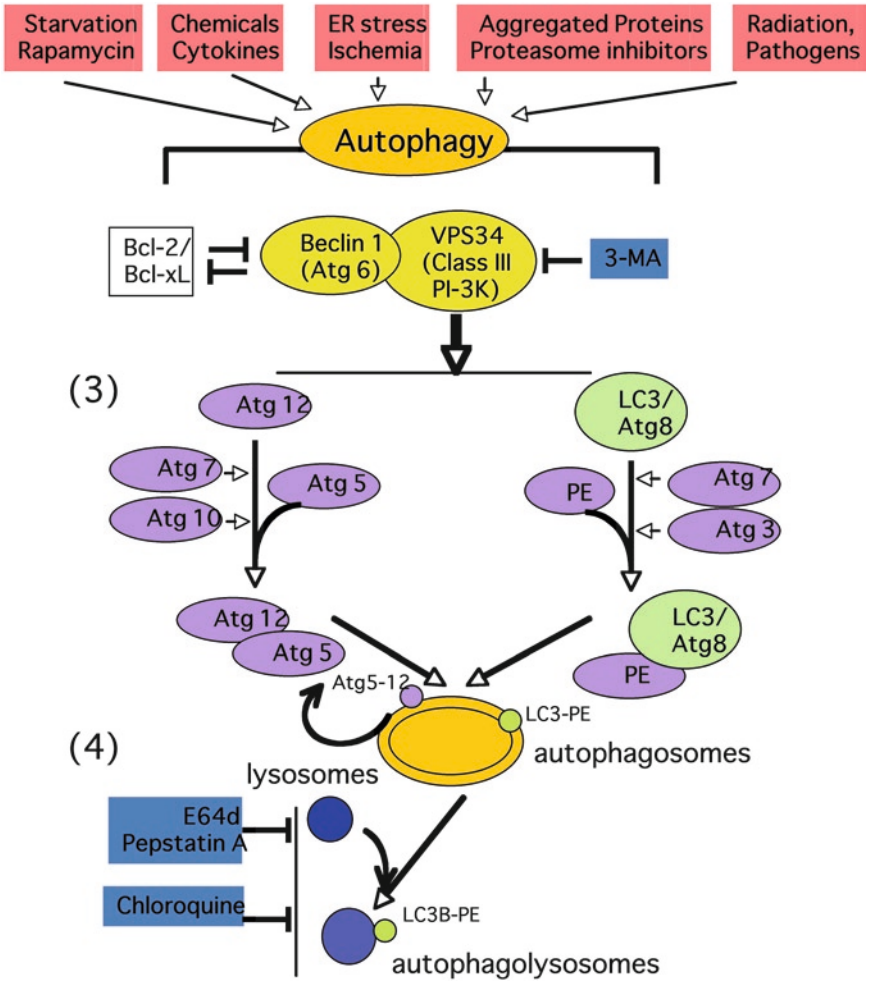

Fig. 25.2 The basic machinery of autophagy. Autophagy could be activated by various pathological conditions. They will lead to the activation of the Beclin 1/VPS34 complex, which in turn positively regulate the Atg5–12 and Atg8/LC3-PE conjugation systems. Atg5–Atg12 targets to the autophagosomes first, but then recycles to the cytosol once LC3/Atg8 is conjugated to the membrane phospholipids (PE) on the autophagosomal precursors. Matured autophagosomes are eventually fused with the lysosomes. Several chemicals known to be able to modulate autophagy are highlighted in blue. See the text for details

During the elongation and expansion of the phagophore, two ubiquitin-like proteins, Atg12 and Atg8 (or a mammalian homolog, microtubule-associated protein 1 light chain 3, LC3), are required [18]. Atg12 is first activated by Atg7, an ubiquitin-activating enzyme (E1)-like protein, and then transferred by Atg10, an ubiquitin carrier protein (E2)-like protein, to Atg5 through a covalent bond. The Atg5–Atg12 complex interacts with Atg16 to form a multimer complex, which then moves to the phagophore in response to the activation of the Ptdins3K complex. Atg8/LC3 is cleaved by Atg4 at its C-terminus to generate Atg8-I or LC3-I. Cytosolic Atg8/LC3-I with the exposed C-terminal glycine residue is conjugated to phosphatidylethanolamine (PE), also via Atg7, and the E2-like enzyme, Atg3 [19]. The conjugated form of Atg8/LC3 (Atg8-II or LC3-II) targets to the autophagosomal membrane following the Atg5-Atg12-Atg16 complex. This association of Atg8/LC3 to autophagosomes is considered important for the membrane extension of the autophagosome and the eventual closure of the membrane to form vesicles. The Atg12-Atg5 complex detaches from mature autophagosomes, whereas Atg8/LC3 stays on the membrane until it is degraded by the lysosome. Atg8/LC3 is thus widely used as a marker for monitoring the autophagy process (Fig. 25.1b). In the mammalian cells, the translocation of LC3-I to the membrane to form LC3-II can be tracked by immunoblot assay or by fluorescence microscopy by immunostaining for LC3 or using GFP-LC3 fusion molecules, which is a sensitive and useful approach to monitor autophagy status [8].

Autophagy is regulated by complicated signaling pathways. In the mammals, both mTOR and AMP-activated protein kinase (AMPK) are sensors of intracellular signaling [18]. The upstream class I PtdIns3K protein kinase B (PKB/Akt) pathway, in response to growth factor stimulation, activates mTOR complex 1 (mTORC1) through inhibiting a downstream protein complex, the tuberous sclerosis complex 1 and 2 (TSC1 and TSC2). mTORC1 then inhibits autophagy. On the other hand, AMPK is a positive regulator of autophagy and is activated by a decreased ATP/AMP ratio through the upstream LKB1(Peutz-Jeghers syndrome protein) kinase. Under metabolic stress, the activated LKB1-AMPK axis can phosphorylate and stabilize $p27^{kip1}$, permitting cells to survive through growth factor withdrawal via autophagy [20].

The Physiological Role of Autophagy in Hepatocytes

Autophagy in Protein Metabolism

Protein metabolism in the body involves both protein synthesis and protein degradation. The regulation of protein degradation or proteolysis is as important as that of protein synthesis. As mentioned before, the autophagy–lysosome and the ubiquitin–proteasome systems are the two major intracellular proteolytic mechanisms. Under physiological conditions, in most visceral tissues like the liver autophagy processes the bulk proteolysis, whereas in peripheral tissues, mainly the skeletal muscle, the proteasome system is regarded to be the main pathway for bulk proteolysis [21].

Autophagy has been extensively studied in the liver under the condition of food restriction or starvation. Autophagy is activated to provide cells with the necessary nutrients through degradation of intracellular materials. In animals, starvation induces the largest protein loss in the liver. In the first 48 h, mice and rats can lose 25–40% of their liver proteins. The loss seems to be mainly in the cytosol with most subcellular constituents being targeted except the nucleus and DNA, resulting in a 25% reduction in cell volume in the first 24 h of starvation [22]. The involvement of macroautophagy and the lysosome was realized when protein loss was found to be associated with stimuli that cause autophagy, such as glucagon administration or deprivation of amino acids, insulin, or serum in the perfused livers and in cultured cells. Amino acid deprivation has since been employed as a standard simulation for autophagic degradation of proteins in mammalian cells.

Hepatic autophagy has been best characterized for the regulation by plasma amino acids. Leucine, tyrosine, phenylalanine, proline, glutamine, histidine, methionine, and tryptophan are eight so-called regulatory amino acids [21]. These amino acids suppress macroautophagy in perfused rat livers at 0.5× and 4× the normal plasma concentration, among which leucine is by far the most effective [22]. Leucine is also the only amino acid that can inhibit protein degradation in myocytes and adipocytes. Alanine has a coregulatory effect, in that it could work with regulatory amino acids at 1× the normal plasma concentration to achieve the maximal inhibitory effect, but has no impact on proteolysis by itself. The coregulatory effect of alanine could also be mimicked by insulin.

It seems reasonable that amino acids suppress autophagy as a feedback mechanism since autophagy regulates proteolysis. However, the molecular mechanism of amino acids regulation is still not clear. Mortimore et al. indicated that in hepatocytes there existed a leucine-binding protein of the plasma membrane to be responsible for effective autophagy inhibition of leucine [23]. Intracellular signaling pathways are also involved in the amino acids regulation. It has been reported that amino acids regulate proteolysis in hepatocytes by mTOR signaling pathway, although the details need further investigation [24]. More recently, Gohla et al. suggested that the heterotrimeric G protein G(i3) specifically transmitted the antiautophagic effects of amino acids and insulin in the liver [25]. But how insulin and amino acids affect G(i3) and how G(i3) in turn affects autophagy are not known.

Glycogen Autophagy and Glucose Homeostasis

Glucagon is perhaps the first hormone known to simulate autophagy. Ashford and Porter reported “autophagy-like” structures in glucagon-perfused rat liver in 1962 [26]. There are two spatially separated cellular locations of glycogen in hepatocytes, the cytosol (hyaloplasmic pool), and vacuolar (autophagosomes). Autophagy actively participates in the overall breakdown of cellular glycogen and can selectively degrade polysaccharides. Autophagic degradation of glycogen in the newborn liver has long been recognized as an important survival mechanism for the newborn to adapt to the postnatal environment [27]. Glycogen autophagy establishes a link between autophagy and glycogen metabolism and is a selective, hormonally controlled and highly regulated process, representing a mechanism of glucose homeostasis [28].

Glycogen autophagy is necessary to maintain life during the period of postnatal hypoglycemia. Glycogen-hydrolyzing acid glucosidase activity is low in the liver and heart at birth, but peaks at 6 h in the livers of newborn rats, which is coincident with the abundance of autophagosomes containing glycogen [29]. Autophagosomal degradation of glycogen is regulated mainly by the cAMP/protein kinase A (induction) and phosphoinositides/mTOR (inhibition) pathways. cAMP and cAMP elevating agents, such as glucagon or adrenalin, induce glycogen autophagy in the liver and heart of newborn rats [28]. Cyclic AMP-lowering agents, such as propranolol, inhibit glycogen autophagy. The cAMP and mTOR pathways can converge on common targets, such as protein phosphatase 2A (PP2A). In nutrient abundance, insulin activates mTOR through PKB pathway, which can inactivate PP2A, thus inhibiting autophagy and glycogen turnover [30].

Autophagy in Lipid Metabolism

The liver plays a key role in lipid metabolism. The main pathways of lipid metabolism are lipogenesis, lipolysis, beta-oxidation, and ketosis. The intracellular storage and utilization

of lipids are critical to maintain cellular energy homeostasis [31]. During nutrient deprivation, cellular lipids stored as triglycerides in lipid droplets (LDs) are hydrolyzed into fatty acids for energy. In contrast, in lipogenesis fatty acids are converted to triglyceride, generally occurring in the liver, adipose tissue, and intestinal mucosa. Abnormal accumulation of LDs in hepatocytes can result in hepatic steatosis.

It has been demonstrated that autophagy plays a critical role in regulating intracellular lipid level (macrolipophagy). It seems that autophagic degradation of LD in the lysosome could provide the necessary energy supply from the recycled fatty acids and inhibition of autophagy in cultured hepatocytes and in mouse increased the amount of LDs [32, 33]. It is not clear how LDs could be captured by the autophagosome and delivered to the lysosomes. LDs and autophagic components could be associated during nutrient deprivation. Interestingly, it has been also shown that the LC3 lipidation system essential for autophagy could be involved in LD formation. LD formation accompanied by accumulation of triacylglycerol was largely suppressed in hepatocytes that could not execute autophagy. LC3 was localized on the surface of LDs and LC3-II was fractionated to a perilipin (LD marker)-positive lipid fraction from the starved liver, suggesting that the LC3 conjugation system is critically involved in lipid metabolism via LD formation [34]. Thus, it seems that the relationship of autophagy and LD during different metabolic status is more complicated than initially thought. The functional role of autophagy in lipid metabolism may have important implications for human lipid deregulation diseases.

The Pathobiological Role of Autophagy in Liver Diseases

A growing body of evidence has now shown that autophagy is involved in the pathogenesis of many liver diseases. Genetically, loss of Atg7 in the liver leads to hepatomegaly and accumulation of abnormal organelles in hepatocytes [35]. Accumulation of ubiquitin-positive aggregates in autophagy-deficient hepatocytes can be clearly defined. All the evidence indicates that autophagy is important for the turnover of abnormal organelles and misfolded proteins in hepatocytes.

Autophagy in Alpha-1-Antitrypsin Deficiency

Alpha-1-antitrypsin (AT), the archetype of the serpin protein family, is a major blood-borne inhibitor of neutrophil elastase, which can degrade all the constituents of the lung connective tissue matrix. AT deficiency is a common autosomal codominant disorder, and it arises from the inheritance of the "Z" allele. The mutant protein, called ATZ, becomes aggregation-prone and accumulated in the ER. This leads to the deficiency of AT in the blood stream, rendering neutrophil elastase to degrade the connective tissue matrix of the lung and causing chronic lung emphysema. In contrast to this loss-of-function phenotype of AT deficiency, the liver presentation of this disease is a gain-of-toxic function due to the accumulation of aggregated ATZ in the ER of hepatocytes, which induces liver inflammation and carcinogenesis.

Abnormal accumulation of ATZ in the ER can cause ER stress and cell death [36]. However, only 8–10% of the homozygotes have developed clinically significant liver disease [37]. The variation in the liver disease phenotype may indicate that there are some putative genetic modifiers or protective responses responsible for the disposal of mutant ATZ in the ER. Autophagy activation seems to be an effective compensatory mechanism. In a transgenic mouse model with constitutive expression of ATZ in the liver, the basal level of autophagosomes occupies ~2.5% of the cytoplasm of liver cells compared to ~0.5% in the C57/BL6 mouse, which indicates that the autophagic response is enhanced in the liver of ATZ mice [38]. Autophagosomes with the content of ATZ were found to be abundant in hepatocytes and in mouse model of AT deficiency and in patients with AT deficiency. Degradation of ATZ was reduced by chemical inhibitors of autophagy including 3-methyladenine, LY-294002, and Wortmannin. In addition, in the yeast model of ATZ degradation, it has also been found that aggregated ATZ is targeted to the autophagy pathway for degradation [39]. It is conceivable that autophagy can play an important role in modulating the liver pathogenesis of AT deficiency. Autophagy may be important for cellular protection against mitochondria injury and hepatocytes apoptosis, which may be responsible for the chronic liver injury in AT deficiency [40].

Autophagy in Hypofibrinogenemia

The protein quality control function of ER is to recognize and remove aberrant proteins from the secretory pathway. A R375W mutant of fibrinogen γ chain (Aguadilla γD) causes misfolding of the molecule, and the abnormal fibrinogen molecule is recognized as aberrant and degraded by ER-associated protein degradation (ERAD), thus leading to blood hypofibrinogenemia. Hepatic ER accumulation of the fibrinogen variant could cause liver cirrhosis. Although the soluble form of the mutant can be degraded by the proteasome via ERAD, autophagy is required to degrade the excessive amount of the soluble aberrant protein and most importantly the insoluble aggregates in the ER [41]. Autophagy has been linked to the ER protein quality control in this disease.

Significantly in other cases where proteasome, and thus ERAD, is inhibited, autophagy is activated in response to ER stress through the unfolded protein response (UPR) pathway [42, 43]. In these cases, autophagy has been found to be important in alleviating ER stress by removing misfolded proteins accumulated due to the blockage of ERAD. In this way autophagy protects against cell death induced by ER stress.

Autophagy in Alcoholic Liver Disease

The histological hallmark of chronic alcoholic liver disease is the formation of Mallory-Denk bodies in hepatocytes, which contain cytokeratin 8, cytokeratin 18, ubiquitin-positive protein aggregates, and p62/SQSTM1. Autophagy is a critical mechanism to clean these protein aggregates. However, autophagy could be suppressed in chronic alcoholic liver disease. In ethanol-perfused rat liver, proteolysis was remarkably inhibited. Electron-microscopic examination of lysosomal components showed that the aggregate volume of autophagosomes was significantly smaller in hepatocytes [44].

The impaired proteolytic activity of hepatic lysosomes, reduced trafficking of lysosomal enzymes, impaired microtubule structures, and alteration of hepatic amino acids may partially account for autophagy suppression [45, 46]. Alcohol-induced lysosomal damage is evidenced by enhanced lysosomal fragility, which could result from either altered lipid metabolism or oxidative stress [46]. The function of lysosomal system can be impaired by lower serum ethanol levels in livers of female ethanol-fed rats. Another possible mechanism of autophagic suppression by ethanol is disruption of protein trafficking in the liver. Trafficking of exogenous proteins into the hepatocyte by endocytosis and the intracellular delivery of proteases to lysosomes are both inhibited by ethanol consumption. Acetaldehyde, the initial product of ethanol oxidation, inhibits the polymerization of tubulin to form microtubules, which is necessary for autophagosome formation [47]. Ethanol-induced suppression of autophagy may also result from alterations in hepatic amino acids levels. In rats, intrahepatic levels of leucine increased by 1.4–1.8-fold over control animals after chronic ethanol exposure [48]. Because leucine is one of the strongest autophagic amino acids, higher levels of intrahepatic leucine may partially explain autophagic suppression in the ethanol-fed state.

In contrast to the chronic status, acute ethanol exposure, particularly the binge exposure, activates autophagy significantly (W.-X. Ding and X.-M. Yin, manuscript submitted). We have found that autophagy is important for the protection against ethanol-induced liver injury and hepatocyte steatosis. Autophagy is central to the removal of damaged mitochondria and accumulated LDs, thus eliminating both a source of reactive oxygen species (ROS) (i.e., damaged mitochondria) and a key ROS amplifier (lipids), which are required for the ethanol-induced liver damage.

Autophagy in Hepatic Carcinogenesis and Liver Cancer

The role of autophagy in carcinogenesis and cancer is intriguing. As a cellular adaptive mechanism to stress, autophagy seems to be able to promote cancer cell survival. Meanwhile, extensive autophagy induced by radiotherapy or chemotherapy can also contributes to cell death [49]. Genetic evidence suggests that autophagy could serve as a tumor-suppressor function. It has been shown that monoallelic deletion of the autophagic gene *beclin 1* promotes tumorigenesis. Heterozygous disruption of *beclin 1* reduces autophagy and increases the frequency of spontaneous malignancies including liver cancer [50]. Thus, *beclin 1* has been demonstrated as a haplo-insufficient tumor-suppressor gene. However, the autophagy regulation by oncogenes or tumor-suppressor genes seems variable. Antiapoptotic proteins such as Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1 can inhibit autophagy, whereas several tumor-suppressor proteins such as BH3-only proteins, death-associated protein kinase-1 (DAPK1), PTEN, TSC1, TSC2, and LKB1 induce autophagy [51]. Furthermore, the tumor-suppressor p53 has dual positive and negative regulatory function in autophagy. Within the nucleus, p53 can act as an autophagy-inducing transcription factor. Within the cytoplasm, p53 exerts a tonic autophagy-inhibitory function, and its degradation is actually required for the induction of autophagy [52].

The basal expression of autophagic genes and their corresponding autophagic activity under conditions of starvation are suppressed in different HCC cell lines with variant malignant phenotype. It seems that the autophagy defect correlates well with the more malignant phenotype of HCC. In HCC tissues, expression of Beclin 1 was extremely low. But Beclin 1 expression level could predict the prognosis of HCC patients only in a Bcl-X_L-positive expression background [53]. These results indicate that autophagy defects, together with a suppressed cell death program, might facilitate tumor progression and poor prognosis of HCC [54].

On the other hand, autophagy has also been demonstrated as a major survival strategy in response to metabolic challenges such as glucose or amino acid deprivation in HCC cells [55]. Given the dual function of autophagy in tumor suppression and tumor cell survival, the exact role of autophagy in hepatic carcinogenesis and liver cancer progression needs further investigation. Efforts to understand and modulate the autophagy pathway in different scenarios will provide new approaches to liver cancer therapy and prevention.

Conclusion

Autophagy is an evolutionarily conserved mechanism responsible for the degradation of macromolecules and subcellular organelles, which is constitutively activated in the liver. Autophagy plays crucial roles not only in the physiology of the hepatocytes, but also in the pathogenesis of a number of liver diseases. Exploration of the therapeutic potential of autophagy manipulations in liver diseases is definitely a promising direction for the future.

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Chapter 26
Hepatic Ischemia/Reperfusion Injury

Callisia N. Clarke, Amit D. Tevar, and Alex B. Lentsch

Introduction

Hepatic ischemia/reperfusion (I/R) injury is a frequently encountered complication in a variety of clinical scenarios including liver transplantation, major hepatic resection, abdominal trauma surgery and hemorrhagic shock. In the late nineteenth century, Dr. James Hogarth Pringle, a celebrated surgeon, developed a technique in which the blood flow through the hepatic artery and portal vein was occluded in order to achieve hemostasis during abdominal surgery for hemorrhage associated with liver trauma. This technique was later named the Pringle maneuver. With the growth in the field of hepatobiliary surgery, this technique of total vascular occlusion was adapted and has enabled surgeons to perform complex procedures such as orthotropic liver transplantation and large liver resections and repairs that otherwise would have resulted in massive hemorrhage and certain death.

Despite the benefits of the Pringle maneuver, there are limitations that can cause substantial morbidity and mortality. These limitations are related to the duration of ischemia occurring during surgery which limits the delivery of oxygen and nutrients to the liver. Prolonged liver ischemia results in a reperfusion injury that has both oxidative stress and inflammatory components. These elements cause significant hepatocellular injury that may progress to organ dysfunction both locally and remotely, poor graft function and/or death. Because the clinical ramifications of this injury process are severe, much attention has been focused towards a better understanding of the cellular and molecular pathways involved in hepatic ischemia/reperfusion injury. Here, we discuss the current knowledge of the mechanisms that induce, propagate, and regulate the injury response to hepatic ischemia/reperfusion. These mechanisms differ to some degree, depending on whether the ischemia occurs in a warm setting, such as surgical resection or trauma surgery, or cold ischemia, as occurs during liver transplantation. As such, we have divided the discussions accordingly.

A.B. Lentsch (✉)
Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH, USA
e-mail: alex.lentsch@uc.edu

Warm Ischemia/Reperfusion Injury

The liver is a highly aerobic organ that is sensitive to interruption of blood flow. Using experimental models, Jaeschke et al. [1] first described and characterized two distinct phases of liver injury after hepatic ischemia/reperfusion injury. The early phase of injury occurs within the initial few hours of reperfusion and is characterized by Kupffer cell activation, proinflammatory cytokine production, and generation of oxygen-derived free radicals. This same laboratory defined the role of complement activation as an amplifier of these processes [2]. During the period of ischemia and early reperfusion, hypoxia leads to impaired adenosine triphosphate (ATP) production and acidosis which protects against necrosis [3, 4]. After reperfusion, the acidosis is corrected and hepatocytes then become susceptible to pH-dependent necrosis [5]. Serum levels of alanine aminotransferase, a specific marker of hepatocyte injury, are modestly elevated during this phase. Likewise, liver architecture assessed histologically shows only minor changes. Despite the limited degree of injury during this phase, the oxidant stress results in the release of a number of proinflammatory cytokines that serve to initiate and propagate an intense secondary inflammatory response. The key mediators of this early phase are discussed below.

Oxidant Stress

Kupffer cells are resident liver macrophages and represent the largest collection of tissue-fixed macrophages. There is considerable evidence to suggest that Kupffer cells are activated early after hepatic ischemia/reperfusion and are a primary source of reactive oxygen-derived free radicals [6, 7].

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_26, © Springer Science+Business Media, LLC 2011

One factor known to contribute to the early activation of Kupffer cells after I/R is complement. While the precise nature of complement activation during I/R is unclear, depletion or receptor antagonism leads to reduced Kupffer cell oxidant production and diminished subsequent liver injury [2]. Studies have shown that administration of gadolinium chloride (an agent that suppresses Kupffer cell activity) in mice attenuates early hepatocellular injury after hepatic I/R [8]. Conversely, chemically up-regulating Kupffer cell activation aggravates cellular injury and production of reactive oxygen species [9]. Kupffer cell generation of superoxide has been shown to be a critical factor in the injury observed in the early reperfusion period [2, 10]. The dismutation of superoxide into oxygen and highly diffusible hydrogen peroxide results in lipid peroxidation and inactivation of antiproteases, contributing to parenchymal cell injury [10, 11].

In addition, oxidants are known to induce activation of transcription factors, such as nuclear factor (NF)-κB and activator protein-1 (AP-1) [12, 13]. Activation of these transcription factors has multiple, cell-specific effects after I/R, as will be discussed below. However, both transcription factors contribute to the regulation of gene expression for multiple cytokines expressed in the liver after I/R [13].

Transcription Factors

NF-κB

Nuclear factor (NF)-κB is a general term used to describe a number of dimeric combinations of proteins of the Rel family which possess transcriptional activating properties [14, 15]. The primary form of NF-κB consists of a heterodimer of p50 (NFκB1) and p65 (RelA) proteins. This complex has the ability to bind with promoter sequences in DNA and to inaugurate transcription (generation of mRNA) for many proinflammatory mediators. However, other combinations of Rel family members have been identified and different configurations of Rel proteins (e.g., p65/p50, p65/p52, etc.) may have preferential sensitivities to different target promoter sequences [16, 17]. In unstimulated cells, NF-κB is sequestered in the cytoplasm by inhibitors of κB (IκB) proteins, of which there are currently at least five known isoforms. IκBs prevent nuclear localization of NF-κB by masking its nuclear localization signal peptide and blocks NF-κB from binding to DNA by allosteric inhibition [18].

Two modes of NF-κB activation have been described to occur in the liver during I/R injury (Fig. 26.1). The first mode is the classical pathway of NF-κB activation in which cell stimulation results in the serine phosphorylation of IκB by the IκB kinase complex (IKK complex). This kinase complex consists of two catalytically active subunits, IKKα and IKKβ, and a nonenzymatic regulatory scaffold protein IκKγ (also known as NF-κB essential modifier, NEMO) [19, 20]. Phosphorylated IκB then becomes the target of ubiquitin ligase which polyubiquitinylates the protein for subsequent proteasomal degradation [14, 21]. In addition to this well characterized pathway, there appears to be an alternative method of NF-κB activation that does not involve the IKK complex, serine phosphorylation or proteasome-mediated degradation of IκB. This alternate mechanism of NF-κB activation was originally described in hypoxic T cells and involves the phosphorylation of IκBα on tyrosine residue 42 leading to its dissociation from NF-κB [22]. However, tyrosine phosphorylated IκB α is not proteolytically degraded. Experimental data suggest that activation of NF-κB via this mechanism occurs predominantly after hypoxia, whereas the classical pathway occurs primarily after cytokine stimulation[23–26]. For both mechanisms of activation, once NF-κB is freed from IκB it translocates to the nucleus where it initiates the transcription of target genes.

Given the various functions of NF-κB, its role in hepatic I/R injury appears multifactorial (Fig. 26.2). In Kupffer cells, NF-κB induces pro-inflammatory cytokine production [27]. In endothelial cells, NF-κB activation leads to the expression of chemokines and adhesion molecules [28, 29]. However, in hepatocytes, the function of NF-κB is more complex. Does NF-κB predominantly induce cytokine production in hepatocytes, leading to more injury or does its ability to activate protective genes have greater impact?

Although the question of whether or not hepatocyte NF-κB activation is harmful or beneficial in I/R is still unclear, experimental data have shown unequivocally that the inflammatory response that occurs after I/R is detrimental to the hepatocytes. Treatments that reduce/block TNF α, interfere with the cytokine cascade, block neutrophil infiltration so that all have a uniform effect of ameliorating liver injury after I/R. This beneficial effect of blocking inflammation is seen even when the "protective" effects of NF-κB activation is completely lacking. Namely, TNF α -/- mice essentially have no NF-κB activation after I/R [30]. In this setting, both the harmful and the beneficial effects of NF-κB are missing. These mice suffer little hepatic injury after I/R despite having limited regenerative capacity. This is in stark contrast to partial hepatectomy, where NF-κB activation is essential for liver recovery. This differential response may stem from the fact that the partial hepatectomy leaves behind healthy hepatocytes that can readily resist the attacks from Kupffer cells, neutrophils, and the proapoptotic effects of TNF α. Similarly in preconditioning, marked by early TNF α production and NF-κB activation, healthy hepatocytes would be resistant to harmful effects of TNF α, while enjoying the benefits of "protective" genes activated by NF-κB. Injured and stressed hepatocytes from I/R, on the other hand, would be far less resistant to the harmful effects of NF-κB activation, namely the production of cytokines including TNFα. This harmful

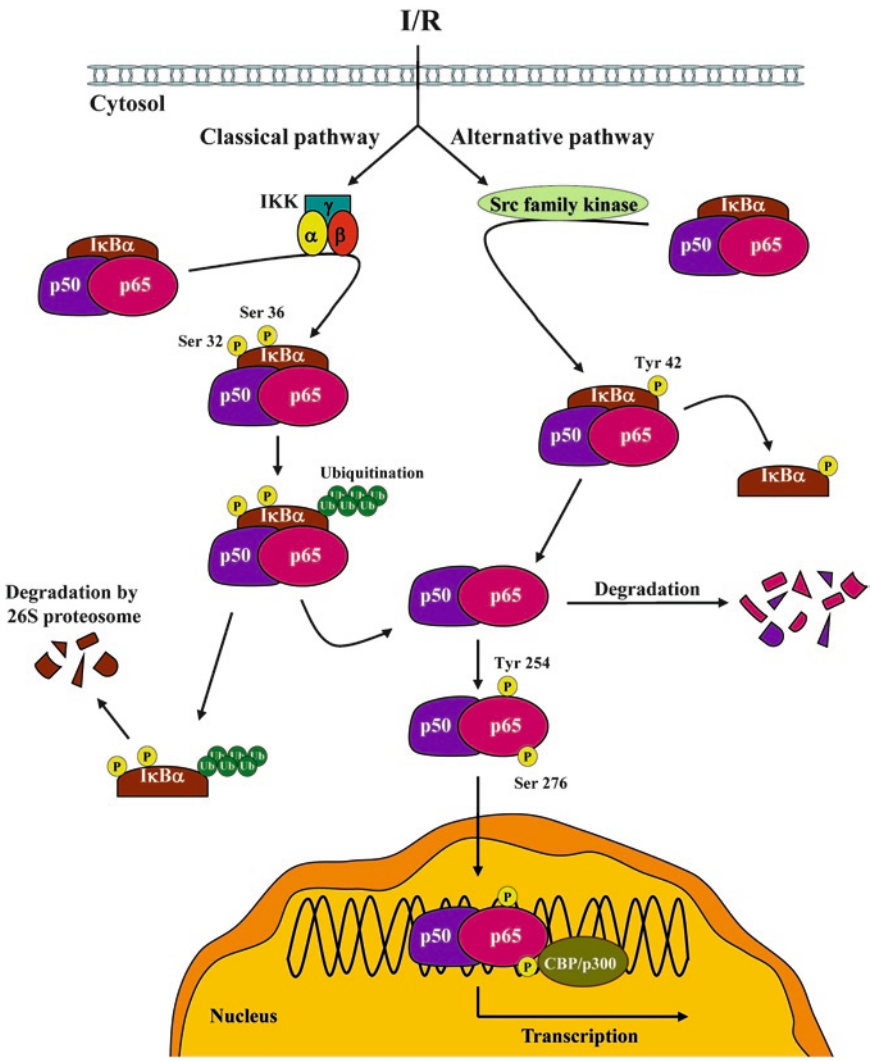

Fig. 26.1 Pathways of NF-κB activation. The classical pathway involves phosphorylation of IκB α on serine residues 32 and 36 and its subsequent degradation. The alternative pathway involves phosphorylation of IκB α on tyrosine residue 42 and dissociation from NF-κB without degradation. Once in the nucleus, the NF-κB complex binds to coactivators, such as CBP/p300 which facilitate transcriptional activation of target genes. From Shin et al. [160]. Used with permission

effect, in the injured hepatocytes, might outweigh any benefit from the induction of "protective" genes.

Hepatocyte NF-κB has been directly targeted in two recent reports. The first study showed that hepatocyte-specific deletion of IKKβ abrogated activation of NF-κB in hepatocytes and greatly reduced liver injury after hepatic I/R [31]. Interestingly, the protective effects of this mutation were associated with a greatly reduced ability of hepatocytes to produce TNF α. However, Kupffer cell production of TNF α was unchanged. The authors suggested that the hepatic inflammatory response to I/R was driven largely by NF-κB activation in hepatocytes. A more recent study by the same laboratory reported that hepatocyte-specific deletion of IKKγ/NEMO resulted in much worse liver injury after I/R [32]. Even more interesting, all hepatocyte IKKγ/NEMO-null mice died within 24 h of reperfusion. The authors suggested that mice with hepatocyte-specific deletion of IKK γ may have some residual NF-κB activation that was protective, whereas the IKK γ/NEMO-deficient livers had no NF-κB activation and were unable to recover from the insult. While this concept is plausible, these studies have not resolved the role of NF-κB in hepatocytes during I/R injury.

Other studies suggest that hepatocyte NF-κB activation is cell-protective during I/R injury. In mice undergoing hypothermic ischemia, NF-κB activation was activated to a much greater degree than those exposed to normothermic ischemia. The augmented NF-κB activation was associated with reduced hepatocellular injury [33]. Similarly, a study examining the effects of age on liver I/R injury found that older mice had decreased hepatic activation of NF-κB, yet more hepatocellular injury [34]. These findings were confirmed in a later study, which identified an age-related defect in the proteasomal processing of NF-κB in hepatocytes [35]. These studies along with others [36], provide compelling evidence that activation of NF-κB in hepatocytes during I/R injury is an important mechanism of protection.

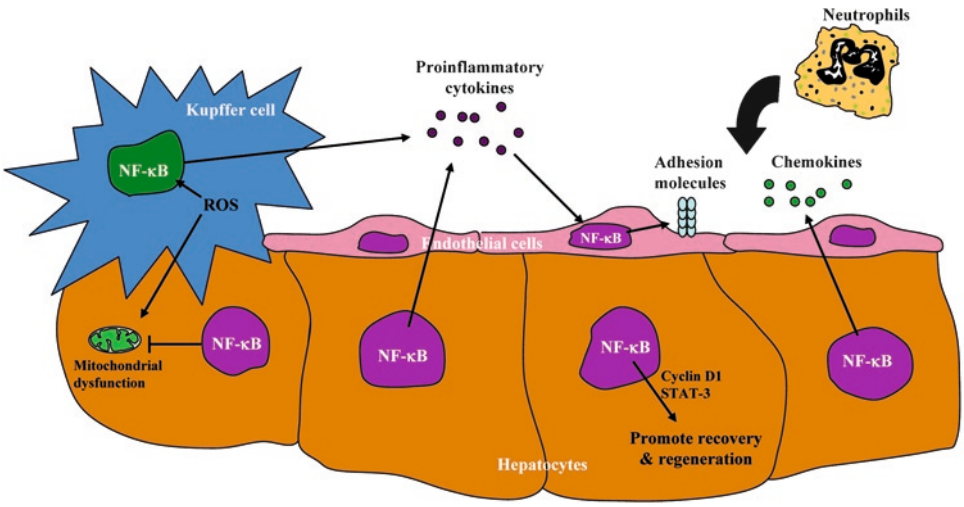

Fig. 26.2 Cell-specific functions of NF-κB in the liver during I/R injury. Ischemic stress results in the generation of reactive oxygen species (ROS) in Kupffer cells. ROS activates NF-κB in Kupffer cells and induces mitochondrial dysfunction in neighboring hepatocytes. Activation of NF-κB in Kupffer cells and hepatocytes results in the production of proinflammatory cytokines which can stimulate other liver cells via autocrine and paracrine mechanisms to produce inflammatory mediators including chemokines and adhesion molecules. Chemokines and adhesion molecules function to facilitate the recruitment of neutrophils and propagate the inflammatory response. NF-κB can also have hepatoprotective functions by promoting hepatocyte recovery and regeneration. From Shin et al. [160]. Used with permission

AP-1

Activator Protein-1 (AP-1) represents a family of proteins that modulate various cellular processes such as programmed cell death, cellular proliferation, and cell survival mechanisms [37]. There are three main protein groups: Jun proteins (c-Jun, JunB, JunD), Fos proteins (c-Fos, FosB, Fra1, Fra2), and activating transcription factors (ATF2, ATF3, and B-ATF). These peptides form dimers that constitute the AP-1 complex. Because of its role in cellular regulation, AP-1 is of particular interest in hepatic ischemia/reperfusion injury. Studies have shown that AP-1 DNA binding complexes and c-Jun N terminal kinase (JNK) are activated after hepatic I/R [38, 39]. AP-1 activation is primarily believed to contribute to the hepatocellular injury seen in hepatic I/R. Zhou et al. showed that recombinant adenoviral delivery of mitochondrial superoxide dismutase (MnSOD) attenuated AP-1 activation and resulted in decrease liver damage after hepatic I/R in mice [39]. More direct studies demonstrated that JNK inhibition in rat liver undergoing hepatic I/R resulted in improved survival and decreased liver damage as evidenced by both decreased serum ALT levels and decreased cellular necrosis and apoptosis [38]. More recent studies have demonstrated that AP-1 is regulated by JunD during I/R. Mice nullizygous for JunD showed increased phosphorylation of c-Jun and activation of AP-1 after I/R [37]. These effects resulted in increased hepatocellular injury [37]. Thus, it appears that AP-1 is an important transcriptional mediator of hepatic I/R injury which can be regulated by expression of AP-1 family proteins such as JunD.

JAK-STAT Pathway

The Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway has been implicated as a key mediator of the protective effects observed in ischemic preconditioning of both heart and liver [40–42]. The JAK family consists of a number of tyrosine kinases that are linked to cytokine receptors. Ligand binding to these receptors results in JAK activation and subsequent phosphorylation of STAT proteins. There are seven STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5b, and STAT6. Phosphorylation of STAT monomers results in formation of homo- or heterodimers which posses the ability to translocate to the nucleus, bind target DNA sequences and induce gene transcription [41]. Among STAT family members, it appears that STAT3, which is a target of IL-6, is the most relevant to hepatic I/R injury. Matsumoto et al. demonstrated that STAT3 and IL-6 where increased after ischemia preconditioning and protected the liver from hepatic I/R injury and that the hepatoprotection and upregulation of STAT3 was not seen in IL-6 −/− mice [42].

STAT4 and STAT6 also play a role in the regulation of liver I/R injury. STAT4 is activated primarily by ligand binding to the interleukin (IL)-12 receptor. IL-12 has been shown to be a proximal mediator of the inflammatory injury response to I/R in the liver [43]. The role of STAT4 in liver I/R injury is somewhat controversial. Knockout of STAT4 has shown to have either no effect on liver injury [44], or greatly reduced injury [45]. The hepatoprotection afforded by STAT4-deletion observed in the latter study was associated with

increased expression of heme oxygenase-1 (HO-1), suggesting that STAT4 contributes to liver injury by suppressing HO-1 expression [45].

In contrast to STAT4, STAT6 appears to be hepatoprotective during I/R injury. Exogenous administration of the cytokines IL-4 and IL-13 results in increased activation of STAT6 in the liver and decreased proinflammatory cytokine production, neutrophil recruitment, and liver injury [46]. However, when these cytokines were administered to STAT6-knockout mice, the protective effects were lost, suggesting that STAT6 may represent as a viable therapeutic target. Subsequent studies confirmed the hepatoprotective role of STAT6 [45].

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) represent a family of hormone nuclear receptors whose activity is regulated by specific ligands including arachidonic acid metabolite, and 15-deoxy-Delta^{12,14}-prostaglandin J_2 (15d-PGJ_2) [47]. There are three known isoforms of the PPAR subfamily in mammals: PPARα, PPARβ/δ, and PPARγ [48]. PPARγ is known to have anti-inflammatory properties as its activation inhibits the release of TNFα, IL-1, and IL-6 by macrophages [49, 50]. Synthetic PPARγ ligands, including thiazolidinediones (TZDs) are widely used as insulin sensitizers in the treatment of diabetes and have been used to treat nonalcoholic steatohepatitis with some success [51, 52]. Studies have demonstrated that TZDs and connecting peptide (C-peptide) activate PPARγ to attenuate I/R injury to the myocardium, intestine, kidney, and lung [53–58].

Recently the role of PPARγ activation during hepatic ischemia/reperfusion was investigated. PPARγ was found to be constitutively activated in hepatocytes. However, during ischemia, PPARγ activation rapidly decreases and remains suppressed during reperfusion [59]. Mice treated with the PPARγ agonists, rosiglitazone and connecting peptide were noted to have increased PPARγ activation and reduced liver injury compared to untreated mice [59]. Furthermore, PPARγ-deficient mice had more liver injury after ischemia/reperfusion than their wild-type counterparts [59]. These studies suggest that PPARγ is an important endogenous regulator of liver ischemia injury and that its activation is hepatoprotective during liver I/R. Furthermore, PPARγ activation has been showed to be age-dependent [53]. Although there is no difference in constitutive expression of hepatocyte PPARγ ligand at baseline among various age groups, there was some variation in the distribution of isoforms between age groups. In addition, PPARγ activation was prolonged after liver I/R in young mice [53]. Consequently, it appears that PPARγ activation may be an important regulatory mechanism of liver I/R injury that is diminished with increased age.

Peroxisome proliferator-activated receptor-α (PPARα) has also been implicated as a key regulator of liver ischemia/reperfusion injury. PPARα has been linked to down-regulation of proinflammatory mediators [60, 61] and is expressed in hepatic parenchymal cells but not in Kupffer cells [62, 63]. Mice nullizygous for PPAR α have significantly greater liver injury after partial hepatic ischemia/reperfusion, than their wild-type counterparts [64]. Pretreatment of wild type mice with PPARα agonist, WY-14643, also resulted in similar results with significantly less parenchymal injury as measured by serum ALT levels than mice receiving vehicle [64]. PPARα -knockout mice also had augmented liver neutrophil accumulation without significant change in proinflammatory cytokines or chemokines [64]. This was also associated with decreased inducible nitric oxide (NO) synthase expression in liver than wild-type mice after ischemia-reperfusion protected against oxidant-induced injury [64]. These data suggest that PPARα is an important regulator of the hepatic inflammatory response to I/R in a manner that is independent of proinflammatory cytokines.

Proinflammatory and Anti-inflammatory Mediators

The cascade of cytokine production that is initiated during the early phase of reperfusion is the driving force for the development of the inflammatory response and the associated significant hepatocellular injury. Much is known about the expression of pro- and anti-inflammatory mediators and their roles in the propagation and resolution of liver injury (Fig. 26.3).

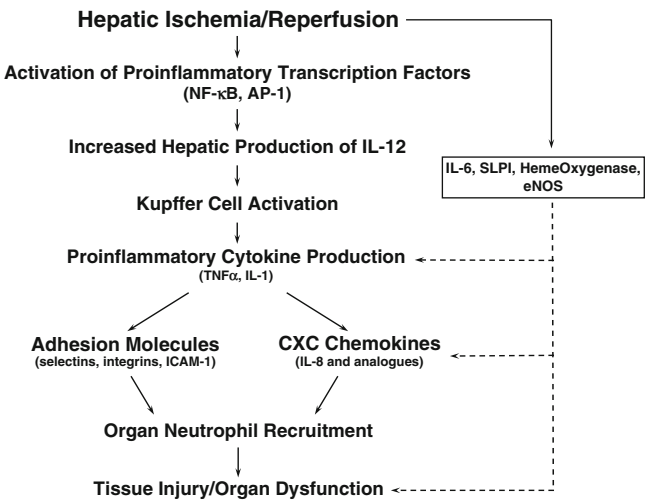

Fig. 26.3 Pro and anti-inflammatory mediator pathways that regulate hepatic I/R injury

Proinflammatory

IL-12

IL-12 is believed to be a proximal cytokine in the inflammatory cascade and critical to the propagation of inflammation after reperfusion. Its proinflammatory properties were first explored in sepsis models where it was found to be a potent Kupffer cell activator and a trigger for hepatic leukocyte accumulation [65]. It is also necessary for optimal clearance of bacteria and bacterial endotoxin [66]. In the context of hepatic I/R injury, studies have demonstrated that IL-12 expression is induced during the period of ischemia and peaks early after reperfusion and rapidly decreases after reperfusion [43]. More importantly, blockade of endogenous IL-12 with neutralizing antibody, significantly reduced subsequent tumor necrosis factor- α (TNFα) and interferon-γ (IFNγ) production. Conversely, administration of exogenous administration of IL-12 increases TNFα production and hence TNFα dependent liver injury [67]. A subsequent study provided evidence that IL-23, a cytokine closely related to IL-12, may also be an important proximal cytokine. As such, it is likely that IL-12 (and/or perhaps IL-23) is an integral mediator of the acute hepatic response to ischemia reperfusion by up-regulating production of other proinflammatory cytokines including, TNFα and IFNγ, thereby propagating hepatic inflammation and subsequent hepatocellular injury.

TNFα

TNFα is a central proinflammatory cytokine which is required for full induction of the inflammatory response to hepatic I/R [68]. Colletti et al demonstrated the neutralization of TNFα after hepatic I/R using antiserum results in reduced injury locally and systemically [68]. Kupffer cells are believed to be the primary generators of proinflammatory cytokines after hepatic I/R, but hepatocytes and extrahepatic sources are also believed to contribute [69, 70]. TNFα expression is rapid and peaks within 1–2 h of reperfusion [68]. It appears that the primary function of this cytokine is to induce and propagate the inflammatory response. It does so through induction of chemokines that serves as potent neutrophil-attractants and up-regulation of endothelial cell adhesion molecules that facilitate neutrophil transmigration into tissue beds [71, 72]. TNFα also potentiates hepatocellular injury by increasing hepatocyte susceptibility to injury [73, 74].

IL-1

IL-1 is a proinflammatory cytokine with similar effects to TNF α. For many years, it was assumed that it functioned in a capacity similar to TNF α. However, it was demonstrated that it differs substantially from TNF α in the role it plays in postischemic liver injury. While expression of TNF α peak at 1–2 h after reperfusion and then rapidly return to baseline levels, IL-1 expression gradually increases for many hours after reperfusion, peaking after 8–12 h [75]. Mice lacking functional receptors for IL-1 had no difference in the extent of liver injury, but did show delayed neutrophil recruitment in association with attenuated early production of the chemokines, macrophage inflammatory protein-2, suggesting that IL-1 may play a role in the sustained recruitment and sequestration of neutrophils after severe hepatic ischemia/reperfusion. Thus, unlike TNF α, IL-1 appears to function as an accessory molecule that facilitates chemokine expression and subsequent neutrophil recruitment, but is not essential for the inflammatory response induced by I/R.

Chemokines

CXC chemokines are critically involved in the recruitment of neutrophils to the liver after ischemia/reperfusion. They represent one of four branches of the chemokine superfamily based on a conserved, cysteine-containing amino acid sequence at the amino terminus of each molecule. CXC chemokines can be further subdivided into two subsets based on the presence or absence of a Glu-Leu-Arg (ELR) amino acid motif at the amino terminus of the peptide. ELR-positive CXC chemokines bind to the receptors CXCR1 and/or CXCR2, while ELR-negative chemokines bind to the receptors CXCR3, CXCR4, CXCR5, and CXCR6 [76, 77].

The ELR-positive CXC chemokines are of particular interest in liver injury, given that CXCR1 and CXCR2 are expressed by neutrophils, endothelial cells, and hepatocytes [78, 79]. TNF α and IL-1, proinflammatory cytokines produced early during warm hepatic I/R, induce CXC chemokine production by a variety of cells. Endothelial cell chemokine production is believed to augment neutrophil activation and adherence while parenchymal chemokine production is essential to create a chemokine gradient that encourages translocation of neutrophils from the blood stream to the site of injury. CXC chemokines are also up-regulated in remote organs after hepatic I/R and mediate remote organ neutrophil recruitment and injury which is observed after hepatic I/R [80, 81].

Platelet Activating Factor

Platelet activating factor (PAF) is a potent phospholipid that has been implicated as a catalyst in various inflammatory processes. It is produced by a variety of cells including neutrophils, macrophages, platelets, and endothelial cells. Its effect on neutrophils has been widely studied and it is now known to

promote chemotaxis, neutrophil priming, and superoxide generation [82, 83]. PAF also plays a critical proinflammatory role by inducing the production of various cytokines including TNFα, IL-6, and IL-8, all known to play central roles in the propagation of hepatic I/R injury [84–86]. Additional studies have shown that antagonism of PAF prior to hepatic I/R protects against injury by decreasing plasma cytokine levels, as well as reducing neutrophil accumulation, priming, and respiratory burst [87, 88]. Other studies have documented similar results with PAF antagonism resulting in decreased endothelial injury, lowered hepatic lipid peroxidation, and higher levels of tissue ATP when compared to control groups [89]. These studies suggest that PAF is an important upstream regulator of the hepatic inflammatory response to I/R.

TLR4 Ligands

Toll-like receptors (TLR) represent a class of evolutionarily conserved proteins that recognize various pathogens and then serve as key regulators of the innate immune system by activating immune cell responses [90–92]. TLRs are expressed on a variety of cells including macrophages, and when activated causes production of proinflammatory cytokines and costimulatory molecules [93]. TLR4, a receptor for lipopolysaccharide, has been implicated as a critical mediator of inflammation [94, 95] Liver I/R often have effects on distant organs, such as the intestine, and therefore may result in the elaboration of TLR4 ligands. Hepatic expression of TLR4 is up-regulated rapidly after I/R and is associated with increased production of TNF α [96]. In TLR4-deficient mice, production of TNF α, hepatic recruitment of neutrophils, and degree of liver injury were all decreased [97, 98]. These studies suggest that TLR4 is an important component of the hepatic inflammatory response to I/R and that it is likely an upstream regulator of this response.

Anti-inflammatory

In order to prevent an overwhelming and potentially lethal inflammatory response to hepatic I/R, endogenous anti-inflammatory mediators are expressed that serve to maintain a homeostatic balance. Several key mediators have been identified and their roles in regulating the inflammatory and injury responses are elucidated (Fig. 26.3).

IL-6

IL-6 is a pleiotropic acute phase reactant with anti-inflammatory properties that has been shown to be protective in hepatic I/R injury and ischemic preconditioning. The liver is a major site of IL-6 production and is the primary organ responsible for its clearance [99]. Ischemic preconditioning confers hepatoprotection as evidenced by improved survival and decreased hepatocellular injury in wild-type mice undergoing hepatic I/R. However, this survival benefit is negated in IL-6-knockout mice [42]. Administration of recombinant IL-6 prior to hepatic I/R was shown to have less cellular injury and faster recovery correlating to increased proliferation [100]. IL-6 is believed to mediate its hepatoprotective effects during hepatic I/R through down-regulation of TNFα and c-reactive protein production, thereby blunting the resulting inflammatory cascade [42, 100]. IL-6 carries out these effects through Jak/STAT pathway activation of STAT3, a transcriptional regulator that reduces apoptosis and increases production of protective antioxidants [42, 101, 102].

SLPI

Secretory leukocyte protease inhibitor (SLPI) is a protease inhibitor produced by a variety of cells [103]. During I/R injury, hepatic expression of SLPI occurs very early in the injury response [104]. Blockade of SLPI with antibody results in significant increase in proinflammatory mediator expression, neutrophil recruitment, and liver injury, demonstrating that SLPI is an important endogenous regulator of the injury response [104]. Administration of SLPI reduced activation of the transcription factor, NF-κB, and decreased production of proinflammatory mediators and reduced local and remote organ injury [104]. SLPI also functions as a potent inhibitor of multiple proteases which contribute to hepatocellular injury [105]. Thus, this mediator appears to protect against direct cellular injury as well as regulate the inflammatory response induced by I/R.

Heme Oxygenase

Heme oxygenase (HO) catalyzes the degradation of heme into carbon monoxide (CO), biliverdin, and free iron. HO exists in two forms; HO-1 is oxidative stress-inducible, while HO-2 is constitutively expressed [106]. HO-1 expression confers anti-inflammatory and antiapoptotic effects through the generation of byproducts such as CO and biliverdin [93]. Kato et al. noted that HO-1 overexpression induced by cobalt protoporphyrin (CoPP) administration reduced hepatocellular injury in rat livers undergoing ex vivo cold ischemia and reperfusion or isotransplantation [107]. Pretreatment with isoflurane, an inhaled anesthetic, also up-regulates HO-1 expression and is hepatoprotective during hepatic I/R [108]. Other studies have shown that up-regulation of HO-1 in response to I/R is less important than high baseline levels of activity prior to the procedure [109]. As mentioned above, the by-products of heme degradation by HO-1 also attenuate

hepatic I/R injury. Biliverdin in particular is believed to confer protection by decreased endothelial expression of adhesion molecules reducing the adherence and translocation of leukocytes to the postischemic liver [110, 111]. Biliverdin decreases production of proinflammatory cytokines and up-regulates antiapoptotic pathways [110, 111]. CO, another byproduct of heme degradation, is a potent anti-inflammatory molecule. When exogenous inhaled CO is administered in murine liver transplant and I/R models, TNFα and ICAM-1 expression are attenuated in association with decreased activation of NF-κB [112].

Nitric Oxide

NO is synthesized from L-arginine through the action of nitric oxide synthase (NOS). This enzyme exists in three forms: inducible NOS (iNOS), constitutive endothelial NOS (eNOS), and neuronal NOS (nNOS). NO production from eNOS is generally thought to be hepatoprotective in hepatic I/R through vasodilation and reduced macrophage infiltration [113, 114]. In contrast, NO derived from iNOS is thought to be hepatotoxic. iNOS-derived NO causes oxidative damage during hepatic ischemia reperfusion [115]. A recent study also demonstrated that iNOS-derived NO induces activation of matrix metalloproteinase-9 (MMP-9) which leads to detachment of hepatocytes from the extracellular matrix and cell death, and may also contribute to neutrophil recruitment [116]. Studies using specific knockout mice have demonstrated that iNOS contributes to hepatic injury, while eNOS is protective [117].

Lymphocytes

The role of neutrophils in hepatic I/R injury is well-known. However, over the last decade there has been increasing evidence of a significant role of lymphocytes in this response. The liver is home to a large population of leukocytes, only second to the spleen, and T lymphocytes make up a significant portion in this group [118]. T lymphocytes are rapidly recruited to the postischemic liver within the 1 h of reperfusion [119–121]. The majority of these lymphocytes are CD4-positive. Interestingly, lymphocyte recruitment precedes neutrophil recruitment. While the exact mechanism of recruitment is unknown, RANTES, a known T lymphocyte activator, is also up-regulated after reperfusion and has been implicated as a major chemoattractant for lymphocytes [122]. The function of $CD4^+$ T lymphocytes in I/R injury remains unclear as treatment of mice with anti-CD4 antibody reduces liver inflammation and injury [119] and pharmacologic inhibition of hepatic lymphocyte recruitment had similar effects [123], while studies employing CD4-knockout mice demonstrate reduced liver inflammation, but increased liver injury [120]. These studies were consistent in the findings that $CD4^+$ T lymphocytes regulate liver neutrophil accumulation. Interleukin (IL)-17 was identified as the CD4 T lymphocyte-derived mediator of neutrophil accumulation as blockade of IL-17 reduced neutrophil accumulation [120]. It appears that IL-17 functions to regulate the expression of the neutrophil chemokine, MIP-2. An analysis of specific $CD4^+$ T lymphocyte subsets found that γδ T cells mediate neutrophil recruitment, while NKT cells directly injure the liver [120]. Thus, it appears that CD4 T lymphocytes mediate both inflammatory and direct injury components of the hepatic response to I/R.

Neutrophil-Mediated Liver Injury

Jaeschke et al. were the first to document the important role of neutrophils in hepatocellular injury occurring after I/R [1]. The mechanisms by which neutrophils are recruited to the liver were the topic of intensive study for many years. Today, many of the details of these mechanisms are known and have been applied in the development of therapeutics targeting leukocyte recruitment. Hepatic I/R results in the local expression of TNF α [124]. With regards to neutrophil recruitment, TNF α has two important effects. First, it stimulates multiple cell types in the liver to produce CXC chemokines (described above) which results in the formation of a chemotactic gradient. Second, it stimulates vascular endothelial cells to up-regulate adhesion molecule expression on their cell surface [71].

There are three main classes of adhesion molecules. The selectin family of adhesion molecules is expressed on both leukocytes and endothelial cells, and function to capture neutrophils through initial rolling and transient adhesion to the endothelium [125–127]. By bringing neutrophils in close proximity to the endothelial cell surface and reducing their velocity, selectins allow other adhesion molecules expressed on neutrophils and endothelial cells to interact. Specifically, integrins (e.g., CD11b) expressed on neutrophil surfaces and immunoglobulin molecules (e.g., ICAM-1 and VCAM-1) on endothelial cells mediate firm adhesion and diapedesis of the neutrophil from the vascular space to the interstitium [128–130]. Neutrophils that are adherent to the vascular endothelium are activated by CXC chemokines expressed at the site of injury. Furthermore, the chemotaxis of these activated neutrophils to the liver parenchyma is directed by a preestablished CXC chemokine gradient. Once there, these primed neutrophils are activated they mediate the significant hepatocellular injury observed many hours after I/R.

Accumulated neutrophils damage hepatocytes via elaboration of oxidants and proteases. Adherence of neutrophils to hepatocytes maximizes and prolongs the release of these cytotoxic elements and is mediated by CD11b on neutrophils and ICAM-1 on hepatocytes [10]. Hepatocyte ICAM-1

expression is up-regulated during the inflammatory response by stimulation with cytokines such as TNFα and IFNγ [10]. Activated neutrophils produce superoxide via NADPH oxidase. This superoxide spontaneously dismutates to oxygen and hydrogen peroxide and is used by neutrophil-derived myeloperoxidase to form hypochlorous acid (HOCl) [124, 131]. HOCl is highly reactive and can react with amino groups to form toxic chloramines [132].

Additionally, release of proteases (e.g., collagenase, elastase, cathepsin G, heparanase) from stored granules occurs during neutrophil activation and contributes to hepatocellular injury [133, 134]. These two mechanisms of neutrophil-mediated hepatocyte injury work synergistically. Oxidant production results in HOCl production and an acidic environment which inactivates antiproteases present in the plasma, thereby prolonging the action of proteases and increasing hepatocyte necrosis [135].

Recovery from Warm Ischemia/Reperfusion

Time Course and Cell Cycle Control

While the mechanisms of liver injury after I/R have been widely studied, the manner in which it clears dead tissue, resolves inflammation and recovers and regenerates its functional mass has not been thoroughly studied. Initial work has characterized the recovery response in the liver after I/R injury. Within 12 h, hepatocellular injury has occurred by the mechanisms discussed thus far. At this point, there is evidence of dead areas of liver being walled-off by recruited leukocytes and over the course of the next 5–7 days, dead tissue is cleared and replaced with healthy hepatocytes. Regulation of cell cycle control proteins occurs during this period and some of these have been characterized [136].

The p53 pathway is usually switched off at rest and is induced when cells are exposed to insults including DNA damage [137]. p53 then transcriptionally activates multiple proapoptotic genes including p21 [137]. p21 is a cyclin-dependent kinase (CDK) inhibitor that regulates the transition from G_1 to S phase of the cell cycle. p21 expression is undetectable in normal liver, but is markedly increased after ischemia and 6–24 h of reperfusion [136]. After 48 h of reperfusion, expression returns to normal levels. The up-regulation of p21 coincides with the injury response after I/R.

Stathmin is an important regulatory protein of microtubule cytoskeletal remodeling. It is critical to cell cycle progression and proliferation through mitosis. Stathmin transcription is negatively regulated by p21 [138]. After hepatic I/R, p21 is induced after 6 h of reperfusion and remains elevated for 24 h and stathmin expression remains suppressed [136]. However, after 48 h, expression of p21 returns to normal and at this time there is substantial induction of stathmin expression in the liver. This increase in stathmin expression was found to colocalize with proliferating hepatocytes in the recovering/regenerating liver [136], suggesting that it is an important contributor to hepatocyte regeneration following I/R.

Chemokine Participation

As described above, CXC chemokines are critical for neutrophil recruitment after hepatic I/R. In endothelial cells, the binding of CXC chemokines to CXCR2 results in proliferation and chemotaxis in a manner facilitating angiogenesis [139, 140]. Similarly, other studies demonstrate that the receptor, CXCR2, is expressed on hepatocytes [141] and that CXC chemokines have direct effects on hepatocytes that are mediated by CXCR2 [142, 143]. In vitro studies demonstrated that the CXCR2 ligand, MIP-2, promotes hepatocyte proliferation [142]. Furthermore, liver regeneration after partial hepatectomy was significantly reduced in CXCR2-knockout mice or wild-type mice treated with neutralizing antibodies to MIP-2 [143]. Collectively, these studies suggest that CXC chemokines, acting through CXCR2, promote liver recovery and regeneration in a model of resection, in which the remnant liver is uninjured.

The role of CXC chemokines on hepatocytes during liver recovery and regeneration after I/R is far different. It has been demonstrated that mice lacking CXCR2 or treated with a CXCR2 antagonist, have enhanced liver proliferation and recovery after I/R [144]. This study suggests that signaling through CXCR2 in hepatocytes exposed to the stress of I/R is detrimental to recovery and limits proliferation. Another aspect of the divergence in function of CXC chemokines signaling through CXCR2 between hepatectomy and I/R may relate to ligand availability. In models of hepatectomy, in which the remnant liver is uninjured, levels of the chemokines MIP-2 and KC are increased three to fivefold 48 h after 70% hepatectomy [142]. In contrast, after ischemia and 48 h of reperfusion MIP-2 and KC levels are more than 100-fold higher than sham-operated controls [144]. Others studies have shown that adenoviral-mediated overexpression of KC in the liver (>100-fold) results in massive hepatocellular necrosis within 48 h [145]. The concept of concentration-dependent effects on hepatocyte proliferation or death were confirmed with in vitro studies that demonstrated that MIP-2 is protective against hepatocyte cell death at low doses, but augments death at high doses [144]. Thus, it seems that low to moderate increases in CXCR2 ligands, as occurs after partial hepatectomy, may promote liver regeneration, whereas much larger increases in expression of CXCR2 ligands, as occurs after I/R injury, may be hepatotoxic and/or oppose hepatocyte proliferation and regeneration. The signaling

pathways utilized by CXCR2 have been well-studied in neutrophils. However, nothing is known regarding the signaling pathways used by this receptor in hepatocytes. Given the potential clinical impact of this ligand/receptor system, this represents an important gap in our knowledge that warrants further investigation.

Cold Ischemia–Reperfusion Injury

Orthotopic liver transplantation (OLT) is the best therapy for end-stage liver disease. Ischemia/reperfusion injury is a primary concern and is the major cause of primary graft non-function after liver transplantation. The devastating consequences of liver I/R and the ability to prevent them have been widely studies for decades. Raffucci et al. [146] in the 1950s documented increased tolerance and survival in hypothermic dogs undergoing hepatic I/R when compared to their normothermic counterparts. Hypothermia mediates its protective effects on hepatocytes during hepatic I/R by altering the hepatic inflammatory response that occurs after I/R. Studies in murine models have demonstrated a decrease in liver injury as determined by serum markers and histology by as much as 89% in hypothermic subjects when compared to normothermia and is accompanied by decreased production of the proinflammatory cytokines TNFα and IL-1β and consequently a decrease in neutrophil accumulation [147]. Several transcription factors and signal transduction pathways have also been implicated in hypothermic protection against liver I/R injury. JNK and AP-1 activation is notably decreased in livers subjected to hypothermic I/R when compared to warm I/R [147]. It is now widely accepted that hepatic hypothermia protects against hepatocyte injury during surgical resection [148] and this technique is now commonly used in a variety of clinical scenarios. However, OLT provides a unique clinical setting in which the period of cold ischemia and the cells affected differ from simple hepatic I/R and/or hepatic resection.

As previously discussed, ischemic liver injury is caused by a deficiency of oxygen causing the loss of mitochondrial respiration, ATP depletion, and deterioration of energy-dependent metabolic pathways and transport processes [149]. Hypothermia and cold preservation after harvesting the liver reduces the metabolic rate in the tissue, thereby prolonging the period during which anoxic cells retain essential metabolic functions [150] enabling regional transport of organs without significant increase in ischemic graft injury. Clinical hypothermia along with a variety of preservative solutions at a temperature of 4°C is optimal for graft storage [151, 152]. Hypothermia itself, however, can induce cell injury through a variety of mechanisms described below.

Sinusoidal Endothelial Cell (Sec) Apoptosis

While hypothermia provides cellular protection to hepatocytes undergoing ischemia, it induces significant apoptotic cell death in liver endothelial cells, breaching the integrity of the liver vasculature [153]. This cold-induced apoptosis is mediated by two separate mitochondrial alterations that occur on subsequent rewarming. The first is a reversible process characterized by mitochondrial shortening. The second results in iron-dependent formation of reactive oxygen species that affect mitochondrial permeability and leads to apoptosis [153]. Hypothermia also directly induces cellular injury to theses endothelial cells by causing an influx of sodium and chloride followed by secondary alterations of cellular calcium homeostasis and cell swelling [154]. SECs are more susceptible to cold ischemia than hepatocytes and only a short period of ischemia (30 min) produces apoptosis only of the SECs where as hepatocytes can tolerate longer periods of ischemia, up to 1 h before significant apoptosis is observed [154]. Typically, SECs will morphologically display cytoplasmic blebbing, nuclear and cytoplasmic condensation, and typical apoptotic bodies in phagolysosomes after cold I/R [154].

Increased Platelet Adhesion

Platelet adhesion to sinusoidal endothelium at the time of organ reperfusion is one of the main deleterious effects of cold preservation as early postoperative liver function post-transplantation is directly related to the extent of platelet adhesion that occurs on reperfusion [155]. On reperfusion, circulating platelets rapidly adhere to SEC and release toxic intermediates that injure hepatocytes as well as induce further SEC apoptosis [156, 157]. Hypothermia is believed to mediate this response by increasing SEC expression of the platelet receptor, von Willebrand factor (vWF), thereby increasing platelet adhesion and activation. This process appears to be at least partly mediated by actin disassembly and MMP secretion by SECs [158].

Recovery and Regeneration

Decreased Liver Regeneration

More recently, it has been demonstrated that cold ischemia decreases the ability of the liver to regenerate after partial liver transplantation in rats [159]. Selzner et al. found that prolonged cold preservation time (10 and 16 h) was associated with a

dramatic decrease in PCNA staining, a marker of cell proliferation and thus, regeneration [159]. There was an associated decrease in TNFα and IL-6 levels in the same group when compared to rats with a shorter period of cold ischemic preservation [159]. Pretreatment with recombinant IL-6 (rIL-6) reversed impaired proliferation as evidenced by normalization of markers of regeneration and significantly improved survival in the 10-h group [159]. These findings suggest that sustained periods of cold preservation significantly impair TNFα and IL-6 production and the regenerative ability of the liver.

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Chapter 27
Inflammation and Liver Injury

Pranoti Mandrekar and Gyongyi Szabo

Introduction

The inflammatory response in the liver contributes to a great extent to the development of acute and chronic liver diseases. The physiological function of the liver is elimination of pathogens and antigens from the blood for which mounting of an immune response is required [1]. To avoid unnecessary activation of the immune system, the liver develops a local immune response followed by induction of peripheral tolerance toward the antigen. When stressful agents such as pathogens or environmental insults challenge the liver for extended periods of time and their elimination is not possible, inflammation follows. The onset of inflammation is followed by fibrosis, cirrhosis, and liver failure. In acute forms of liver injury caused by viruses, drugs, or liver resection, inflammatory responses play an important role leading to cytotoxic injury. Cytokines produced by cells of the innate and adaptive immune system in the liver are key mediators of the inflammatory response and are the driving force in inflammatory disease [2]. The primary activation of innate immune cells in the liver occurs through recognition of pathogen-derived signals such as endotoxin and other exogenous or endogenous ligands. These danger signals enter the liver via portal circulation and are recognized by pattern recognition receptors (PRR) that activate intracellular signaling pathways resulting in cytokine and interferon gene expression [3, 4]. Endogenous danger signals from damaged or dying cells can also stimulate the same inflammatory pathways. In this review, we will describe the various immune cell types involved in the hepatic inflammatory diseases, discuss the hepatocellular receptors involved and signaling pathways, and finally highlight the mediators of inflammation such as cytokines, chemokines, and immunoregulatory cytokines activated during liver injury.

G. Szabo (✉)
Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA
e-mail: gyongyi.szabo@umassmed.edu

Cells in the Liver and Inflammatory Response

Immune Cells in the Liver

The function of the liver as an "immune organ" is ascribed to the fact that a host of immune cells reside in the liver to perform a wide array of immune responses. The liver has the unique potential to sense and attack invading pathogens, warn the host of pathogens through production of inflammatory mediators, and regulate the induction of pathogen-specific adaptive immune responses. In addition to pathogens, endogenous danger signals from damaged or dying cells are also recognized by innate immune pathways in the liver and induce "sterile" inflammation. All these and many other functions are executed by the different cell types in the liver including the parenchymal cells or hepatocytes, but more importantly the nonparenchymal innate immune cells such as tissue-resident macrophages or Kupffer cells (KCs), different types of dendritic cells (DCs), natural killer (NK) cells, and NKT cells all enable appropriate immune responses against pathogens. In addition, infiltrating cells such as neutrophils, T cells, regulatory T cells, and bone marrow-derived stem cells play an important part during acute and chronic liver inflammatory diseases. Liver inflammation is categorized under acute hepatitis identified by acute inflammation and damage to hepatocytes or chronic liver injury recognized by steatohepatitis or liver fibrosis. Different liver cell types (see Fig. 27.1) contribute to liver injury based on the hepatotoxin, duration of the insult, and direct or indirect exposure to the hepatotoxin.

Monocytes/Macrophages

Tissue-resident macrophages or KCs comprise about 35% of the nonparenchymal liver cells in normal adult mice and constitute the first macrophage population in the body to come in contact with invading pathogens, toxins, and tumor cells leading to their clearance and mounting of a systemic immune response to the local inflammation [5]. A more comprehensive

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_27, © Springer Science+Business Media, LLC 2011

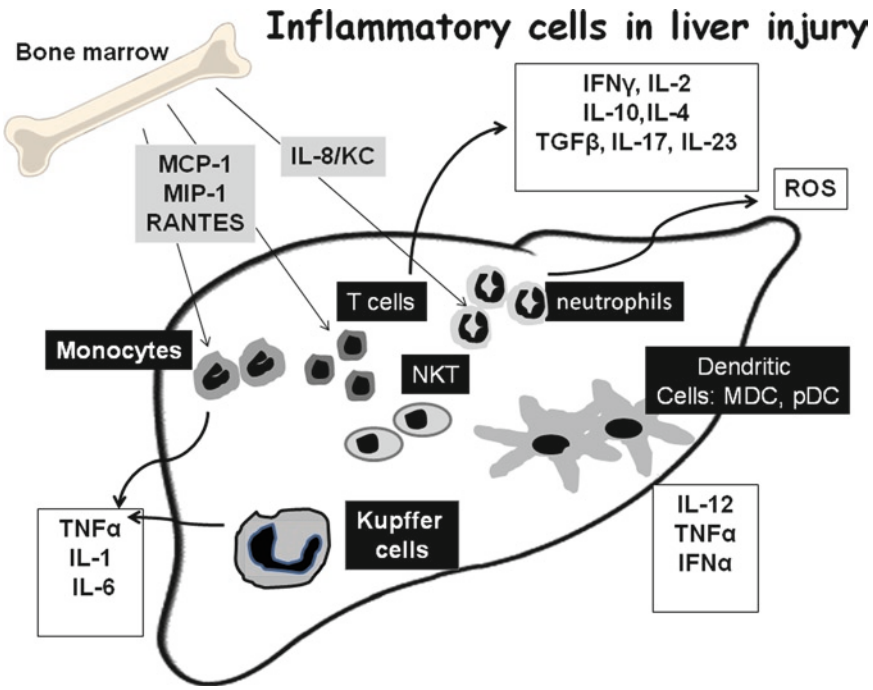

Fig. 27.1 Inflammatory cells involved in liver injury. Immune cells types such as monocyte/macrophages, T cells and NKT cells, dendritic cells, neutrophils, and the cytokine/chemokine mediators produced resulting in liver inflammation are depicted

account on this cell type is provided in Chap. 6. Activation of KCs leads to modification of its morphology and surface expression of classical macrophage markers such as CD68 (ED1) and CD163 (ED2) as well as production of a large number of immune mediators including cytokines such as IL-1, TNFα, and IL-6, chemokines such as IL-8, MCP-1, MIP-1, and RANTES, and reactive oxygen species (ROS) [4, 6–9]. KCs represent an important component of innate immunity in the liver and express toll-like receptors (TLRs) 1–6 and TLR8 that are pivotal in liver homoeostasis [10]. While the existence of pathogens and toxins below a certain threshold results in protective effects of KCs, it has been shown that higher thresholds or extended duration of exposure to environmental insults can lead to pronounced secretion of inflammatory mediators and ultimately to KC-mediated liver injury. The role of KCs in ischemia-reperfusion injury, acetaminophen injury, viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, and fibrosis has been well studied [2, 11, 12].

Dendritic Cells

Hepatic DCs, classical antigen-presenting cells in the liver, are normally found in the portal triad and the central veins, maintain an immature state, and efficiently capture and process antigens in the liver [13, 14]. Upon antigen capture in the sinusoidal area or exposure to environmental insults such as alcohol, circulating DCs can transcytose into the inflamed liver and subsequently migrate to hepatic lymph nodes [15– 18]. Owing to the efficient antigen-presenting function, hepatic DCs can readily tip the balance between an active immune response and immune tolerance [19]. The immunotolerogenic capacity of the liver, at least partially, is owed to the predominant presence of immature DCs [20]. Immature DCs can induce T cell tolerance, whereas mature DCs promote antigen-specific T cell activation [21]. Maturation of DC is associated with up-regulation of MHC II, CD80, and CD86, with CD205 being an additional marker [14]. While only less than 1% of the total nonparenchymal cells in the liver are DCs, two major subtypes present are myeloid and plasmacytoid [22–24]. Myeloid dendritic cells (MDC) are $CD14^{-}$, BDCA-1^{+}, $CD11c^{+}$, $CD83^{+}$, $CD33^{+}$, and HLA-DR^{bright} cells and produce cytokines including IL-12 and IL-10 and little IFNα in response to pathogens [14, 15]. All TLRs except for TLR7 and TLR9 are functional in human MDC, whereas TLR9 is expressed on MDCs in mice [25]. Plasmacytoid DCs (PDCs), potent producers of Type-I IFNs, are HLA-DR^{bright}, BDCA-2^{+}, BDCA-4^{+}, and $CD123^{bright}$ cells, but lack myeloid markers CD11c and CD33 [13, 14, 25]. PDCs are present in the normal liver and their functional activation is fundamental for defense against viral infections [26]. Viral sensing TLRs, TLR7/8, and TLR9 are expressed on PDCs triggering large amount of IFNα production in these cells [27–29]. Immature MDCs and PDC exist in normal liver and additional recruitment of these cells occurs in response to infectious stimulation [15–17]. In hepatitis C infection, functional defects were noted in both plasmacytoid

and myeloid DC populations in human peripheral blood and myeloid DCs were further impaired by alcohol administration in vitro [28, 30, 31]. Animal data mirror human findings such that the mice that express HCV core protein exhibit impaired DC function, which was further diminished by administration of alcohol [32]. Alcohol even in the absence of infection was shown both in humans and animal models to inhibit DC capacity to induce T cell proliferation [33, 34].

Neutrophils

Polymorphonuclear leukocytes or neutrophils are vital to host defense and are involved in an early response to tissue injury to eliminate microorganisms and apoptotic cells [35–37]. Neutrophil recruitment at the site of tissue injury occurs in response to inflammatory mediators particularly chemokines such as IL-8, MIP-2, and CINC-1 and also TNFα, IL-1α, and IL-1β [38–43]. Inflammatory mediators not only recruit neutrophils, but also prime the cells for increased ROS production [44], creating an oxidative environment in the liver leading to mitochondrial injury and hepatocyte damage [45]. Activation of neutrophils results in increased myeloperoxidase activity, activation of NADPH oxidase, and iNOS contributing to tissue damage [36, 37].

NK and NKT Cells

The liver is enriched in natural killer (NK) and NK-like T cells (NKT), which fulfill functions of host defense against invading pathogens, T cell recruitment, and modulation of liver injury. While human NK cells are identified based on their CD56 expression, murine NK cells are identified by expression of marker, NK1.1. NK cells are regulated by cytokines derived from KCs such as IL-12 and IL-18, produce large amounts of antiviral interferon gamma (IFNγ), and modulate T cell responses in the liver [46, 47]. The percentage of NK cells in the liver may increase in hepatic diseases such as viral infections, hepatis steatosis, ischemia-reperfusion injury, and liver cancer [5, 48, 49] and is shown to inhibit hepatocyte proliferation [50] via IFNγ. An unconventional population of T cells that expresses markers of NK and T cells is also identified in high frequencies during hepatic inflammation in alcoholic liver disease, hepatitis models, steatosis, and fibrosis [49, 51]. These cells are termed NKT cells and can promote acute liver damage by release of IL-4, IFNγ, and Fas-mediated apoptosis [49]. Both NK and NKT cells are important antiviral effectors due to their contribution to virus elimination via direct killing of virus-infected hepatocytes and cytokine production [48, 52].

Regulatory T Cells

Regulatory T cells (Tregs) not only play an important role in immune homeostasis by suppressing autoreactive T cells, but also impair effective immune responses to some pathogens and tumor cells [53]. Of critical importance are the CD4+CD25+FoxP3+ Tregs which maintain peripheral tolerance by suppression of T cell responses. Tregs are induced during various liver diseases such as hepatitis B and C virus infection and hepatocellular carcinoma (HCC) and correlate with disease progression, suppression of the activity of $CD8^+$ T cells, and survival time in patients [54, 55]. Tregs produce IL-10 and TGFβ to create a regulatory milieu and result in antigen-specific as well as by-stander immunosuppression [56]. In addition, inhibitor molecules such as CTLA-4 and PD-1 expressed on Tregs further contribute to suppression of T cell responses [56, 57]. On the other hand, low Treg frequency and loss of function correlate with induction of autoimmune hepatitis indicating their function in maintenance of hepatic tolerance and homeostasis [58, 59]. In liver transplantation, however, Tregs have clinically beneficial effects as they play a major role in maintaining spontaneous immune tolerance [60, 61].

Th17 Cells

Interleukin-17 (IL-17)-producing $CD4^+$ T cells (Th17) have recently been identified as important "players" in inflammation-associated diseases due to their capacity to clear specific pathogens and induce autoimmune tissue inflammation [62]. Transforming growth factor β (TGFβ) with IL-6 or IL-23 initiates differentiation while IL-23 stabilizes the generation of Th17 cells [63]. Th17 cells express the transcription factor retinoid-related orphan receptor (ROR)γt, RORα, and signal transducer and activator of transcription-3 (STAT3). TGFβ, which is a well-identified mediator of liver fibrosis, is essential for the development of both Th17 and regulatory T cells (Treg) which raises the possibility of a developmental link between Th17 and Tregs; however, while Th17 cells are highly proinflammatory, Tregs inhibit tissue inflammation and maintain self-tolerance [64]. The imbalance between Th17 and Tregs has been shown to contribute to increased inflammation and autoimmune tissue damage in primary biliary cirrhosis in humans [65, 66]. Reduced Treg and increased Th17 cell ratios and increased serum levels of IL-17/IL-23 are also associated with acute rejection in liver transplantation [67]. Periductal IL-17 production was associated with increased proinflammatory cytokine expression (IL-6, lL-1β, IL-23p19 mRNA) in biliary epithelial cells in livers with PBC [68]. In alcoholic hepatitis which is a condition with high degree of proinflammatory activation, a recent study

showed increased IL-17 levels in human alcoholic liver disease [69]. In chronic HBV infection, the frequency of Th17 cells was also increased in the blood and in the liver [70]. IL-17 promoted activation of myeloid DCs and monocytes and enhanced production of proinflammatory cytokines [70]. Together, these observations suggest that increased Th17 cell recruitment and activation in the liver is associated with several types of liver inflammation.

Bone Marrow-Derived Stem Cells

Bone marrow-derived stem cells comprise of hematopoetic stem cells (HSC) and mesenchymal stem cells (MSC) and hepatic progenitors (HPC) are reported to give rise to hepatocytes by transdifferentiation and cellular fusion [71]. While bone marrow cells contribute to restoration of liver function by hepatocyte regeneration, in the context of disease, these cells can also play an important role in liver injury based on the inflammatory milieu to which they are recruited. A growing body of evidence indicates that bone marrow cells can contribute to fibrogenic potential [72]. During progression of hepatic inflammation to fibrosis, activated stellate cells appear to be myofibroblasts which may be derived from epithelial-to-mesenchymal cell transition or derived from bone marrow precursors [73]. Portal fibroblasts and circulating mesenchymal cells derived from bone marrow are also important sources of matrix proteins in fibrosis. Cytokines and chemokines released by KCs, particularly TGFβ and platelet-derived growth factor (PDGF), activate stellate cells resulting in production of extracellular matrix proteins and the fibrous scar. Among the infiltrating immune cells to the site of injury, mature neutrophils that reside in the bone marrow are rapidly mobilized during an inflammatory episode or in response to an infection [74]. In acute and chronic liver inflammation, chemokines such as IL-8/KC and MIP-2 provide the positive signal for neutrophil migration, whereas CXCL12/SDF-1α retained neutrophils in the bone marrow via activation of the SDF-1α/CXCR4 chemokine axis. Studies have also shown a role for IL-17 in the neutrophil recruitment from the bone marrow in inflammatory conditions via induction of G-CSF and CXC chemokines [75, 76].

Signaling Pathways and Liver Inflammation

Sensing Danger Signals

Inflammation is the natural protective response of innate immune cells to danger signals to eliminate the cause and maintain homeostasis. The source of danger signals in the.liver could be endogenous molecules derived from damaged cells, named damage-associated molecular patterns (DAMPs), or pathogen-associated molecular patterns (PAMPs) that are recognized by receptors in the PRR family. The role of PRR including TLRs, peptidoglycan receptors, and helicases, all expressed on immune cells as well as on some parenchymal cells [4, 77] in hepatic inflammation, is increasingly evident (see Table 27.1). Engagement of PRR and cytokine receptors on parenchymal and nonparenchymal cells in the liver leads to activation and interaction of various intracellular signaling molecules. Hepatic inflammation in acute and chronic liver diseases involves recognition of circulating endotoxin/lipopolysaccharide by the PRR or TLRs and finally production of cytokines and chemokines. In innate immune cells, binding of TLRs to their ligands is associated with activation of

Table 27.1 Pattern recognition receptors

	Ligands			
Receptors	PAMP	DAMP	Cellular localization	Adapters
Toll-like receptors (TLRs)				
TLR 2	Peptidoglycan (gram +) Lipoteichoic acid (gram +) Phospholipomannan *(Candida)*, lipoarabinomannan *(Mycobacteria)* Glycolipids *(Treponema maltophilum)* *Neisseria* porins Viral envelop proteins of measles virus, human CMV, and HSV type I Zymosan (fungi) Glycoinositolsphingolipids (*Trypanosoma cruzi*)	Heat shock proteins (Hsp60, Hsp70, HSPB8, αA crystalline, gp60) HMGB1 Hyaluronan fragments (ECM) Biglycan (ECM)	Membrane	MyD88/Mal
TLR 2/1 heterodimer	Tri-acyl lipoproteins		Membrane	MyD88/Mal
TLR 2/6 heterodimer	Di-acyl lipopeptides		Membrane	
TLR 3	Double-stranded RNA (virus), poly I:C, 3pRNA (RIG-I)	Endogenous mRNA	Endosome	TRIF/Tram

(continued)

Table 27.1 (continued)

Receptors	Ligands		Cellular localization	Adapters
	PAMP	DAMP		
TLR 4	Glycoinositolsphingolipids (*Trypanosoma cruzi*) Lipopolysaccharide (LPS) (Gram-) Flavolipin (Flavobacteriae) Mannan F protein of respiratory syncytial virus (RSV) Murine retroviral envelope protein	Heat shock proteins (Hsp60, Hsp70, HSPB8, αA crystalline, gp60) HMGB1 Hyaluronan fragments (ECM) Biglycan (ECM) Fibronectin fragments (e.g. Fn-EDA) Heparan sulfate, SP-A Fibrinogen Minimally modified (mm)LDL Mrp8, Mrp8-Mrp14 complex (S100 proteins) Murineβ-defensin-2	Membrane	MyD88/Mal TRIF/Tram
TLR 5	Flagellin *(Legionella, Salmonella, Listeria, Pseudomonas)*		Membrane	MyD88/Mal
TLR 6	Lipoteichoic acid (gram +) Zymosan (fungi)		Membrane	MyD88/Mal
TLR 7/8	Single-stranded RNA (virus); synthetic: imidazoquinoline		Endosome	MyD88/Mal
TLR 9	DemEthylated CpG (bacterial and viral); Malaria pigment hemozoin	HMGB1	Endosome	MyD88/Mal
TLR 10 (humans only)	Unknown		Membrane	
TLR 11	Uropathogenic bacteria; *Toxoplasma gondii* profilin-like protein		Membrane	MyD88/Mal
NLR receptors				
NOD 1	*Meso*-lanthionine; *meso*-DAP *(Chlamydia* spp*; enteroinvasive E.coli)*γ-d-Glu-*meso*-DAP (iE-DAP) *(Helicobacter pylori, Listeria monocytogenes)* l-Ala-γ-d-Glu-*meso*-DAP (TriDAP) D-lactyl-l-Ala-γ-Glu-*meso*-DAP-Gly (FK 156) *(Pseudomonas* spp*., Shigella flexneri)* Heptanoly-γ-Glu-*meso*-DAP-d-Ala (FK565)			
NOD 2	Muramyldipeptide (MDP) M-TRILys			
NLRC3	M-TRILys			
NLRC4 (IPAF)	Flagellin *(Legionella, Salmonella, Listeria, Pseudomonas)*			
NALP1	MDP Anthrax lethal toxin			ASC
NALP2	MDP			
NALP3	Lipopolysaccharide (LPS) (Gram-) Maitoxin Viral and bacterial RNA Aerolysin Nigericin	Uric acid crystals, (silica, aluminum, asbestos, cholesterol crystals) Extracellular ATP		ASC
NAIP	Flagellin *(Legionella, Salmonella, Listeria, Pseudomonas)*			MyD88/Mal
Helicase receptors				
RIG-I	Double-stranded RNA (virus), poly I:C, 3pRNA (RIG-I)	Endogenous mRNA		MAVS (IPS)
MDA5	Double-stranded RNA (virus), poly I:C, 3pRNA (RIG-I)	Endogenous mRNA		MAVS (IPS)

The toll-like receptors (TLRs), NOD-like and helicase receptors, their ligands, subcellular localization, and intracellular adapters are enumerated

intracellular adapters and kinases such as IKK and MAP kinases followed by DNA binding of transcription factors such as NFκB. Additionally, inflammatory responses in the liver also induce intrinsic and extrinsic apoptotic pathways contributing to liver damage. Finally, the function of nuclear receptors such as peroxisome proliferator receptors PPARα and PPARγ and their role in liver injury will be discussed in this section.

Toll-Like Receptors (TLRs)

Of the ten human TLRs [78], TLR4, the receptor for lipopolysaccharide, expressed on the surface of all innate immune cells and also on hepatocytes, endothelial cells, and stellate cells [6, 79–81] is extensively studied. TLR2 is expressed on most innate immune cells and hepatocytes in the liver [82]. Endosomal TLRs including TLR3, TLR7/8, and TLR9 are intracellularly localized and recognize pathogen-derived nucleic acid sequences [78, 83, 84]. While TLR3 senses dsRNA, TLR7/8 recognizes ssRNA and TLR9 senses unmethylated CpG motif in DNA [83, 84]. TLR8 is functional only in humans and not in mice [85]. Ligand engagement of all TLRs, except for TLR3, leads to recruitment of the common TLR adapter, MyD88, to the TIR domain of TLRs triggering downstream signaling events (see Fig. 27.2) [83, 86]. Subsequently, downstream signaling events involve formation of the IRAK4/TRAF6/IRAK1 complex leading to IRAK1 phosphorylation and activation of the IKK complex leading to NF-κB activation and induction of inflammatory genes including TNFα, IL-12, IL-1, IL-6, and chemokines [83]. On the other hand, TLR3 utilizes the adapter TRIF and activation of the TBK1/IKKε (epsilon) complex resulting in phosphorylation, dimerization, and nuclear translocation of IRF3, a nuclear regulatory factor involved in Type I IFN induction [86]. Among all TLRs, it is noteworthy that TLR4 can trigger both the MyD88- and the TRIF-induced pathways resulting in rapid NF-κB-mediated inflammatory gene activation and a somewhat delayed TRIF-mediated Type I IFN induction [83, 87].

The liver is an immuno-privileged site with a relatively high resistance against organ rejection after transplantation [88, 89]. Low TLR4 expression by liver DCs correlates with reduced capacity to activate allogeneic T cells in response to endotoxin [90]. Stimulation with TLR3 or TLR7 ligands changes the tolerogenic liver environment to promote autoimmunity, suggesting that activation of innate immune cells can alter the immunotolerant profile of the liver [89]. TLR activation and liver injury are preceded by changes in gut permeability leading to constant exposure to endogenous TLR ligands, activation of TLRs in liver cells, and augmentation of innate immune responses. In various forms of hepatitis induced by alcohol use, metabolic dysbalance, or viral infection, up-regulation of TLRs has been found in the liver that likely contributes to perpetuation of a chronic inflammation [30, 91].

Helicase Receptors

In addition to TLRs, helicase-containing intracellular receptors can also recognize viral nucleic acid sequences. Double-stranded viral RNA is sensed by retinoic-acid-inducible gene I, (RIG-I) and melanoma-differentiation-associated gene 5,

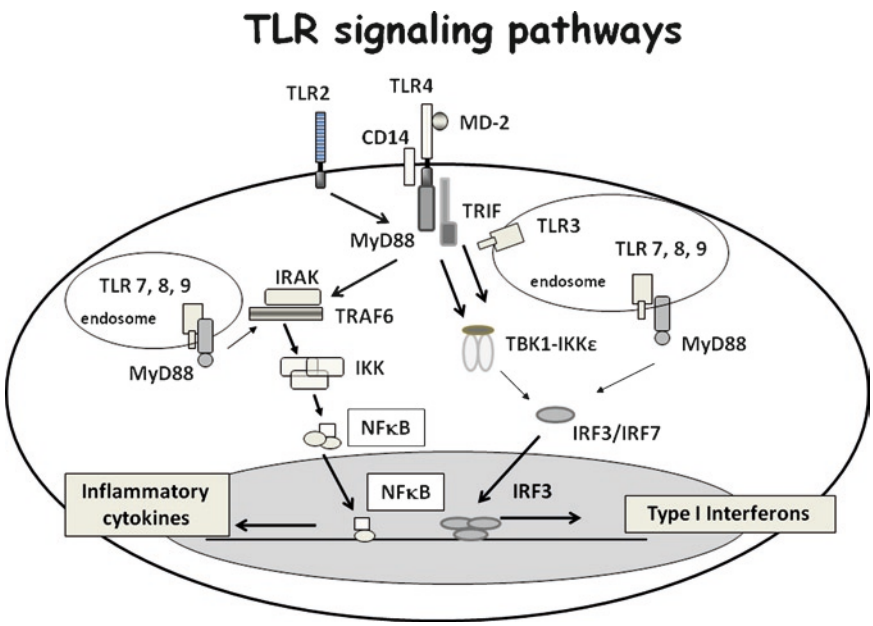

Fig. 27.2 Toll-like receptors, their intracellular adapters, kinases, and signaling pathways leading to activation of transcription factors and production of cytokines are illustrated

(MDA5) [92]. The adaptor protein for RIG-1 and MDA5 is localized in the mitochondria and it has been referred to as IFNβ promoter stimulator-1 (IPS-1), MAVS, VISA, or Cardiff. Activation of this pathway results in both NF-κB activation leading to proinflammatory cytokine production and phosphorylation of IRF3 or IRF7 triggering type-I IFN production. The role of RIG-I has been studied in HCV and it has been shown that HCV NS3–4a serine protease can disrupt the Type-I IFN induction by cleaving the adaptor molecule [93, 94]. In addition to HCV, RIG-I virus recognizes other negative-strand RNA viruses such as sendai virus VSV and influenza A [95]. MDA5 is the principal receptor for synthetic poly(I:C) in hepatocytes [96].

NLRs and the Inflammasome

The intracellularly localized human nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) contain 22 members, many of them form a complex with caspase-1 and the adapter molecule ASC (apoptosis-associated speck-like protein containing a CARD), termed the inflammasome [97, 98]. The inflammasome complex contains procaspase-1 and activation of NLRs results in caspase-1 activation that cleaves cytosolic IL-1β, IL-18, and IL-33, which are then secreted extracellularly [99]. The role of NLRs in various liver diseases and the cell-specific role of the inflammasome in the liver are yet to be fully explored. A recent study demonstrated that the IL-1β-deficient mice were protected in a model of acetaminophen-induced liver injury [100, 101]. Inflammasome activation was also present in a model of liver fibrosis induced by carbon tetrachloride and mice deficient in NLRP3 had reduced fibrosis [102]. Direct activation of hepatic stellate cells with monosodium urate crystals resulted in inflammasome and caspase -1 activation [102]. The role of NLRs and the inflammasome in other liver diseases awaits investigation.

Intracellular Signaling Molecules

While various signal transduction pathways play a role in hepatic inflammation, nuclear factor-kappaB (NF-κB) and the mitogen-activated protein kinase (MAPK) family members have been extensively studied. Liver macrophages (KCs) and hepatocytes through TLR activation activate members of the MAPK family, such as extracellular receptor-activated kinases 1/2 (ERK1/2), p38, and c-jun N-terminal kinase (JNK) [44, 103]. Whereas activation of MAPK-ERK1/2 contributes to LPS-induced TNFα mRNA production in chronic alcohol-exposed macrophages [104], p38 MAPK activation is linked to the stabilization of otherwise short-lived mRNA such as TNFα mRNA [105]. JNK activation is associated with apoptotic signaling in liver disease and recent studies show that JNK1 and not JNK2 promotes steatohepatitis [106] and its progression to fibrosis [107]. The ubiquitous transcription factor NF-κB activates transcription of a wide variety of genes, including those coding for cytokines (TNFα and IL-1β), adhesion molecules (VCAM-1, ICAM-1), and various immunoreceptors [108]. Increased NF-κB DNA binding in the liver is primarily associated with KC activation [109]. Numerous studies provide evidence for a role for ROS, rather than LPS alone, in the in vivo activation of NF-κB during hepatic inflammation [110, 111]. NADPH oxidase in KCs increases ROS leading to NF-κB activation and TNFα production [111]. Inhibition of NF-κB activation using an IκBα superrepressor resulted in resolution of inflammation demonstrating a functional significance for NF-κB activation in hepatic inflammatory disease [112]. The exact role of NF-κB activation in the different cell types in the liver and their contribution to liver disease needs further investigation.

Apoptotic Pathways

Cellular homeostasis in the liver requires a balance of extrinsic or intrinsic apoptotic pathways. Hepatocyte apoptosis has a major role in hepatic injury subsequent to the onset of inflammation in a wide range of liver diseases such as viral hepatitis, alcoholic hepatitis, ischemia-reperfusion injury, fulminant hepatitis, and cancer [113]. For additional information on apoptosis, please see Chap. 25. Studies show that apoptotic pathways in the liver occur via receptor-dependent or receptor-independent mechanisms. The receptor-dependent or extrinsic pathway is triggered by engagement of death receptors such as CD95 or FasL, TRAIL (TNF-related apoptosis inducing ligand), and TNFα (tumor necrosis factor-α) or TGF-β (transforming growth factor beta) receptors on the cell surface. The receptor-independent or intrinsic pathway is initiated in the mitochondria by oxidative stress and leads to alteration in mitochondrial membrane potential and ultimately cytochrome C release. Studies in liver disease patients and animal models show a prominent role for TNF and Fas-mediated apopotosis and triggering of cell death in the liver [113]. TNFα facilitates programmed cell death by activation of caspases via the TNF receptor-1 (TNF-R1). Both extrinsic and intrinsic apoptotic pathways are intertwined and converge on the mitochondria where mitochondrial dysfunction is a prerequisite for hepatocyte apoptosis [114].

In acetaminophen-induced acute liver injury and fulminant hepatic failure, an acute inflammatory response is followed by hepatic cell death via TNFα and Fas-signaling leading to

increased mitochondrial permeability and cytochrome C release [115]. Alterations in mitochondrial transition permeability have been observed in hepatocytes undergoing apoptosis in alcoholic liver disease [116] and apoptotic hepatocytes are often colocalized with infiltrating neutrophils. In chronic hepatitis C, hepatocyte cell death is linked to Fas-mediated apoptosis and strongly correlates with the severity of inflammation [117]. Similar to viral hepatitis, nonalcoholic steatohepatitis livers are also sensitized to Fas-mediated apoptosis and correlate with inflammation as well as fibrosis [118, 119]. In addition to TNFα and Fas-mediated death, emerging data also suggest sensitization to TRAIL-mediated apoptosis in several models of viral hepatitis and nonalcoholic fatty liver disease [120, 121]. While inflammatory mediators such as TNFα, Fas-ligand, and TRAIL derived from innate immune cells contribute to hepatocyte apoptosis, expression of stress ligands interacting with receptor NKG2D on NK and NKT cells may also contribute to apoptosis in stressed hepatocytes [122].

Nuclear Receptors

Nuclear receptors are ligand-dependent transcription factors that play an important role in hepatic metabolism, inflammation, and liver homeostasis. The main classes of nuclear receptors functionally significant in the liver are: the classic steroid or thyroid hormone receptors such as glucocorticoid receptor (GR), the nuclear orphan receptors such as constitutive androstane receptor (CAR) and pregnane X receptor (PXR), and the retinoid X receptor (RXR), peroxisome proliferators-activated receptors (PPARs), and liver X receptor (LXR). The protective role of glucocorticoids which regulated their anti-inflammatory action via activation of the GR is studied in ischemia-reperfusion [123] and alcoholic hepatitis [124]. Recent reports have suggested a link between inflammation-mediated cell signaling and regulation of nuclear receptors in the liver. LPS signaling induces the localization of hepatic RXRα from the nucleus to the cytoplasm without any effect on RXRα mRNA levels. In KCs IL-1β production activates the JNK pathway, which phosphorylates RXRα at the Ser260 site and this alteration triggers the rapid export of the receptor from nucleus to cytoplasm and its consequent degradation [125]. Thus, a marked suppression of RXRα-targeted inflammatory gene expression has been observed [126]. LPS or TNFα-activated NFκB represses RXRα-mediated gene expression through the direct interactions between the DNA-binding domain of RXRα and p65 [127]. In the liver, activated PXR inhibits the activity of NFκB and its target genes. The functional significance of PPARs in liver inflammation is extensively investigated in nonalcoholic and alcoholic liver disease. While PPARα and PPARγ are both anti-inflammatory, PPARα also regulates hepatic fatty acid metabolism [128], and PPARγ is an essential regulator for adipocyte differentiation and lipid storage in mature adipocytes [129, 130]. PPARα stimulates fatty acid catabolism by the peroxisomal, microsomal, and mitochondrial β-oxidation systems and therefore allows the use of fatty acid by hepatocytes as energetic substrate [131]. PPARs exert their anti-inflammatory effects by negatively interfering with NFκB, STATs, and AP-1 signaling pathways in hepatocytes and monocytes/macrophages [132]. PPARα and PPARγ expression was reduced during viral hepatitis B and C infection and was associated with pathogenesis of the disease [133, 134]. The use of fibrates and thiazolidinediones, PPAR agonists are being developed and used to reduce triglyceride synthesis in the liver [135].

B7 Family Members of Cell Surface Molecules

Cell surface molecules also modulate inflammation in the liver and other tissues through cell–cell interactions. Programmed cell death-1 (PD-1), the receptor for the negative regulatory costimulatory molecules B7-H1 (PD-L1) and B7-H2 (PD-L2), plays an important role in regulation of antigen-specific immune responses as well as in regulation of inflammatory cell activation [136]. For example, in chronic HCV infection increased PD-1/PD-L1 expression has been shown to contribute to decreased T cell activation and inhibitory feature of DCs [137]. In livers with chronic inflammation, liver biopsies revealed increased expression of B7-H1 and B7-DC expression on KCs, liver sinusoidal epithelial cells, and leukocytes. In all forms of liver inflammation, including chronic HBV, HCV, autoimmune hepatitis, and nonalcoholic fatty liver disease, the degree of necroinflammation correlated with expression levels of PD-1 family members suggesting that these molecules contribute to the regulation of liver inflammation [138].

Liver Injury and Mediators of Inflammation

Oxidative Stress

ROS generated during oxidative stress have been implicated in many liver diseases, particularly in the context of liver inflammation. A more detailed account of this topic is presented in Chap. 29. The role of ROS has been shown in alcoholic and nonalcoholic steatohepatitis and in viral hepatitis [139]. In alcoholic liver disease, the source of ROS includes mitochondria, MEOS, mostly through CYP2E1 induction, and depletion of antioxidants such as glutathione [140]. ROS derived

from NADPH oxidase also play a crucial role in hepatitis in alcoholic and nonalcoholic fatty livers [141]. In animal models of alcoholic liver disease, pharmacologic inhibition of NADPH oxidases in wild-type mice or a p47phox-deficient phenotype prevented liver steatosis in a mouse model of alcoholic liver disease [142]. In addition to inflammatory cells such as neutrophil leukocytes and monocyte/macrophages, members of the NADPH oxidase family are expressed in various cell types in the liver including hepatocytes and stellate cells [111]. NADPH oxidase activation in these other cell types can lead to hepatocyte injury or stellate cell and myofibroblast activation and fibrinogenic actions [143].

In drug-induced liver injury (DILI) oxidative stress and drug-induced redox changes have been shown to contribute to increased hepatocyte susceptibility to the cytotoxic actions of cytokines such as TNFα via modulation of the JNK and NF-κB pathways [144]. Viral hepatitis, particularly chronic HCV infection, is associated with increased ROS and decreased antioxidant levels in patients [139, 145]. The source of increased ROS in chronic HCV infection is both activated immune cells and hepatocytes. It has been shown that HCV core protein increases ROS and RNS in infected hepatocytes and that this is related to disturbances in calcium flux and mitochondrial oxygen transport [146].

Chemokines

Infiltration of leukocytes contributes greatly to the development and progression of liver injury. Chemokines are chemotactic cytokines professionalized to recruit leukocytes to the sites of injury and inflammation. Following liver injury, various resident hepatic cell types secrete CXC chemokines such as IL-8, MIP-2, and CINC-1 resulting in attraction of NK, NKT, $CD4^+$ T cells, and neutrophils [147–149]. MCP-1, a CC-chemokine, recruits $CCR2^+$ monocytes and is an important pathogenic factor during acute liver injury [150]. Leukocyte migration from the vascular lumen into the liver tissue occurs during ischemia/reperfusion, endotoxin-mediated liver injury, acute liver failure, alcoholic liver disease, and viral hepatitis [35–37, 147, 151]. Chemokines such as MCP-1 and RANTES are increased in activated stellate cells during liver fibrosis and recruit inflammatory cells which in turn activate stellate cells, perpetuating a vicious cycle in which inflammatory and stellate cells stimulate each other [152]. Recent studies using animal models of chronic liver diseases have used selective blockade of chemokines or chemokine receptors to understand the distinct effects on recruitment of immune cells and their possible development as therapeutic strategies in liver diseases. These studies showed that blocking of CCR2, CCR1, and CCR5 protects from liver fibrosis [153, 154].

Inflammatory and Immunoregulatory Cytokines

Intrahepatic inflammatory and immune cells as well as hepatocytes in a limited way are both sources and targets of cytokines [10]. Proinflammatory cytokines including TNFα, IL-1, and IL-6 are increased in most types of liver inflammation. TNFα plays a major role in liver homeostasis because it can activate both proapoptotic (caspases) and antiapoptotic (NF-κB) pathways in hepatocytes, contribute to liver regeneration, promote steatosis, and exert metabolic effects [155]. Increased levels of TNFα in the serum or in PBMCs were shown to correlate with disease severity in alcoholic hepatitis [156]. Serum TNFα levels are also upregulated in patients with NASH [157] and in a mouse model TNF receptor deficiency was protective from fibrosis in experimental NASH [158]. While inhibition of TNFα as a potential therapeutic approach is attractive based on its biological effects, clinical trials with anti-TNFα antibodies in acute alcoholic steatohepatitis failed due to severe side effects including infections [159].

IL-6 acts on immune cells, but it also binds to the 80 kD membrane glycoprotein (gp80) that complexes with the gp130 signal transducer molecule in hepatocytes [160]. IL-6 in the liver induces acute phase response [161] and in fulminant hepatic failure IL-6 expression in the serum and in the liver correlates with disease progression [162]. However, IL-6 also has important hepatoprotective effects. In animal models with ConA-induced hepatitis, IL-6 was protective [163] and IL-6 treatment was also in protective transplanted fatty livers [164].

It has been proposed that the balance of Th1 and Th2 cytokines in the liver microenvironment may determine disease outcome. For example, a predominant Th1-type immune response characterized by predominant IFNγ and IL-12 induction has been associated with clearance of acute HCV infection [165]. In a prospective study, changes in Th1/Th2 cytokine profile were associated with HCV clearance [166]. In normal homeostasis, there is cross-regulation between the Th1 type proinflammatory and Th2 cytokines. For example, IL-10 inhibits production of TNFα and IL-12 and inhibits antigen presentation [167]. IL-10 neutralization led to more severe liver damage in the ConA hepatitis model, while IL-10 administration limited necrosis [168]. A Th1/Th2 cytokine dysbalance has also been shown after chronic alcohol administration [3, 169, 170]. The immunoregulatory cytokine, TGFβ, acts on immune cells as well as stellate cells [171]. TGFβ inhibits monocyte production of TNFα, but it augments Th17 cell differentiation [63] while promotes fibrin deposition in stellate cells [171]. Given the pleiotropic effects and multiple cellular sources of all of these pro- and anti-inflammatory cytokines, therapeutic strategies that aim at cytokines have proven to be difficult.

Adipokines

Adipokines are polypeptides secreted in the adipose tissue that have a broad autocrine and mostly paracrine effect on metabolism including fat regulation, insulin resistance, and inflammation. Leptin, adiponectin, and resistin have been most studied and summarized in the context of liver diseases, but, in addition to these classical adipokines, fat tissue can also be the source of proinflammatory mediators and cytokines including TNFα, IL-6, IL-8, and MCP-1 [172]. Studies from animals suggest that leptin deficiency or impaired leptin receptor function results in liver steatosis, acts as a proinflammatory agent, and has a protective role in alcoholic liver disease [173, 174]. Leptin-deficient mice show impaired liver regeneration after partial hepatectomy and the lack of leptin reduces angiogenesis and paraneoplastic foci in steatohepatitis [175, 176].

Adiponectin has effects opposite of leptin and it inhibits inflammation [177]. Weight reduction increased the ratio of adiponectin to TNFα and improved NASH [178]. Adiponectin is anti-inflammatory, reduces body fat, ameliorates insulin resistance, and has hepatoprotective functions [177]. The anti-inflammatory role of adiponectin by inhibition of TNFα production has been shown in experimental alcoholic [179, 180] and nonalcoholic liver disease [181]. In NASH, adiponectin exerts its effect via the liver adipoR2 signaling pathway, making it a promising target in treatment [179]. Contrary to adiponectin, resistin, another adipokine, seems to play a causative role in hepatic steatohepatitis [182]. A role for proinflammatory resistin action has been indicated not only due to its expression in monocytes, but also resistin-mediated increase in TNFα and IL-12 production in macrophages by a NFκB-dependent mechanism [183] and also expression of IL-6 and IL-β [184]. Resistin also seems to have a proinflammatory action on hepatic stellate cells, thus participating in liver fibrogenesis [185].

The Impact of Chronic Inflammation on the Liver

Inflammation and Fibrosis

While the liver can recover from many forms of inflammation that last for a limited time (acute viral hepatitis, drug-induced hepatitis, ischemic liver damage, etc.), chronic inflammation predictably triggers the process of fibrosis which leads to progressive liver damage and clinical deterioration over time. Investigation of the role of immune cells in regulation of fibrosis revealed that cytokines derived from activated KCs and macrophages have profibrotic effects including TNFα and TGFβ that activate quiescent stellate cells [186]. However, direct activation of stellate cells can also occur through mediators of inflammation such as ROS or LPS/TLR4 interaction on stellate cells which has been shown in the context of alcoholic and nonalcoholic steatohepatitis [143]. In a mouse model of TAA/leptin-induced fibrosis, DCs were shown to play a central role in liver inflammation and fibrosis via TNFα [187]. Other innate immune cells, particularly NK cells, however, were shown to control fibrosis by killing activated stellate cells [188]. Thus, both soluble mediators and activated immune cells contribute to the tight control of stellate cell activation and fibrosis in the liver.

Inflammation and Liver Cancer

Increasing evidence suggests that persistent inflammation provides an environment that favors development of malignancy [189, 190]. Inflammatory reaction at the tumor site can promote tumor growth and progression. HCC usually arises in chronically inflamed cirrhotic livers that usually have excessive leukocyte recruitment. In this peri-tumor liver environment, there is increased representation of activated monocytes and interleukin-17 producing cells [191]. Tumor-associated activated monocytes secreted proinflammatory cytokines that triggered the proliferation and Th17 cells, further amplifying the inflammatory and antitumor effect [191]. Other studies also supported the important role of tumor-associated activated macrophages in malignancies [192]. In hepatocytes, IKK beta contributes to hepatocarcinogenesis and a recent role for TLR4 signaling has also been demonstrated in animal models of HCC [193, 194].

Future Directions

The innate and adaptive immune cells and resident cells in the liver, receptors expressed, and signaling pathways leading to production of mediators such as cytokines/chemokines have been extensively studied in the past decade. Future studies identifying new drug targets to inflammatory signaling molecules limiting production of inflammatory cytokines without their complete elimination will open new avenues for combating a variety of liver diseases involving increased inflammation. Additionally, establishing strategies to test therapeutics using clinical and translational approaches in humans will make these treatment regimens more easily and quickly available to the patient.

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Chapter 28
Oxidative Stress and Liver Injury

Francisco Javier Cubero and Christian Trautwein

Introduction

Oxidative stress and liver injury are strongly associated. Oxidative stress in the liver can be triggered during different conditions and by specific etiologies, including hepatotoxins (acetaminophen [1]), viruses (e.g., hepatitis C virus [2]), nonalcoholic steatohepatitis (NASH) [3], hepatocellular carcinoma [4], alcoholic liver disease (ALD) [5], ischemia-reperfusion, and liver fibrosis [6]. Oxidative stress is a state of imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant defense neutralizing the reactive intermediates and triggering damage. Generation of ROS can be induced by a variety of enzymes including CYP2E1, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, lipoxygenase and cyclooxygenase, and in some instances, by damaged mitochondria as found in ALD [7–11]. Liver cells contain superoxide dismutase (SOD) that dismutates (superoxide anion) to yield H_2O_2 (hydrogen peroxide). H_2O_2 and $O_2^{\bullet-}$ can interact via the Fenton reaction to produce more powerful and cytotoxic radicals, such as $OH^{\bullet}$ (hydroxyl radical) [12] (Fig. 28.1). To counteract these radicals, glutathione (GSH), SOD, glutathione peroxidase, and catalase are the major endogenous antioxidant enzyme systems, which limit intracellular accumulation of $O_2^{\bullet-}$ and H_2O_2 during the process of normal aerobic metabolism [12]. Hepatocytes are exposed to high levels of exogenous ROS produced by other cells such as Kupffer cells that become activated during liver injury which release tumor necrosis factor-alpha (TNF) and lead to hepatocyte death.

Oxidative stress can cause hepatocyte injury and death via production of peroxides and free radicals that damage all components of the cell, including proteins – mainly oxidation to sulfinic and sulfonic acids, nitrosylation, glutathionylation, and the formation of disulfide bonds, lipids, and DNA. Recent reports have demonstrated that oxidant-induced liver injury is triggered by the direct effects of ROS on signal transduction pathways [13]. Depending on the form of injury, the cell type, and the duration of the oxidative stimulus, common regulatory pathways of hepatocyte injury have been identified including the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase ½ (ERK1/2), c-Jun N-terminal kinase (JNK), and the nuclear factor-kappa B (NF-κB) pathway. In vitro and in vivo models of hepatocyte ROS-induced toxicity have shown that ERK1/2 typically induces resistance to oxidant stress, whereas JNK promotes cell death [14]. The effects of NF-κB activation are more complex and cell type-specific. Thus, a thorough understanding of the regulation of the signaling pathways induced by ROS which trigger liver injury may be of utmost interest for the development of new therapies.

Oxidative Stress and Signaling Pathways

Mitogen-Activated Protein Kinases

MAPKs are critical regulatory proteins of the hepatic response to oxidative stress. MAPKs regulate cell death, injury, proliferation, and differentiation. These enzymes are a family of serine/threonine kinases which include ERK1/2, JNK, p38 MAPK, and the big MAP kinase (BMK1) pathways [15], sometimes grouped together and referred to as the stress-activated protein kinases. The pathways in which these different MAPKs are activated also share similar homology. All four pathways operate in a cascade fashion with a MAP kinase kinase kinase (MAPKKK) phosphorylating and activating a MAP kinase kinase (MAPKK) and the MAPKK phosphorylating and activating a MAP Kinase (MAPK), respectively. Activation occurs in response to a variety of stimuli including oxidative stress-derived ROS, growth factors, and cytokines (Table 28.1) [16–22]. The duration of the MAPK activation as well as the subcellular localization of the activated kinase are regulatory mechanisms within the

F.J. Cubero (✉)
Department of Internal Medicine III, University Hospital Aachen (RTWH), Aachen, Germany
e-mail: fcubero@ukaachen.de

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_28, © Springer Science+Business Media, LLC 2011

cell that explain the diversity of effects resulting from the activation of individual MAPKs [23, 24]. Current research is targeting the mechanisms of MAPKs that modulate hepatocyte injury and cell death by the action of oxidative stress. Until now, MAPKs have been thought to respond to a variety of extracellular stimuli by phosphorylating and activating downstream transcription factors which regulate the expression of critical genes.

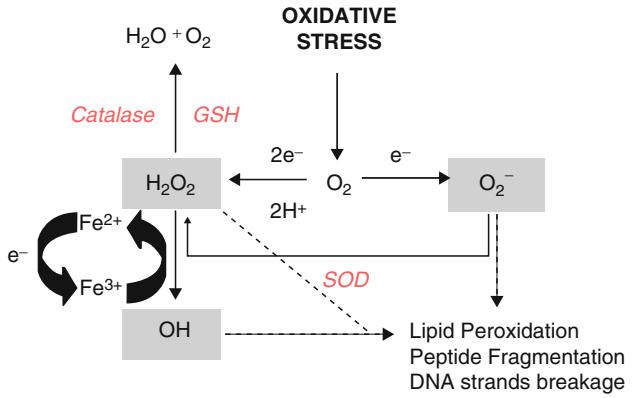

Fig. 28.1 Oxidative stress produces large amounts of cytotoxic free radicals (H_2O_2, $O_2^{\bullet-}$ and OH•). Free radicals can attack the double bonds of unsaturated phospholipids in cell membranes which eventually degrade the structural integrity of cell membranes and impair the functions of enzymes by causing fragmentation of polypeptide chains or the cross-linking of proteins or strand breaks in DNA. Cells have an antioxidant enzyme system (catalase, GSH or SOD) to neutralize free radicals and prevent damage

The RAS-RAF-MEK-ERK Pathway

The RAS-RAF-MEK-ERK signal transduction cascade was the first network to be discovered, defined by extracellular signal-regulated kinase-1 (ERK1) and ERK2 (Fig. 28.2). The ERK cascade has an important function in cellular proliferation, differentiation, and survival. Impaired activation of this pathway has been reported to be a common cause of human cancers. The 42 kDa ERK2 and the 44 kDa ERK1 Ser/Thr kinases, for which this signaling pathway is named, are activated by MEK by phosphorylation of critical threonine and tyrosine residues. MEK is found immediately upstream of ERK1 and 2 in this signaling pathway [25–27]. ERK is inactivated by multiple phosphatases suggesting that the duration and extent of ERK activation are controlled by the balanced activities of MEKs and these phosphatases. Also, the RAF (RAF-1, A-RAF, and B-RAF) proteins are well known to be activated by RAS and in turn can active MEK1 and 2, the most important isoforms in ERK activation. The RAS-RAF-MEK-ERK cascade represents the most studied pathway that leads to the activation of the ERK

Table 28.1 Activators and inhibitors of the MAPK signaling pathway

Target molecule	Activator	Inhibitor
ERK	High concentrations of menadione	PD98059 [16]
	ROS (H_2O_2, NO_2^-)	U0126 [16]
	LPS	FR180204 [17]
	Alcohol	
	PDGF receptor	
	EGF receptor	
	Inhibition of phosphatase activity	
JNK	Fas receptors	Dihydroquinone[a]
	TNF	Dihydronaphtidine[a]
	ROS (NO_2^-)	Azoles and benzazoles [18]
	LPS	Sulfonyl aminoacid[a]
	Activation of MEKK1/ASK1	Sulfonamide derivatives[a]
	Phosphorylation by MKK4/7	Sulfonyl hydrazides[a]
		3-oxomidooxindole analogues[a]
		4-substituted isoxazole analogues[a]
		4–5 pyridazinone compounds[a]
		4-(4-pyrimidinyl)-5-phenylimidazole[a]
		Methylthiopene[a]
		Benzoylamino[a]
p38	ROS (H_2O_2, $O_2^{\bullet-}$, NO_2^-, NO_3^-, $ONOO^-$)	SB202190 [19]
	LPS	SB239063 [19]
	Alcohol	SB220025 [19]
	MEKK3/6	SB203580 [19]
		PD169316 [19]
		Benzimidazolone [20]
BMK1	ROS (H_2O_2, $O_2^{\bullet-}$?)Growth factors (EGF)	U0126 [21]PD98059 [22]

[a]Patented molecules

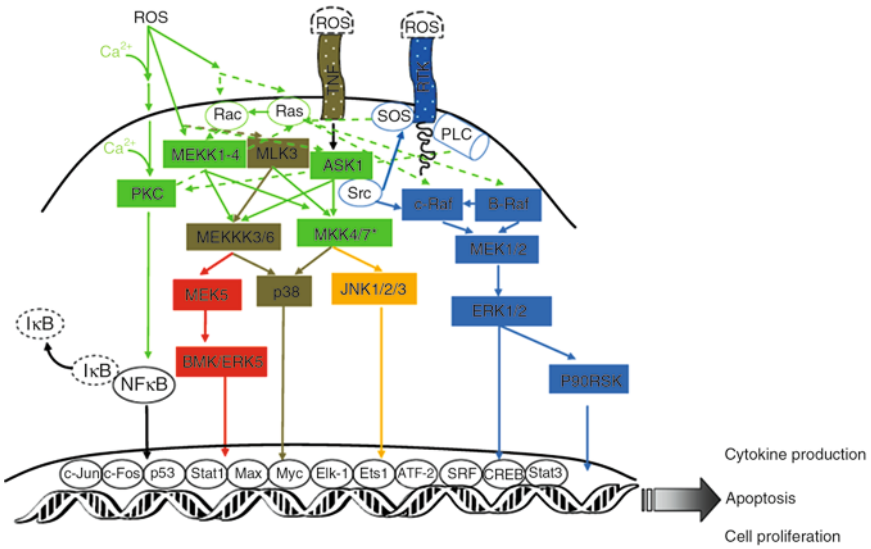

Fig. 28.2 The MAPKs signaling pathway. The major components of the ERK1/2 (*blue*), JNK (*orange*), p38 MAPK (*brown*), and BMK1 (*red*) are shown along the transcription factors that these pathways are known to regulate. ROS activate calcium channel, receptor tyrosine kinases, TNF receptor, and Src, activating these pathways (**Green* represents shared molecules for different pathways)

kinases. Activation of ERK by oxidative stress has been reported in hepatocytes [28]. The activation actions of ROS on ERK do not appear to be direct, but instead seem to be upstream of ERK. MEK1/2-inhibitors such as U0126 and PD98059 block oxidative stress-induced ERK activation (Table 28.1). In some instances, ROS can act directly on receptors–such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) (Table 28.1) – which trigger activation of RAS and the subsequent activation of the RAF/MEK/ERK cascade. Also, ROS can activate certain Src kinases which could potentiate ERK activity. In other instances, nitric oxide causes the nitrosylation of a reactive cysteine residue in RAS and leads to increases in RAS activity, inducing ERK activation. Finally, increases in intracellular Ca^{2+} are reported to induce ERK activation via the CaM kinases pathway. Although activation of the ERK pathway has been shown in many cases to have a role in cell survival, there is increasing evidence that it can also have a role in the induction of apoptosis indicating that in some cases the ERK signaling pathway can promote cell death [29, 30].

The SAPK/JNK Pathway

The JNK pathway, also called the stress-activated protein kinase pathway (SAPK), comprises a cascade very similar to the ERK pathway with a MAPKKK activating a MAPKK, and the MAPK subsequently activating JNK (Fig. 28.2). JNK activation can occur after phosphorylation on critical Thr/Ser residues by MKK4 and MKK7 (MAPKKs) [31]. MEKK1, 2, 3, and 4-specific for the JNK pathway-, MLK, and ASK1 are activators of MKK [32–35]. As shown in Table 28.1, TNF and Fas receptors are well known to activate the JNK signaling pathway upon binding to their ligands [36, 37]. Activation upon TNF and Fas receptors as well as oxidative stress occurs via ASK-1, a MAPKKK which is found reduced in the inactive form but becomes activated when oxidized by ROS. Deletion of ASK1 has shown to prevent ROS-induced cell death, but not death receptor-induced apoptosis [35]. Moreover, growth factor receptors have been reported to be involved in the activation of JNK via RAS-RAC-MLK [38]. The JNK pathway involves a MAPKK subsequently activating JNK. Downstream targets of JNK include c-Jun, ATF-2, Elk1, and p53 [39, 40]. The kinetics of JNK activation have been proposed to play a role in determining whether JNK promotes or inhibits apoptosis. The theory behind ROS and JNK is that low levels of ROS leave phosphatase activity intact, resulting in a transient activation of JNK and an inhibition of apoptosis. Higher levels of ROS may activate the pathway, but also inactivate the phosphatases resulting in prolonged activation of JNK activity, and leading to cell death.

The p38 Pathway

Phosphorylation on a Tyr/Thr residue by MKK3 or MKK6 promotes activation of p38 MAPK (Fig. 28.2). Both growth factors and receptors are known to activate this pathway via activation of MLK3, a downstream kinase or ASK-1. As mentioned earlier, certain growth factors can activate the p38 pathway via activation of RAS-Rac. Nuclear targets of p38

kinase activity include transcription factors such as p53, STAT1, Max, Myc, Elk-1, Ets-1, factors involved in cytokine production, and apoptosis. There has been some controversy on the role of the p38 pathway and the induction of apoptosis; however, the work by Birkenkamp et al. [41] suggests that although the activation of the p38 pathway may be required for apoptosis, in the presence of high enough levels of ERK activation, p38 activation may not be sufficient itself for apoptosis to occur. $O_2^{\bullet-}$ [42–44], H_2O_2 [45], NO_2^- (nitrite) [46], and $ONOO^-$ (peroxinitrite) [47] all activate the p38 signaling pathway most likely via ASK1 [48, 49].

The BMK1 Signaling Pathway

The BMK1 signaling pathway represents the most recently discovered MAPK signaling pathway (Fig. 28.2). It has been involved in proliferation [22] and differentiation [50], but it might not have a role in apoptosis induction. BMK is activated by cellular stress including ROS (H_2O_2) [51] and growth factor receptors such as EGF (Table 28.1) [49].

Crosstalk Between the MAPK Signaling Pathways in Oxidative Stress

Different studies have demonstrated that MAPKs are crucial in the regulation of hepatocyte death from ROS in acute and chronic oxidative stress. Menadione, a powerful superoxide generator, below toxic concentrations induced low levels of transient ERK1/2 and JNK activation, but failed to activate p38 MAPK [23]. In contrast, toxic concentrations of menadione led to markedly increased and prolonged activation of ERK1/2 and JNK (Table 28.1). When ERK1/2 was pharmacologically inhibited, hepatocytes showed a prolonged activation of JNK. These data clearly indicate that the MAPK ERK1/2 and JNK perform critical and opposing functions in the regulation of hepatocyte survival after oxidative stress: at low levels of ROS, ERK1/2 inhibits JNK activation; however, higher levels of oxidative stress bring about JNK overactivation resulting in cell death. In a parallel study, Rosseland et al. [24] added H_2O_2 to primary rat hepatocytes. Concomitant with the menadione study, ERK1/2 activation increased in proportion to concentrations of H_2O_2 and pharmacological inhibition of ERK1/2 led to a significant reduction in hepatocyte survival in the presence of H_2O_2 [14]. Oxidative stress has also been shown to phosphorylate and activate various protein kinase C (PKC) isoforms, involved in the activation of the Ras-Raf-MEK1/2-ERK1/2 signaling pathway [52, 53]. A similar approach was used to determine the impact of chronic oxidative stress on the MAPKs signaling pathway. The data revealed that resistance to chronic oxidative stress occurred by the same mechanism as for acute oxidative stimulus [14].

Nuclear Factor κB

A second signaling cascade that has been implicated in hepatocyte injury from oxidative stress is the Rel/NF-κB pathway (Fig. 28.2). The Rel/NF-κB family of transcriptional factors regulates the expression of numerous cellular and viral genes involved in inflammation, immunity, acute phase response, proliferation, and apoptosis [54–57]. In the cytoplasm, NF-κB is sequestered, in its inactive form, bound to specific inhibitors, IκB. In response to proinflammatory cytokines such as TNF and Interleukin-1 (IL-1), bacterial lipopolysaccharide (LPS), or viral double-strand RNA (dsRNA), the IκB are phosphorylated by specific kinases called the IKK. The IKK complex consists of two catalytic subunits: IKK1, also called IKKα, and IKK2 or IKKβ, and the nonenzymatic regulator NEMO (NF-κB essential modulator) or IKKγ [57]. The IKK complex subunits have a variety of physiological functions. IKK1-deficient mice die shortly after birth [58, 59]. Both IKK2 and NF-κB (NEMO) knockout mice die embryonically due to massive liver apoptosis [60–62]. While murine embryonic fibroblasts from IKK2 knockout mice show residual NF-κB expression in response to TNF and bacterial LPS [60, 62], murine embryonic fibroblasts from NEMO knockout mice exhibit complete lack of NF-κB activation after these stimuli [61]. Recently, our group described that IKK2 conditional and hepatocyte-specific knockout mice show normal NF-κB activation and no liver damage in response to TNF [63]. Production of proinflammatory and oxidative stress mediators (like TNF or inducible nitric oxide synthase [iNOS]) in different models of induced-liver injury is directly related to NF-κB activation [64, 65]. The direct evidence that ROS level may be able to regulate NF-κB was provided by exposure of cells to H_2O_2. In certain cell types [66, 67], H_2O_2 has been shown to be an effective inducer of NF-κB activation [68–70]. However, the activation of NF-κB by H_2O_2 is highly dependable on the cell type and the stimulus.

Different studies have shown that intracellular levels of reduced GSH, a major antioxidant defense system of the cell, are crucial for the H_2O_2-induced NF-κB response [69, 71, 72]. Still the activation of NF-κB by H_2O_2 is inefficient in comparison with physiological inducers such as TNF. Based on the current evidence for NF-κB, ROS might be a common and critical intermediate for triggering different NF-κB-activating signals and it is clear that the key regulatory step is activation of the IKK complex. However, the role of

NEMO in hepatocyte-specific NF-κB-dependent signaling during liver injury remains to be elucidated. By using a large number of antioxidants and overexpression of various antioxidant enzymes, inhibition of NF-κB activation has been accomplished. Upon phosphorylation of IκB by the IKK complex, NF-κB is released and translocates into the nucleus, where it activates gene transcription [57, 73]. NF-κB may potentially modulate ROS-induced liver injury through upregulation of the expression of antiapoptotic genes including c-FLIP, Bcl-XL, and nitric oxide synthase [74–76]. The activation of the NF-κB pathway has shown to provide cells and organism with potent antiapoptotic defense. A study of the regulation of the antiapoptotic gene expression program activated by NF-κB in response to ROS will provide additional insight into the molecular basis of the oxidative-derived liver injury [76].

Oxidative Stress and Liver Injury

Oxidative Stress and Acute Liver Injury: Acetominophen-Induced Oxidative Stress

Acetaminophen (APAP) is a commonly used analgesic/antipyretic drug. High doses of APAP trigger oxidant stress in humans [77], which produces centrilobular liver necrosis and renal failure [78, 79]. In hepatocytes APAP is metabolized via CYP2E1 to form the reactive metabolite, NAPQI [80]. At therapeutic doses, NAPQI is efficiently detoxified by GSH. However, at high doses, the detoxification pathway becomes saturated and the intermediate metabolite covalently binds to cysteine residues in proteins. As a consequence, APAP adducts are formed triggering oxidative stress reactions and $ONOO^-$ formation which induce the dysfunction of mitochondria and extensive DNA fragmentation damage rather than apoptosis. Thus, multiple events contribute to the development of cell death [79, 81, 82]. Recent investigations by three research groups [83–85] demonstrated that JNK plays a pivotal role in APAP-induced liver injury. The inhibition of JNK resulted in protection against APAP. Thus, the activation of specific signal transduction pathways involving JNK is necessary for hepatocyte death to occur with APAP treatment [86]. JNK2 seems to be the main isoform involved in hepatocyte death as inhibition of JNK2, but not JNK1, function decreased liver injury, also corroborated in models of liver injury with LPS or galactosamine [14, 87]. These findings indicate that prolonged activation of JNK2 promotes liver injury. Even though mitochondrial death is the triggering mechanism for the JNK2 effect, the downstream mediator of this JNK2 effect has not been elucidated.

Oxidative Stress and Chronic Liver Injury

Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD), emerging as the most important cause of chronic liver disease in relation to the increasing incidence of obesity and type 2 diabetes in the population, includes a broad spectrum of liver disorders ranging from hepatic lipid accumulation to NASH [88] (Fig. 28.3). NASH is a more severe form of NAFLD in which fat accumulation in hepatocytes is accompanied by necroinflammatory response. According to the "two-hit" hypothesis for NAFLD progression, dietary triglycerides accumulation in hepatocytes is the first hit. This response is due to oxidative stress – including nutritional factors and impaired hepatic lipid metabolism – as a result of either gene polymorphisms or insulin resistance (IR) [89]. Thus, IR can exert a positive feedback mechanism on the oxidative stress status of the liver and promote necroinflammation and progression of the disease [90]. Once steatosis has been established, a "second-hit" is required for hepatocellular injury (Fig. 28.3). Secondary to IR, an increase of free fatty acids (FFA) and depletion of polyunsaturated fatty acids (PUFA), particularly of the n-3 series, are observed [91]. This second hit is most likely coming from ROS, produced at complex I of the mitochondrial electron-transport chain and induced by overproduction of FFA, which produce changes in the mitochondrial membrane potential affecting the mitochondrial integrity [92]. The redox imbalance of the cell is characterized by depletion of GSH, reduced SOD activity, low catalase activity, induction of iNOS, increase in CYP2E1, stimulation of NADPH oxidase activity of Kupffer cells or polymorphonuclear cells that infiltrate the liver, and enhanced free radicals activity, mainly lipid peroxidation products (malondialdehyde and 4-hydroxynonenal) which can directly activate the IKK kinase complex or JNK to activate transcription of proinflammatory cytokines such as TNF or LPS via TLR-through NF-κB (perhaps involving COX-2 [93, 94] or AP-1

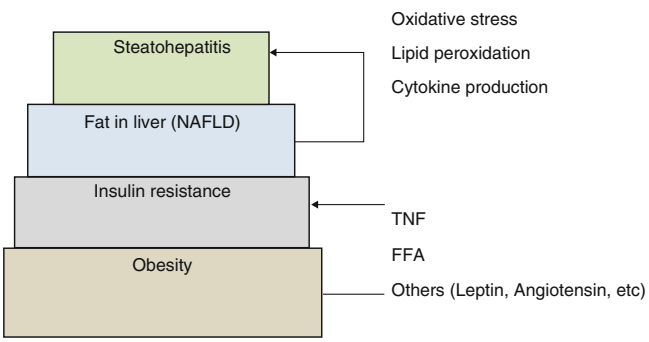

Fig. 28.3 Schematic representation of the pathogenesis in NASH and NAFLD

[14, 92, 95, 96]). Although early and transient activation of JNK promotes cell survival, sustained activation induced by ROS via inactivation of MAPKs contributes to apoptosis. Here, JNK1 seems to be involved in the development of both hepatic steatosis and liver injury [97].

Alcoholic Liver Disease

Kupffer cells, as part of the innate immune system, are the primary effector cells of response within the liver in the pathogenesis of ALD. Ablation of Kupffer cells significantly blunts serum transaminase levels, fatty changes, inflammation, and necrosis caused by chronic ethanol and prevents the generation of ROS after ethanol treatment [98, 99]. Gut-derived LPS, one of the components of the outer wall of gram-negative bacteria, is involved in the activation of Kupffer cells [100] (Fig. 28.4). Several studies demonstrate a correlation between alcohol administration, plasma levels of LPS, activation of Kupffer cells, and ALD [101–103]. LPS binds CD14 once associated with lipopolysaccharide-binding protein (LBP) and the LPS/CD14 complex interacts with TLR4 and thus initiates NF-κB activation resulting in a subsequent increase in TNF levels [5].

Among the MAPK, ERK1/2 activation has been involved in the production in response to alcohol. Kupffer cells from ethanol-fed rats had increased ERK1/2 activation after LPS stimulation, which increased TNF levels [104]. The transcription factor Egr-1 mediated this effect, and ROS might be involved in this process [5]. Although hepatic activation of the JNK downstream effector AP-1 has been described [105], there is no clear evidence for JNK activation or involvement. Thus, the function of MAPKs in ALD remains unclear.

In addition to TNF, Kupffer cells also produce large amounts of ROS and reactive nitrogen species (RNS), which

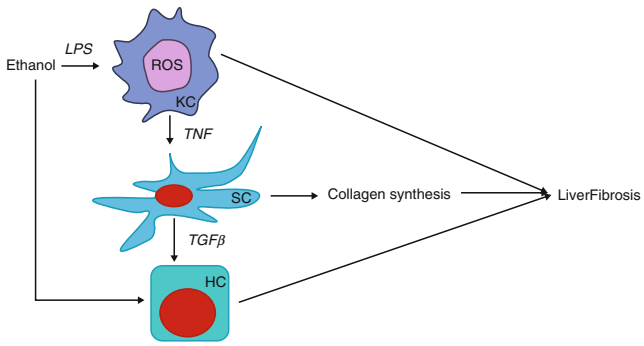

Fig. 28.4 Schematic representation of the pathogenesis of ALD (*KC* Kupffer cells; *SC* hepatic stellate cells; *HC* hepatocytes)

can regulate Kupffer cell activation and signaling to control the fibrogenic response via stellate cells [106]. Activation of NADPH oxidase involves the induction and recruitment of several cytosolic subunits to the plasma membrane, producing ROS [107]. Kupffer cell-derived ROS mediate hepatocellular damage and contribute to fibrosis in patients with ALD [106–109]. PUFA synergize with ethanol in the activation of Kupffer cells, increasing the release of critical mediators that may eventually affect matrix synthesis and degradation [106]. Whether the predominant centrilobular necrosis characteristic of ALD is due to the prevalence of CYP2E1 expression in the pericentral region of the liver or the lower oxygen tension and transient hypoxia that would occur locally is a matter of debate [12, 103]. Either of these factors could substantially impede the gradual progression of the ordered sequence of events in apoptosis and enhance a shift toward necrosis.

Future Perspectives

An exact understanding of the regulation of the signaling pathways induced by ROS which trigger liver injury is of high interest for the development of new therapies. A major aim is the identification of novel downstream molecules which regulate the expression of critical genes. Additionally, it will be important to define the factors controlling the balance in favor of either cell survival or cell death after activating the MAPKs signaling pathways such as ERK1/2, e.g., during NASH or ALD. Here the exact role of the p38 and the BMK1 pathways for the induction of apoptosis must be further investigated. It will be attractive to design novel drugs that specifically target either the signaling molecules–including novel isoforms – or the MAPK pathway. This will enable modification of hepatocyte injury and cell death and maybe more useful than modulating ROS levels, which may be beneficial but also detrimental as they release a wide array of factors critical for hepatocellular proliferation. Furthermore, changing ROS levels besides activating different pathways might also have an impact on phosphatases, which, for instance, result in prolonged JNK activation and consequently hepatocellular damage. Another important aim is to define the role of NEMO as part of the IKK complex during liver injury. NF-κB may potentially modulate ROS-induced liver injury through up-regulating the expression of antiapoptotic genes. Hepatocyte-specific NEMO knockout mice will be an attractive animal model to screen and develop potential drugs, which control and prevent liver-related diseases, but potentially may also have an effect on other acute disorders and chronic degenerative conditions.

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Chapter 29
Fatty Liver

Jaideep Behari

Introduction

The liver plays a central role in maintaining energy balance in the body. After a meal, dietary lipids are transported to the liver in the form of chylomicrons by the lymphatic system. The liver then releases the dietary lipids by the action of lipoprotein lipase and incorporates them into very-low-density lipoproteins (VLDL), which are then secreted from the liver and transported to adipose tissue for storage. The liver also takes up glucose when its plasma concentrations are high and stores it in the form of glycogen. On the other hand, during periods of fasting, the liver maintains blood glucose levels through glycogenolysis and gluconeogenesis. The liver also takes up free fatty acids released from adipose tissue and uses them for energy production through fatty acid oxidation. Thus, the liver plays a critical role in both glucose and lipid metabolism. These topics are independently discussed in Chaps. 8 and 10.

Tremendous advances have been made in the understanding of how the liver handles lipids and the underlying molecular circuits that regulate this process. Yet, significant gaps remain in understanding of the process. Given the complex and finely tuned mechanisms regulating hepatic carbohydrate and lipid metabolism, it is not surprising that multiple etiologies can lead to abnormal accumulation of fat in the liver. This chapter provides an overview of the role of the liver in lipid metabolism and the conditions that can lead to abnormal accumulation of fat in the liver. Detailed descriptions of the molecular processes underlying specific disease states are provided in other chapters in the book.

J. Behari (✉)
Department of Medicine, Division of Gastroenterology, Hepatology, and Nutrition, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: beharij@upmc.edu

What is Fatty Liver Disease?

Fatty liver (also called hepatic steatosis) refers to the abnormal accumulation of lipids within hepatocytes. The term is used when the hepatic lipid quantity is greater than 5% of liver weight. During the process of formalin fixation and paraffin embedding of liver sections for histological review, lipids are dissolved and appear as vacuolated (empty) areas within the cytoplasm of hepatocytes. Thus, fatty liver can also be diagnosed on microscopic examination by the visualization of vacuolated areas representing lipid droplets that are present in more than 5% hepatocytes. Lipid accumulation within hepatocytes can also be visualized by the use of special stains such as Oil red O, which stains neutral lipids red. Fat accumulation in the liver is called "macrovesicular" when the lipid droplets are large and push the hepatocyte nucleus to one side or "microvesicular" when there are multiple small lipid droplets that are smaller than the nucleus.

Clinically, there are no symptoms, signs, or laboratory abnormalities that are diagnostic of fatty liver, although certain etiological factors such as heavy alcohol ingestion may have a pattern of liver injury enzyme elevation (high serum aspartate aminotransferase to alanine aminotransferase ratio and elevated serum gamma glutamyl transpeptidase level) that is suggestive of the cause. Liver biopsy and microscopic examination of liver sections is the "gold standard" for diagnosing and staging fatty liver disease, although the presence of fat in the liver can be visualized by noninvasive imaging modalities such as ultrasound, computed tomography, and magnetic resonance imaging. However, the sensitivity of these methods in diagnosing fatty liver is relatively low and they do not provide information on associated hepatic inflammation or fibrosis.

What are the Causes of Fatty Liver Disease?

The association between excessive alcohol intake and development of fatty liver was recognized nearly 200 years ago [1]. A more comprehensive account of alcoholic liver disease is

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_29, © Springer Science+Business Media, LLC 2011

available in Chap. 36 in this textbook. However, the characteristic histopathological changes seen in alcoholic fatty liver disease may also occur in a number of conditions not associated with significant alcohol intake and the condition is then called nonalcoholic fatty liver disease (NAFLD). A partial list of some common causes of fatty liver is given in Table 29.1. When there is hepatic lipid accumulation alone, the term hepatic steatosis is used. However, up to one-third of patients with liver steatosis have accompanying hepatic inflammation, when the condition is called alcoholic steatohepatitis (ASH) or nonalcoholic steatohepatitis (NASH). Both ASH and NASH can progress to liver fibrosis, cirrhosis, and liver cancer.

While NAFLD has been associated with many drugs, genetic defects in metabolism, and abnormalities in nutritional states, it is most commonly associated with obesity and the metabolic syndrome [2]. For more details on the molecular pathology of NAFLD and NASH, please see Chap. 35. The metabolic syndrome is a group of clinical features linked to overnutrition and visceral obesity (Table 29.2) [3].

Table 29.1 Some conditions associated with fatty liver disease

Metabolic
Obesity
Type 2 diabetes mellitus
Dyslipidemia
Drugs/toxins
Alcohol ingestion
Amiodarone
Methotrexate
Glucocorticoids
Tamoxifen
Antiretroviral therapy
Infections
Hepatitis C
HIV
Nutritional
Jejuno-ileal bypass surgery
Total parenteral nutrition
Malnutrition (Kwashiorkor)
Rapid weight loss
Miscellaneous
Lipodystrophy
Weber-Christian disease
Wilson's disease

Table 29.2 Metabolic syndrome

Central obesity: waist circumference ≥102 cm or 40 in. (male); ≥88 cm or 36 in. (female)
Dyslipidemia: triglycerides ≥1.695 mmol/L (150 mg/dL)
Dyslipidemia: HDL-C <40 mg/dL (male); <50 mg/dL (female)
Hypertension: blood pressure ≥130/85 mmHg
Fasting plasma glucose ≥6.1 mmol/L (110 mg/dL)

The US National Cholesterol Education Program Adult Treatment Panel III requires the presence of at least three of the five criteria [3]

NAFLD is strongly associated with the metabolic syndrome. It has been proposed that NAFLD should be considered the hepatic manifestation of the metabolic syndrome [4]. As the prevalence of obesity, the metabolic syndrome, and type 2 diabetes mellitus are increasing as a consequence of overnutrition and decreased physical activity that characterize the modern lifestyle, there has been a parallel increase in the prevalence of NAFLD. Since the initial description of NAFLD by Ludwig et al. in 1980, the realization of the public health importance of NAFLD has grown tremendously [5]. Not only is NAFLD now considered the most common liver disease in the United States, it is also becoming a worldwide problem. The disease is a common cause of cirrhosis and within a few years is projected to replace hepatitis C as the most common indication for liver transplantation in the United States [6].

Histological Features of Fatty Liver Disease

The histological features of steatohepatitis consist of fatty change, lobular inflammation, and pericellular fibrosis (Fig. 29.1) [7]. Since these histological features are also seen in both alcoholic and NAFLD, clinical evidence of absence of significant alcohol use is needed to make a diagnosis of NAFLD. Ballooning degeneration, the accumulation of fluid within liver cells characterized by swelling of hepatocytes and rarefied cytoplasm, is an important histological finding that has been associated with higher risk of developing progressive fibrosis, cirrhosis, and liver-related death [8, 9]. Fibrosis is staged separately based on Trichrome staining for hepatic collagen [10].

Overview of the Molecular Pathogenesis of Fatty Liver Disease

The precise molecular mechanisms leading to the development of fatty liver are still incompletely understood. However, recent advances have revealed significant overlaps in the molecular pathogenesis of alcoholic and nonalcoholic liver disease. While the clinical consequences of alcohol ingestion or obesity-related fatty liver are most obvious in the liver, the pathogenesis clearly involves other organs such as the visceral adipose tissue and the gut. Similarly, inflammatory cytokines and chemokines are involved in the pathogenesis of both alcoholic and NAFLD, and pathways of lipid handling in the liver are disrupted by ingestion of alcohol as well as obesity. A brief overview of some of the players and processes relevant to hepatic fat metabolism is given below (Fig. 29.2).

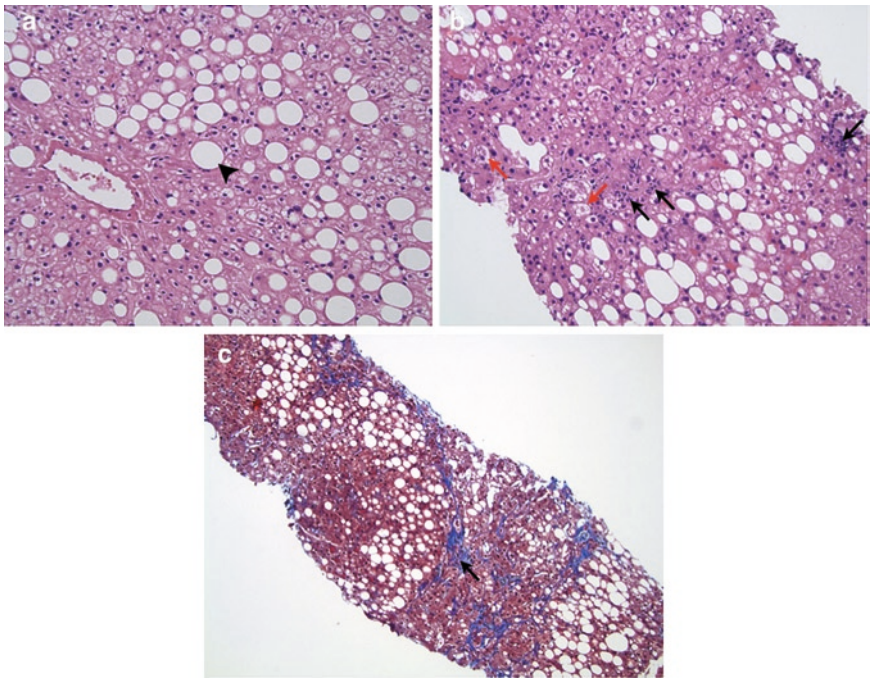

Fig. 29.1 Histological features of nonalcoholic fatty liver disease. (**a**) Simple steatosis. Section shows steatosis involving 40–50% of the hepatocytes. No significant lobular inflammation is seen. The *arrowhead* points towards a lipid-laden hepatocyte (hematoxylin and eosin; 200× magnification). (**b**) Active steatohepatitis (NASH). In addition to mixed micro and macrovesicular steatosis, hepatic lobules show active spotty lobular inflammation (*black arrows*). Some of the hepatocytes show ballooning degeneration (*red arrows*). (H & E, 200× magnification). (**c**) Liver section from the same patient as in (**b**). The hepatic lobules show patchy areas of centrizonal pericellular fibrosis and early central-to-central bridging fibrosis, marked by the *arrow* (trichrome stain, 100× magnification). Images courtesy of Dr. Eizaburo Sasatomi, Department of Pathology, University of Pittsburgh

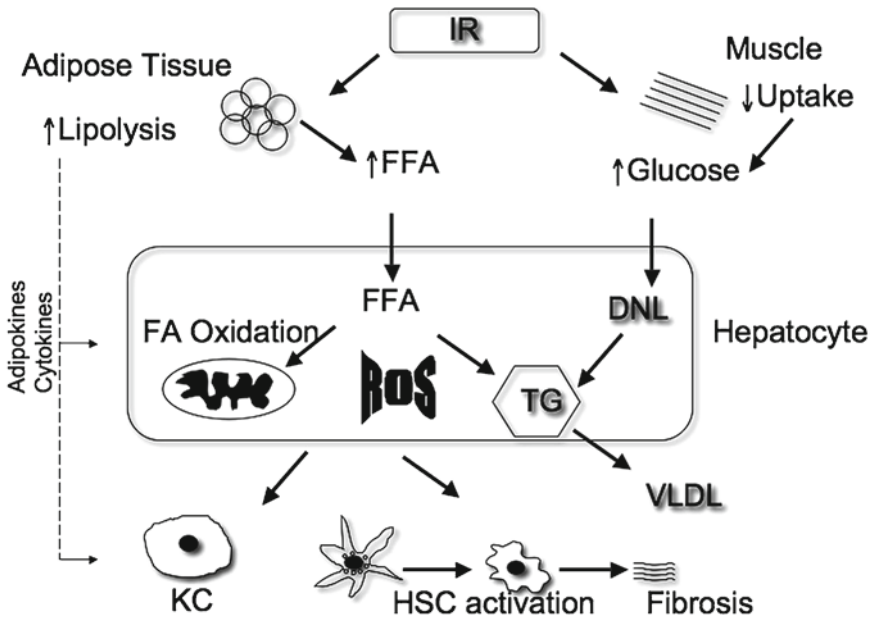

Fig. 29.2 Overview of the molecular pathogenesis of fatty liver disease. Insulin resistance (IR) caused by obesity causes increased lipolysis in adipose tissue and decreased glucose uptake in the skeletal muscle, resulting in increased circulating free fatty acids (FFA) and glucose. FFA taken up by the liver undergoes oxidation in the mitochondria and other organelle or is esterified to triglycerides (TG). The liver also packages lipids for export as very low density lipoprotein particles (VLDL). Reactive oxygen species (ROS) generated in the liver due to lipotoxicity, FFA oxidation, and organelle dysfunction leads to ROS generation. ROS, along with adipokines from visceral adipose tissue and cytokines and chemokines from the liver and adipose tissue amplify the inflammatory signal by activating the resident liver macrophages, kupffer cells (KC), and lead to hepatic fibrosis by activating hepatic stellate cells (HSC)

Physiologic Effects of Insulin Relevant to Liver Metabolism

Insulin plays a critical role in regulating blood glucose and circulating free fatty acid levels after a meal. Insulin is secreted by pancreatic β-cells and binds to the cell surface dimeric insulin receptor on target tissues. Autophosphorylation of the insulin receptor, followed by tyrosine phosphorylation of insulin receptor substrate activates phosphatidyl inositol 3-kinase and protein kinase B, and results in translocation of the glucose transporter 4 (GLUT4) from intracellular vesicles to the cell membrane. In turn, GLUT4 facilitates glucose uptake and utilization by peripheral tissues. In the liver, insulin stimulates glycogen synthesis from glucose and suppresses gluconeogenesis, thereby decreasing hepatic glucose output in the postprandial state. The net effect of these changes is to normalize blood glucose levels after a meal [11].

Insulin also acts on adipose tissue and decreases lipolysis by inactivating hormone sensitive lipase and promotes lipogenesis. In the liver, glucose and free fatty acids are taken up in an insulin-independent manner. However, insulin redirects excess glucose towards de novo lipogenesis (DNL) by activating transcription factors (TF) that regulate fatty acid synthesis (see below). Formation of malonyl-coA by acetyl coA carboxylase, a key enzyme in fatty acid synthesis, inhibits fatty acid oxidation in the liver during this period of energy excess.

Role of Insulin Resistance in the Development of Fatty Liver

Insulin resistance (IR) refers to the decreased responsiveness of target tissues to the physiologic effects of insulin. Insulin resistance is associated with obesity, the metabolic syndrome and Type 2 diabetes mellitus. In the setting of obesity, fat-laden myocytes become resistant to the signaling effects of insulin resulting in decreased glucose uptake from the circulation on stimulation by insulin, contributing to elevated blood glucose levels and increased pancreatic insulin secretion, with metabolic consequences in the liver as described below [12]. Within adipocytes, there is defective inhibition of hormone sensitive lipase by insulin which results in increased lipolysis as well as decreased lipogenesis due to reduced activity of adipogenic TF such as PPARγ. The net effect of these changes resulting from peripheral IR is an increase in the circulating free fatty acid pool and increased fatty acid load to the liver [13]. Recent studies in a murine model of steatohepatitis have suggested that high FFA levels may be toxic to hepatocytes and triglyceride accumulation in the liver might be a protective response in response to elevated FFA levels. When the final step in triglyceride biosynthesis, diacylglycerol acyltransferase 2, is inhibited by antisense oligonucleotides, hepatic steatosis decreases but there is associated increased in hepatic FFA levels and increase in makers of inflammation and hepatocyte injury and fibrosis [14].

In the liver, IR leads to defective suppression of glycogenolysis and gluconeogenesis by insulin which causes increased hepatic glucose production and contributes to hyperglycemia and hyperinsulinemia. High carbohydrate levels contribute to steatosis via increased DNL in the liver. In addition, there is evidence that NAFLD is associated with mixed IR in the liver, in which there is loss of insulin inhibition of gluconeogenesis, but retained ability to suppress fatty acid oxidation. Thus, increased lipogenesis combined with decreased fatty acid oxidation leads to the development of hepatic steatosis.

The molecular mechanisms that result in IR have not been fully elucidated, but recent studies points towards the importance of inflammation in its pathogenesis. Since the initial discovery that TNFα, a proinflammatory cytokine, is produced by adipose tissue and has systemic metabolic effects including on the liver [15, 16], many other adipokines and cytokines produced by adipose tissue have been identified, including leptin, adiponectin, resistin, interleukin-6, retinol-binding protein-4, and others. In obese individuals, production of some of these bioactive substances from adipose tissue is increased (e.g., TNFα) while that of others is decreased (e.g., adiponectin). In addition, secretion of chemokines such as monocyte chemoattractant protein-1 leads to recruitment of inflammatory cells including macrophages, which amplifies the inflammation [17].

There is also evidence that accumulation of lipids in the liver leads to increase in inflammatory gene expression, including NFκB, IL-6, TNFα, and IL-1 [18]. This increase in the hepatic inflammatory response may be either from hepatic steatosis itself, similar to the increase in bioactive production from fat-laden adipocytes, or may be initiated due to the release of proinflammatory cytokines in the portal circulation by visceral adipose tissue. The liver is rich in immune cells such as lymphocytes, NK cells, and dendritic cells that may participate in the process of liver inflammation leading to the development of NASH [17]. A more detailed account on inflammation and liver injury is available in Chap. 28.

Cross Talk Between the Liver and other Organs in Fatty Liver Disease

Adipose tissue, particularly visceral fat, plays an important role in the pathogenesis of IR and development of NASH. Visceral adipocytes overexpress 11 β-hydroxysterol dehydrogenase and more readily mobilize fat and are less mature

than subcutaneous adipocytes [19]. As discussed above, adipocytes are hormonally active and produce several adipokines that contribute to insulin resistance and lead to significant alterations in glucose and fat metabolism in the liver. Among the important adipokines are leptin and adiponectin [20]. Leptin levels are increased in the plasma of patients with NAFLD. However, leptin has an antisteatotic role and is protective. Therefore, it has been suggested that NAFLD may be associated with leptin resistance and a defective response to leptin [21]. On the other hand, adiponectin levels are lower in patients with NASH and more severe liver injury is associated with lower adiponectin levels [22, 23]. Adiponectin has been shown to increase insulin sensitivity, regulate FFA metabolism, inhibit gluconeogenesis, and exhibit anti-inflammatory effects mediated by suppressing TNFα [24–26].

Studies have suggested that the gut plays a role in the pathogenesis of fatty liver. Gut-derived endotoxin has been suggested as a possible factor in the development of fatty liver in animal and human studies. Recently, it was shown that patients with NAFLD have small intestinal bacterial overgrowth and disruption of intracellular tight junctions in the intestines. These findings correlated with the severity of hepatic steatosis but not with the presence of NASH. It was therefore proposed that increased intestinal permeability may play an important role in the development of fatty liver [27].

Role of Signaling Pathways of Inflammation, Proinflammatory Cytokines and Adipokines

Inflammation in the setting of fatty liver results from cross talk between hepatocytes and nonparenchymal cells, including activated Kupffer cell (KC), stellate cells and sinusoidal endothelial cells. This cross talk is mediated by a variety of proinflammatory adipokines and cytokines released from visceral adipose tissue. The NF-κB pathway has been extensively studied for its role in steatohepatitis and is up-regulated in NASH patients and in animal models of the disease. However, its role in pathogenesis of NASH is complex and while activation of NF-κB signaling in liver cells induces inflammation and steatosis, its inactivation also causes steatohepatitis and liver cancer in a mouse model [18, 28, 29]. Deletion of the JNK1 isoform of c-Jun N-terminal kinase (JNK), a mediator of TNF-induced apoptosis, is protective in a mouse model of steatohepatitis, suggesting that JNK signaling is important in the pathogenesis of NASH [30].

Chronic ethanol feeding also causes changes in expression of several inflammatory mediators, including reactive oxygen species (ROS), cytokines, adipokines, and chemokines [31, 32]. Hepatic KCs are an important source of TNFα. In animal models, administration of TNFα results in activation of SREBP, increased fatty acid synthesis, and development of fatty liver [33]. Others studies have shown lipopolysaccharide-mediated increased ROS production by KCs with chronic alcohol feeding and normalization of this response by adiponectin [34, 35]. Chronic alcoholic liver disease is associated with increased serum and hepatic IL-6 levels. However, IL-6 may actually be protective, since IL-6 deficient mice exhibit increased susceptibility to alcohol-mediated liver injury and exposure while IL-6 treatment improves hepatic steatosis.

Overview of Lipid Handling in the Liver

While the physiological aspect of lipid handling by the liver are discussed elsewhere in this textbook (Chap. 10), here we discuss the broad pathological mechanisms that lead to fat accumulation within hepatocytes.

Fatty Acid Uptake by the Liver

Nonesterified fatty acids (NEFA) are released from adipose tissue by the action of lipases. Circulating NEFAs are taken up by the liver via diffusion or by transporters that include the fatty acid transport protein (FATP) family members as well as fatty acid translocase (also known as CD36). Within the liver, NEFAs are bound by members of the fatty acid binding protein (FABP) family and subsequently undergo either oxidation or get esterified by the addition of glycerol. There is evidence from animal studies that blocking the esterification step leads to accumulation of fatty acids within hepatocytes and increase in liver injury from toxic fatty acids [14].

De Novo Lipogenesis

Humans have limited capacity to store excess energy in the form of carbohydrates. Therefore, when carbohydrate intake exceeds utilization, the liver, along with adipose tissue, becomes an important site of DNL, or the process of fatty acid synthesis from carbohydrate-derived acetyl-coenzyme A. The process of DNL is regulated by insulin and a number of TF and nuclear receptors (see below) and there is evidence of its dysregulation in the setting of insulin resistance and in fatty liver disease.

Oxidation of Fatty Acids

In the setting of energy deprivation and fasting, fatty acids undergo oxidation either in the mitochondria or peroxisomes

to generate adenosine triphosphate (ATP). During the process of β-oxidation of fatty acids in mitochondria, which consists of a recurring series of steps, fatty acids are converted to acetyl-coenzyme A. The process is linked to the generation of FADH2 and NADH which leads to ATP synthesis. Fatty acids can also undergo peroxisomal β-oxidation that leads to ATP yield albeit at lower levels. A third form of fatty acid oxidation consists of ω-oxidation that takes place in microsomes and involves the conversion of very long chain fatty acids to dicarboxylic acids. Blockade of any of the steps results in a net gain in fatty acids within the cells to contribute to fatty liver.

Export of Lipids from the Liver

The process of lipid export from the liver is regulated by the microsomal triglyceride transfer protein (MTTP). The liver synthesizes a type of lipoprotein called very-low-density lipoprotein (VLDL) that contains apolipoprotein B100 for transport of triacylglycerol, cholesterol, cholesterol esters, and phospholipids from the liver to peripheral tissue. Disruption of this complex and highly regulated process can also lead to hepatic lipid accumulation and has been shown to be dysregulated in fatty liver disease.

Liver Transcription Factors and Nuclear Receptors

A number of nuclear TF play important roles in the control of hepatic carbohydrate and lipid metabolism and their dysregulation contribute to hepatic lipid accumulation as well as inflammation. Among the TF that have been extensively studied are the members of peroxisome proliferator-associated protein (PPAR) family- PPARα and PPARγ; liver X receptor (LXR); sterol regulatory element-binding protein 1 (SREBP1); carbohydrate responsive element-binding protein (ChREBP); and hepatocyte nuclear factor alpha (HNF4α).

PPAR Family

The PPAR family has three members, PPARα, PPARγ, and PPARδ. PPAR family members function as intracellular lipid sensors. PPARα is activated by free fatty acids, eicosanoids, and fibrate drugs [36]. It contains a ligand-dependent transactivating domain, an N-terminal ligand-independent transactivating domain, and zinc-finger DNA-binding domain [37]. PPARα heterodimerizes with retinoid X receptor (RXR) and binds to peroxisome proliferator-responsive elements of its target genes such as mitochondrial carnitine palmitoyl transferase-I and–II, mitochondrial HMG-CoA synthase, cytochrome P450 enzymes, and acyl-CoA oxidase. Important metabolic processes regulated by PPARα include mitochondrial and peroxisomal β-oxidation of fatty acids, ketogenesis, and plasma HDL metabolism [38, 39].

PPARα knockout mice have defective fatty acid oxidation and exhibit severe hepatic steatosis [40], while stimulation of PPARα by its ligands improves hepatic steatosis in experimental models of NAFLD [41, 42]. Besides its role in fatty acid oxidation, PPARα also modulates inflammation. It increases anti-inflammatory chemokines and downregulates proinflammatory cytokines [43, 44] and by interacting with p65, inhibits translocation of NF-κB to the nucleus [45].

The second PPAR family member with an important role in lipid homeostasis is PPARγ. It is expressed at high levels in adipose tissue where it causes adipocyte terminal differentiation and proliferation of subcutaneous adipocytes. Activation of PPARγ causes increased uptake of fatty acids by adipose tissue, increase in adiponectin levels, and increase in hepatic adiponectin receptor expression. Thus, by increasing insulin sensitivity and decreasing the circulating free fatty acid pool, PPARγ protects liver and pancreatic beta cells from fatty acid-mediated toxicity [25, 46–49]. In addition, PPARγ has a potential beneficial effect on liver fibrosis by maintaining stellate cells in a quiescent state [50]. Therefore, PPARγ agonists such as thiazolidinediones have been proposed as therapeutic agents in the treatment of NAFLD.

PPARδ, the third member of the PPAR family, also has important regulatory roles in adipose tissue, liver, and skeletal muscle. It increases fatty acid transport and oxidation, decreases hepatic glucose output, and decreases inflammation. It has also been shown to improve plasma HDL levels [51–53].

ChREBP and SREBP

ChREBP is a basic helix-loop-helix-leucine zipper TF. It is activated by glucose and translocates from the cytoplasm to the nucleus, where it binds to the E-box motif in the promoter of liver-type pyruvate kinase (L-PK), an important glycolytic enzyme [54, 55]. L-PK catalyzes the formation of pyruvate, which enters the Krebs cycle and generates citrate and ultimately acetyl-CoA, the building block for DNL. Studies with ChREBP knockout mice also revealed that ChREBP independently stimulates the transcription of fatty acid synthetic enzymes. Therefore, in the setting of IR and hyperglycemia, ChREBP mediates the conversion of excess glucose to fatty acids by up-regulating both glycolysis as well as lipogenesis [56].

Hepatic fatty acid synthesis is also regulated via a second TF, SREBP-1c, also a member of the b-HLH-Zip family of

TF [57]. There are three SREBP isoforms of which SREBP-1c is the major hepatic isoform. SREBP1 activates expression of fatty acid biosynthetic genes and stimulates lipogenesis [57, 58]. Mice overexpressing SREBP-1c exhibit increased lipogenesis and develop hepatic steatosis, while genetically obese (Ob/ob) mice show reduction in liver triglyceride after disruption of the Srebp-1 gene [59, 60]. Since SREBP-1c is activated by insulin, it would be expected to be inactive in the setting of IR. Surprisingly, the protein is activated by insulin even when there is IR, resulting in increased fatty acid biosynthesis in the liver in NAFLD [61].

LXR

The two isoforms of LXR, α and β, are intracellular receptors for oxysterols and are important regulators of cholesterol and bile acid metabolism, via promoting conversion of cholesterol to bile acids, increasing reverse cholesterol transport, and decreasing intestinal absorption of sterols. LXRα is expressed mainly in the GI tract, including the liver, kidneys, and adipose tissue. LXRβ is expressed in most tissues [62–65]. Interestingly, LXRs regulate SREBP-1c and ChREBP, and activation of LXR leads to increased liver fatty acid synthesis and development of hepatic steatosis in mice [66–68].

PXR, CAR, and FXR

The pregnane X receptor (PXR) regulates severe genes involved in the cellular response to oxidative stress and hydroxylation and metabolism of bile acids and other toxic substances. PXR is activated by bile acids while constitutive androstane receptor (CAR) is believed to be indirectly activated by bile acids and bilirubin [69]. Activation of PXR by a pharmacologic agonist pregnenolone 16α-carbonitrile was found to induce hepatic steatosis by reducing ketogenesis and fatty acid β-oxidation and increase in triglyceride synthesis [70]. These effects of PXR may be affected by modulating the activity of another TF, FoxA2 [71].

CAR regulates sulfation of bile acids and bilirubin metabolism [72, 73]. Similar to PXR, activation of CAR by treatment with its agonist phenobarbital increases hepatic steatosis by decreasing expression of genes involved in fatty acid oxidation and also causes increased steatosis, inflammation, and fibrosis while targeted disruption of CAR is partially protective against development of steatohepatitis in an animal model of diet-induced steatohepatitis [74].

The farsenoid X receptor (FXR) is the intracellular receptor for bile acids [75]. FXR regulates bile acid uptake into hepatocytes and bile acid biosynthesis. FXR also regulates gluconeogenesis and fatty acid β-oxidation. FXR also decreases fatty acid synthesis via induction of a nuclear receptor small heterodimer partner (SHP) that inhibits LXR and therefore SREBP-1 expression [76, 77]. In support of this model, *Fxr*-null mice exhibit hepatic steatosis on a high fat diet [78]. In addition, in experimental models, FXR agonists have been shown to decrease hepatic fibrogenesis [79].

As discussed above, intriguing roles for these TF have been demonstrated in the regulation of hepatic carbohydrate and lipid metabolism, making them attractive targets for NAFLD therapies. However, due to the complex network formed by the cross-regulation of these TF and the unintended consequences of modulating their activity, further studies will be needed to identify effective and safe therapeutic agents that can be used in patients.

Oxidative Stress and Lipid Peroxidation

Short-lived, pro-oxidant chemicals including hydroxyl radicals, singlet oxygen molecules, hydrogen peroxide, and superoxide anions are called ROS. It has been hypothesized that ROS act as the "second hit" that converts simple steatosis to steatohepatitis [80]. ROS cause oxidative damage to macromolecules in the cell when they overwhelm the protective antioxidant mechanisms of the cell [81, 82].

Functional inactivation of essential cellular biomolecules causes either cell death or production of inflammatory mediators, mediated by redox-sensitive TF such as Nrf-1 and nuclear factor-κB [83, 84]. NASH is associated with increase in serum markers of oxidative stress, markers of oxidative damage for DNA, and proteins [85–88]. Polyunsaturated fatty acids (PUFAs) in the cell can undergo peroxidation by ROS, resulting in the formation of malondialdehyde and trans-4-hydroxy-2-nonenal byproducts [89]. These lipid peroxides contribute to liver injury by causing proinflammatory cytokine production, inflammatory cell recruitment, and depletion of protective antioxidants like glutathione [90–93].

Dysfunction of Intracellular Organelle in Fatty Liver Disease

Mitochondria are the source of ATP and heat generation using metabolic intermediates that are derived from fat and carbohydrate metabolism. They are the primary site for fatty acid oxidation and oxidative phosphorylation. During periods of prolonged fasting, fatty acid oxidation in the liver leads to formation of ketone bodies that are an important energy source for the brain when blood levels of glucose are low. Several lines of evidence suggest that hepatic mitochondrial dysfunction contributes to the pathogenesis of fatty liver.

Ultrastructural studies have shown the presence of hepatocyte megamitochondria with paracrystalline inclusions in patients with NASH [94]. Functional differences that have been reported in mitochondria include impaired ability to synthesize ATP after a fructose challenge, which causes transient liver ATP depletion and lower expression levels of mitochondrial DNA-encoded proteins and lower activity of complexes of the mitochondrial respiratory chain [95, 96].

The mechanisms that result in mitochondrial dysfunction in fatty liver disease are not fully understood. One possible mechanism may be the increased mitochondrial oxidation of fatty acids resulting in increased respiratory chain electron delivery. In this setting, mitochondrial respiratory chain dysfunction may cause interruption in electron flow, transfer of electrons to molecular oxygen, and production of hydrogen peroxide and superoxide radicals [97, 98].

With progressive decrease in mitochondrial fatty acid oxidation, alternative pathways of fatty acid oxidation, peroxisomal β-oxidation and microsomal ω-oxidation, become active and contribute to the generation of ROS by transfer of electrons to molecular oxygen. This creates a vicious cycle of increasing mitochondrial dysfunction. Mitochondrial dysfunction is also worsened by activation of cytochrome P450 2E1, 4A10 and 4A14-mediated microsomal ω-oxidation via generation of dicarboxylic acids that uncouple oxidative phosphorylation and add to oxidative stress [99–101].

Recent Advances

MicroRNA and Fatty Liver Disease

MicroRNA (miRNA) is a 21–23 nucleotide long noncoding single-stranded RNA molecules important in posttranscriptional gene regulation. Several recent papers have demonstrated an important role for miRNA in regulating a wide variety of important metabolic functions such as insulin secretion, lipid metabolism, differentiation of adipocytes, immunity, and apoptosis [102–104]. In the liver, miRNA-122 is a strongly expressed species of miRNA that is also important in regulation of lipid metabolism [105]. In animal models of obesity, inhibition of miRNA-122 expression improved hepatic steatosis and decreased expression of lipogenic genes as well as plasma cholesterol levels [103].

Endocannabinoids

Endocannabinoids are endogenous lipid compounds that have been extensively studied as neuromodulatory substances that are involved in physiological processes such as appetite and food intake and pain-sensation. There are two cannabinoid receptors, CB1 and CB2, which endocannabinoids can bind to. One of the ligands of cannabinoid receptors, anandamide, can increase hepatic fatty acid synthesis by up-regulating SREBP1c. Consistent with this finding, mice with targeted disruption of CB1 receptor are resistance to diet-induced hepatic steatosis. Furthermore, endocannabinoid system has been shown to be involved in hepatic fibrogenesis, making this system an exciting area for research for the development of targeted therapies [106, 107].

The CB1 receptor has also been implicated in the pathogenesis of alcoholic fatty liver. Alcohol feeding causes up-regulation of endocannabinoids and increased expression of the CB1 receptor in the liver while targeted disruption of the receptor leads to resistance of alcoholic fatty liver. Jeong et al. have shown that alcohol causes release of 2-arachidonoylglycerol from stellate cells in the liver which binds to CB1 receptor. In turn, CB1 receptor activation results in increased fatty acid synthesis via activation of SREBP1 [108].

Autophagy

Recently, it was shown that the process of autophagy is important for lipid metabolism and regulation of intracellular lipid content. A more detailed account on the topic is available in Chap. 26. During times of nutrient depletion, triglycerides stored in intracellular lipid droplets are hydrolyzed into fatty acid and undergo oxidation for energy production. Starvation also causes induction of autophagy, a process in which vesicles called autophagosomes consisting of intracellular proteins and organelles are degraded in lysosomes to obtain energy. Singh et al. showed recently that during periods of energy deprivation, intracellular lipid droplets and autophagic components associate and there is increase in triglyceride storage in lipid droplets if autophagy is inhibited [109]. This finding has important implications in the pathogenesis of fatty liver disease and represents another promising arena for the development of targeted therapies.

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Chapter 30
Hepatic Fibrosis and Cirrhosis

Rebecca G. Wells

Introduction

Liver fibrosis is the presence of scar tissue in the liver. Although it varies in location within the liver, especially in early disease, the liver scar uniformly represents both an excess of extracellular matrix (ECM) and a shift in the quality of that matrix. Cirrhosis is the term applied to the final stage of fibrosis, the common end result of progressive fibrosis from all etiologies. Although it is in one sense the far end of the fibrosis spectrum, the term cirrhosis reflects architectural rearrangements rather than the quantity of abnormal matrix, specifically the formation of parenchymal nodules surrounded by scar tissue [1].

Fibrosis and, at the extreme, cirrhosis, results in global liver dysfunction at levels ranging from individual cells to the whole organ. More than just the deposition of abnormal ECM proteins by a set of fibrogenic cells, fibrosis represents a marked disruption in the soluble and mechanical milieu of the liver and altered function of almost every cell type present, often with the influx of additional cell populations from the circulation. Similarly, alterations in the ECM represent changes in multiple classes of matrix molecules in varied regions of the lobule.

Fibrosis is a pathological wound healing response and results specifically from chronic injury to the liver rather than acute injury, which heals without fibrosis. In the setting of repeated cycles of injury and repair, there is a failure of the matrix degradation and architectural remodeling that represent the last stage of wound healing. To what extent, and when, the process becomes self-perpetuating is not known. Some forms of fibrosis advance so rapidly in the later stages that self-perpetuation seems almost certain [2]. Nonetheless, the potential for regression of fibrosis suggests that in most cases continued injury is required for progression of disease.

R.G. Wells (✉)
Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
e-mail: rgwells@mail.med.upenn.edu

In many ways, including the nature of the fibrogenic cells, the composition of the scar, and the mediators of fibrogenesis, fibrosis of the liver is similar to fibrosis in other organs, including kidney, lung, and skin. There are, however, many aspects of liver fibrosis and injury repair that are unique to the liver. The liver is highly regenerative, able to restore its mass completely even after a three-quarters hepatectomy, and regeneration is an important component of liver wound healing, particularly early after injury. For a detailed discussion of liver regeneration, please see Chap. 18. Regeneration also plays a role in the evolution of cirrhosis, and a failure of normal regenerative mechanisms and the resulting contribution of progenitor cells to repair are important components of cirrhosis [3, 4]. Another defining characteristic of the liver is its unique vasculature, and fibrosis of the liver (as opposed to fibrosis in other organs) is notable for disruption of the normal vascular architecture, with shunting and marked hemodynamic abnormalities.

Epidemiology and Etiology

Fibrosis and cirrhosis are common in the United States and worldwide. The prevalence of noncirrhotic liver fibrosis is difficult to estimate because there are no accurate noninvasive diagnostic tests and the disease is typically asymptomatic. Although cirrhosis is often asymptomatic as well, autopsy studies suggest a worldwide incidence as high as 9.5% [5]. Cirrhosis is the fourteenth leading cause of death globally (higher in the developed world) and is increasing [5]. In the United States, cirrhosis is the twelfth leading cause of death; however, morbidity figures likely significantly underestimate the impact of the disease given the increasing use of liver transplantation to prevent death [5]. The incidence of fibrosis in almost all forms of chronic liver disease is higher for men than for women (Fig. 30.1).

Chronic liver injury with fibrosis and cirrhosis results from multiple etiologies, including infectious, toxic, congenital, metabolic, autoimmune, and vascular (Table 30.1). Chronic hepatitis B infection is the most common cause of cirrhosis in the developing world, while alcohol abuse and,

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
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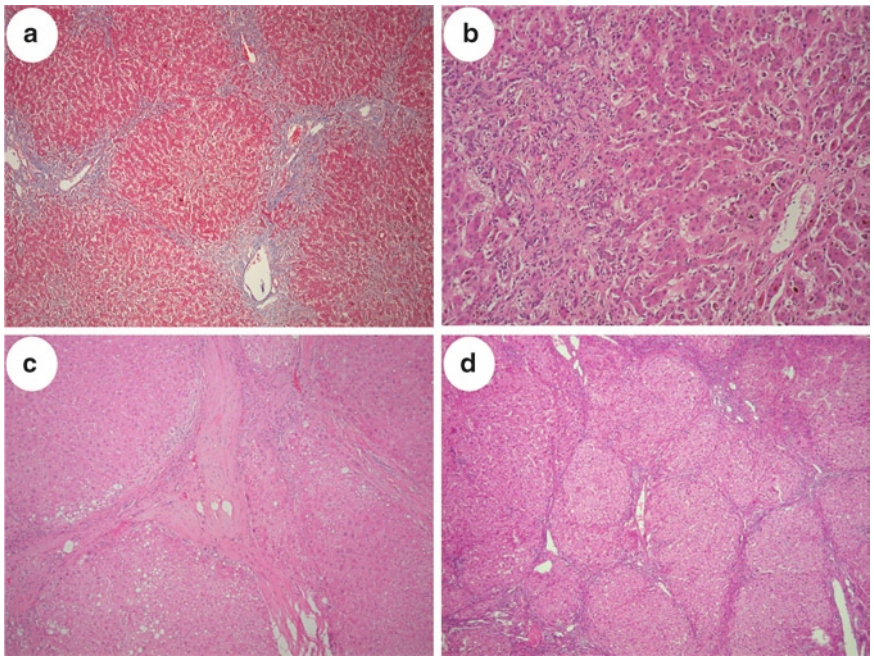

Fig. 30.1 Histology depicting hepatic fibrosis and cirrhosis. (Figure is courtesy of Dr. Tong Wu, Chairman of Pathology, Tulane University, New Orleans, LA.) (**a**) Trichrome staining shows severe bridging fibrosis in a patient receiving TPN. (**b**) Severe cholestasis with bridging fibrosis in a patient receiving long-standing TPN. There is marked hepatocellular and canalicular cholestasis with pericellular fibrosis. The portal tracts show prominent bile ductular proliferation and fibrosis with sparse lymphocytic inflammation. (**c**) Cirrhosis seen in a patient with nonalcoholic steatohepatitis (NASH). (**d**) Cirrhosis in a patient with alcoholic liver disease.

Table 30.1 Common etiologies of fibrosis

Infectious
Chronic hepatitis B
Chronic hepatitis C
Schistosomiasis
Toxic
Alcohol
Drugs, including amiodarone, methotrexate, and vitamin A
Metabolic/inherited
Nonalcoholic fatty liver disease (NAFLD)
Wilson's disease and other copper overload syndromes
Hemochromatosis
Autosomal recessive polycystic kidney disease
Cystic fibrosis
α-1 antitrypsin deficiency
Progressive familial intrahepatic cholestasis syndromes
Multiple inherited disorders of metabolism and storage
Vascular
Budd-Chiari syndrome
Sinusoidal obstruction syndrome
Chronic right heart failure
Autoimmune
Autoimmune hepatitis
Primary biliary cirrhosis
Other/unknown
Biliary atresia
Secondary biliary obstruction
Primary sclerosing cholangitis

increasingly, chronic hepatitis C infection and nonalcoholic fatty liver disease (NAFLD) are the leading causes in the developed world [5, 6]. NAFLD is rapidly becoming the most common reason for liver disease in the United States, and with up to one third of the population demonstrating liver steatosis, NAFLD-associated cirrhosis will likely increase as well [7].

Rates of progression differ with the nature of the injury as well as host factors and are highly variable. Patients with the pediatric disease biliary atresia, for example, may become cirrhotic within weeks, and patients with recurrent hepatitis C postliver transplant within months. In contrast, patients with chronic hepatitis C infection require an average of 30 years to progress to cirrhosis, although there is significant variability in this population both as a function of host factors and the duration of infection [2, 8]. Etiologic factors appear to be synergistic, such that concurrent alcohol abuse, iron overload, and the metabolic syndrome can enhance the rate of fibrosis from other causes [9].

Fibrosis Scoring Systems

Histology is the gold standard for assessing the degree of fibrosis. Although new, noninvasive diagnostic tests are

increasingly used in clinical practice, their accuracy is poor and all currently refer back to histological scoring systems.

Except in the setting of autopsies or explants, biopsies (in particular needle biopsies) are the most common means of obtaining tissue for histological analysis. Needle biopsies, however, sample only about 1/50,000 of the liver, and significant inaccuracy is introduced through sampling error. Even with large samples 15–25 mm long, 25–30% of biopsies are not representative of the whole liver, with underscoring particularly common in cirrhosis [10, 11].

Histology-based fibrosis scoring systems are an important component of clinical trials (for which they were designed), as well as the care of individual patients, and provide important information about prognosis. Cirrhosis on the biopsy of an asymptomatic patient portends the development of symptoms with continued deterioration and potentially death. Similarly, patients with bridging fibrosis – the formation of fibrous septae connecting combinations of portal and central regions – have a worse prognosis than patients with less advanced disease.

There are multiple scoring systems available. Two in frequent use are the METAVIR and Ishak systems. Both were developed for the assessment of chronic hepatitis C and are commonly used for chronic hepatitis B as well, and both incorporate separate scores for grade (degree of necroinflammatory activity) and stage (degree of fibrosis) [12]. The METAVIR system comprises four grades, ranging from no activity through severe activity (defined as different combinations of periportal and lobular necrosis), and five stages, F0 through F4, ranging from no fibrosis to cirrhosis [13]. The Ishak system (a modification of the Knodell system) is more complicated, with a complex grading scheme and seven stages of fibrosis, and may be more suitable for clinical trials and research purposes [14]. Scores in both systems, as well as the similar Desmet/Scheuer system, reflect a progression from periportal to septal fibrosis, then to nodule formation and cirrhosis, but do not take into account histological changes that may occur during regression (see below) [15]. Neither the METAVIR nor the Ishak systems incorporate scores for perisinusoidal fibrosis; thus, although both are used clinically for scoring alcoholic and nonalcoholic steatohepatitis (NASH), they are not ideal for this purpose.

A scoring system for NASH has been proposed by the NASH Clinical Research Network. This includes a NAFLD activity score (NAS) which incorporates steatosis, hepatocellular ballooning, and lobular inflammation, as well as a separate score for fibrosis which takes into account perisinusoidal and portal/periportal fibrosis [16, 17]. There is also a staging system for primary biliary cirrhosis in common use, although the focal nature of the lesions in this disease makes sampling errors particularly problematic [18, 19].

New techniques to analyze biopsies may improve their prognostic relevance in the future. Quantitative morphometry of sections stained with the collagen dye picrosirius red or with antibodies against the myofibroblast marker α-smooth muscle actin (α-SMA) may yield improved assessments of fibrosis [20, 21]. Methods to determine ongoing matrix deposition and degradation offer the possibility that biopsies in the future could provide not just an assessment of the static state of fibrosis, but also a more dynamic and thereby clinically relevant picture of fibrogenesis and fibrolysis [21].

Improved staining and morphometry are particularly needed in cirrhosis. In all of the current scoring systems, cirrhosis occupies the final category, without further subdivisions. Cirrhotic patients and their livers are, however, highly heterogeneous and morphometric studies have shown that fibrosis continues to evolve and progress even in cirrhotics [20]. Clinically, cirrhosis has been divided into four stages, defined based on the presence or absence of complications such as ascites, varices, and variceal bleeding. Stages one and two represent compensated cirrhosis and stages three and four decompensated cirrhosis; this categorization has prognostic import [22, 23]. An expanded clinical/histological scoring system which subdivides METAVIR stage F4 disease into three substages has recently been proposed (Fig. 30.2) [24, 25]. These investigators suggest that degrees of clinical decompensation and elevations in the hepatic venous pressure gradient (HVPG) correlate with histological features including septal thickness and cellularity, matrix cross-linking, and nodule size. This expanded scoring system has not yet been validated, although it has been shown that septal thickness and nodule size correlate with HVPG in cirrhotics [26, 27], and that collagen in cirrhotic livers from humans and rodents is highly cross-linked [28, 29]. Measuring these parameters is thus far too difficult for routine clinical use. Additionally, it is not known whether these categories provide information about the potential for fibrosis regression, or whether there is a point of irreversibility that can be defined histologically. Regardless, a system to subcategorize cirrhotics will clearly be essential in future clinical trials of antifibrotics.

Matrix Proteins of the Normal and Fibrotic Liver

The ECM has key roles in the normal and the fibrotic liver. Matrix proteins are the scaffold proteins of the liver, providing rigidity and elasticity. They also have important functions in signaling, serving as ligands for signaling receptors (primarily integrins) and regulating local concentrations of soluble factors. Although individual matrix proteins of

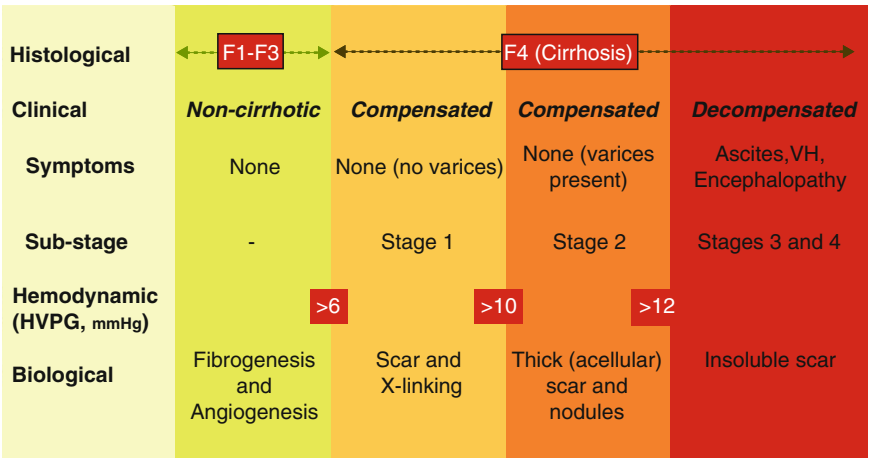

Fig. 30.2 Proposed expanded classification system for cirrhosis. Adapted from Garcia-Tsao et al. [25]. Used with permission.

particular importance to fibrosis are highlighted here, many matrix proteins have multiple domains and most interact with a large number of other proteins. There is a complex interrelationship between different ECM proteins in the liver that undergoes significant, yet poorly understood, changes in fibrosis.

The ECM of the liver is unique. Although the organization of ECM in the liver capsule and the basement membranes surrounding the bile ducts and vessels is similar to that found in other epithelial organs, the perisinusoidal matrix is unlike any found elsewhere in the body. Hepatocytes and sinusoidal endothelial cells lack the continuous basement membrane of networked type IV collagen, laminin, entactin, and perlecan found underlying epithelial populations elsewhere. Instead, their basolateral surfaces face the space of Disse, a narrow (<1 μM) space containing discontinuous deposits of type IV collagen which is not associated with laminin or perlecan. Another basement membrane collagen, type VI, is laid down homogeneously, increasing from portal to central regions, and there are discontinuous deposits of type III collagen [30, 31]. Plasma fibronectin, which is secreted by hepatocytes, is abundant [30]. Structure is provided by a network of thick cables of type I collagen that arise in the portal tracts and extend into the lobules [32].

The specialized matrix organization of the space of Disse – the absence of a typical basement membrane – is required to maintain the normal differentiation state of hepatocytes and sinusoidal endothelial cells as well as other resident nonparenchymal cells of the sinusoid [32–34]. One of the first and most important changes in fibrosis is the capillarization of the sinusoids in which a continuous, organized basement membrane replaces the sparse, atypical matrix of the space of Disse and there is a precipitous decline in the area of sinusoidal endothelial cell fenestration [30, 35, 36]. This results in hepatocyte dedifferentiation, impaired solute and nutrient exchange, and loss of the portal to central gradients of ECM that normally contribute to phenotypic differences between periportal and pericentral regions [37]. A switch in the isoform composition of type IV collagen also contributes to hepatocyte dedifferentiation [38]. Additional early changes of fibrosis include increased deposition of the cellular fibronectin splice variants EDA and EDB (see below), which contribute to myofibroblastic differentiation and altered function of hepatic stellate cells [39–41]. Interestingly, there are differences in the pattern of matrix deposition in early fibrosis between adults and neonates, particularly in the perisinuosidal space, which may contribute to differences in the progression of pediatric and adult liver disease [42].

As fibrosis progresses, there is accumulation of abnormal matrix, in particular the fibrillar collagens (types I, III, and V), additional cellular fibronectin, and proteoglycans, resulting in total liver ECM up to ten times that found in the normal liver. There are also marked, albeit poorly understood, changes in matrix protein modifications, including cross-linking, glycosylation, and glycosaminoglycan side chain sulfation [43, 44].Although the location of ECM deposition, particularly early in the disease, reflects the location of injury, these distinctions blur as fibrosis advances toward cirrhosis: dense fibrous septae form, becoming increasingly cross-linked and acellular, and the normal architecture of the liver is eventually lost [28].

Collagens in the Liver

Collagens are the major structural proteins in the normal and fibrotic liver. All collagens are characterized by the presence of stretches of Gly-Xaa-Yaa repeats, where Xaa and Yaa are proline and hydroxyproline (or to a lesser extent hydroxylysine),

respectively. Individual collagen molecules (α-chain protomers) self-assemble to form hetero- or homotrimeric triple helices, depending on the specific type of collagen; this provides some collagens with a significant amount of functionally important isoform diversity [45].

Synthesis of the collagens, in particular the fibrillar collagens, is highly regulated at both the transcriptional and translational levels; transcription of the α1(I) chain, for example, is regulated via a 5′ stem loop and 3′ UTR binding proteins [29]. Posttranslational processing involves more than ten different enzymes and results in proline and lysine hydroxylation (at the Yaa position) and N-linked and O-linked glycosylation [46]. The fibrillar collagens, once secreted from the cell, also undergo proteolytic cleavage of globular N- and C-terminal propeptide domains. Some of these degradation products, particularly the N-terminal propeptide of type III collagen (known as PIIINP), may have clinical utility as serum markers of fibrosis [47].

Triple-helical collagens assemble into large supramolecular arrays that derive mechanical stability from interchain cross-linking. There are three major families of collagen cross-linking enzymes. Tissue transglutaminase (transglutaminase 2) mediates the formation of ε-(γ-glutamyl lysine) cross-links in collagens and other matrix proteins, rendering them resistant to proteolysis [48]. Transglutaminases are increased in fibrosis, although they play a complex role: mice null for transglutaminase 2 have increased mortality after induction of fibrosis, suggesting a protective role for the enzyme, although fibrotic septae in advanced disease demonstrate increased transglutaminase-mediated cross-links and are resistant to degradation [28, 49–51]. The lysyl oxidases (LOX) catalyze the oxidative deamination of lysine and hydroxylysine in collagen (and lysine in elastin), leading to the formation of aldehydes that condense with neighboring groups to form covalent cross-links. LOX family expression increases rapidly after injury, before the appearance of fibrogenic myofibroblasts [52, 53]. Inhibiting LOX activity inhibits fibrosis, suggesting that these enzymes play a causal role in the development of fibrosis, potentially through their mechanical effects on collagen or by preventing its degradation [54–57]. Members of a final family of collagen cross-linking enzymes, the lysyl hydroxylases, convert lysine into hydroxylysine, increasing the protease resistance of collagen and determining the route of LOX-mediated cross-linking [29, 58, 59].

Of the more than twenty different collagens described, at least nine are expressed in the adult liver, and all are believed to increase in fibrosis. The most important, and best studied, are the fibrillar collagens (types I, III, and V). These collagens have long central regions of more than 300 uninterrupted Gly-Xaa-Yaa repeats which enable the formation of rigid triple-helical fibrils that contribute tensile strength to the liver [45]. Type III collagen is the first collagen to increase in chronic liver disease and it remains highly expressed, although supplanted by type I collagen as fibrosis progresses [60]. Molecules of the different fibrillar collagens do not form separate arrays, but are instead incorporated into composite fibrils, or alloys, that vary according to the local environment, with the relative contribution of each collagen determining the mechanical properties and protease resistance of a given fibril [33, 45, 61, 62]. The fibrillar collagens also interact with other collagens including type VI collagen and other matrix molecules including fibronectin and proteoglycans, resulting in complex supramolecular assemblies.

There are several groups of nonfibrillar collagens found in the liver. These collagens have interruptions in the Gly-Xaa-Yaa domains, enabling them to form a variety of different three-dimensional structures. Although less well understood than the fibrillar collagens, the nonfibrillar collagens nonetheless have important roles in the pathogenesis of liver fibrosis. Many, in particular the network-forming collagens (types IV, VI, and VIII), which have multiple interruptions in their triple-helical chains, are associated with the basement membrane and potentially regulate both portal fibrosis and the capillarization of the sinusoids [63]. Type IV collagen, the major collagen of the basement membrane, stabilizes other basement membrane proteins, including perlecan and laminin. It is synthesized in the liver primarily by sinusoidal endothelial cells, implicating these cells in the matrix changes of early fibrosis [64]. Type VI collagen is also found in the sinusoids as well as around blood vessels and may, via interactions with type IV collagen, anchor other structures in place [65, 66]. Type VI collagen is closely associated with latent forms of matrix metalloproteinases (MMPs) and regulates matrix remodeling; the α2 chain sequesters latent MMPs, blocking their matrix degrading activity [67]. An important function of all of the network-forming collagens is to facilitate interactions between other matrix molecules [68].

The FACIT (fibril-associated with triple helices) collagens (XIV, XIX) have alternating triple-helical and nonhelical domains, and as a result, are highly flexible. Type XIV collagen is widely expressed in the liver, especially in the portal tract. It is associated with dense, mature fibrils of types I and III collagen in regions of established fibrosis under stress and contributes to the mechanical properties of the fibrillar collagens [69–72]. Type XIV collagen has antiproliferative activity and induces myofibroblast precursors in the liver including fibroblasts and hepatic stellate cells to become quiescent; this may be important in defining the course of established fibrosis [33, 73].

The MULTIPLEXIN (multiple-triple-helix domains and interruptions) collagen type XVIII is a heparan sulfate proteoglycan found in basement membranes and in a continuous layer along the hepatic sinusoids [74, 75]. It links the basement membrane to the underlying matrix and is likely to be

particularly important during capillarization of the sinusoids. This collagen, which interacts with multiple other matrix molecules, participates in ECM breakdown and remodeling as part of the ductular reaction and fibrosis-associated angiogenesis [76–78].

Other Matrix Proteins Important in Fibrosis

Fibronectin is a multifunctional, multidomain glycoprotein that is a central component of the fibrillar matrices surrounding cells. It consists of repeats of type I, type II, and type III domains and contains a heparin-binding N-terminus, a collagen-binding domain, and an Arg-Gly-Asp (RGD) motif mediating integrin binding. It is the product of a single gene, but undergoes extensive alternative splicing to yield two major forms, plasma fibronectin, a soluble disulfide-linked dimer secreted by hepatocytes directly into the circulation, and cellular fibronectin, a multimeric form produced by a wide variety of cells and assembled into cell-associated fibrils via integrin-mediated interactions. There are multiple isoforms of cellular fibronectin including fibronectin EDA and fibronectin EDB, which encode the extra type III domains A and B, respectively [79].

In the normal liver, fibronectin is found surrounding basement membranes in the portal region and in the perisinusoidal space. It is the most abundant ECM protein in the normal space of Disse, coating hepatocytes and collagen fibrils [30]. After injury, fibronectin (especially the cellular fibronectin isoforms EDA and EDB) is one of the first matrix proteins to increase, especially in fibrotic bands, the space of Disse, and the portal region [39]. The growth factor TGF-β stimulates sinusoidal endothelial cells to produce fibronectin EDA, which in turn induces the myofibroblastic activation of hepatic stellate cells and other myofibroblast precursors [40, 41]. Although there are no reports that mice lacking the fibronectin EDA splice variant have been studied for their response to liver injury, these mice have disordered skin wound healing and decreased pulmonary fibrosis in experimental models, indicating a general importance of EDA in wound healing [80, 81].

Fibronectin is closely associated with tenascin C and fibulin, adhesion modulators that antagonize cell interactions with fibronectin and thereby inhibit cell spreading and cell-mediated contraction of the matrix [82]. Tenascin C is synthesized and secreted by hepatic stellate cells and is normally found in the sinusoids, although not the portal tract. In rat models of fibrosis, it is expressed transiently at sites of tissue injury and is located in early septae (at the interface between the parenchyma and the scar) rather than in organized areas of fibrosis, suggesting that it participates in the early deposition of ECM [83–86]. Tenascin-C-deficient mice have decreased fibrosis [87]. Fibulins 1 and 2 also increase in human and rodent chronic liver disease, in particular in myofibroblasts in fibrous septae, and they are believed to be involved in tissue remodeling [88, 89].

Elastin and fibrillin, the proteins that constitute elastic fibers, provide the liver with resilience (in comparison to the collagens, which provide tensile strength). Both increase significantly in liver fibrosis in regions of injury [90–93]. Elastic fibers are formed when fibrillin arrays are cross-linked by transglutaminase to form microfibrils; in some cases, these bind to tropoelastin (the precursor of mature elastin) and undergo stabilization via LOX-mediated cross-linking [94]. Fibrillin-1, the only fibrillin family member expressed in the liver, is deposited by hepatic stellate cells, portal fibroblasts, and liver myofibroblasts and is broadly distributed in vessel walls, in the portal tract, around the limiting plate, and lining the sinusoids [90, 91, 95]. In fibrosis, it is found in newly deposited matrix, surrounding myofibroblasts that are invading the parenchyma, and is also localized to the dense septae of mature matrix [90, 91]. Elastin, on the other hand, is expressed by portal fibroblasts and myofibroblasts primarily in the portal tract and is absent from the sinusoids [90, 91, 95]. The function of elastic fibers and their constituent proteins, beyond their mechanical role, is not well understood. They bind to multiple other proteins including collagens, proteoglycans, and latent TGF-β family members, which are closely related to the fibrillins, and potentially mediate the spatial integration of a variety of soluble and mechanical signals [94, 96]. Fibrillin may be involved in regulating sinusoidal hemodynamics as well as, by virtue of its antiadhesive function, cell-matrix interactions and migration [90]. It also plays a crucial role in regulating signaling by bone morphogenetic proteins and TGF-β family growth factors [91, 94, 96].

Proteoglycans (proteins modified by glycosaminoglycans), like elastic fibers, serve both structural and signaling purposes. They retain water, form a highly negatively charged barrier to the passage of other molecules, sequester and target growth factors, and regulate collagen organization. Proteoglycans increase up to fivefold in fibrosis, with particularly striking changes in chondroitin and dermatan sulfate proteoglycans expressed by myofibroblasts [97, 98]. The basement membrane includes several proteoglycans, among them collagen XVIII (above) and perlecan, a large heparin sulfate proteoglycan synthesized by sinusoidal endothelial cells and hepatic stellate cells which surrounds cells of the ductular reaction and is a key contributor to capillarization of the sinusoids [97, 99–101]. The small leucine-rich proteoglycans decorin, biglycan, lumican, and (less well studied) fibromodulin also increase in liver fibrosis; they modulate the assembly and mechanical properties of the fibrillar collagens [102–106]. Decorin and biglycan have signaling functions as well, binding to TGF-β and regulating its signaling. Hyaluronic acid is a pure carbohydrate polymer and not by

definition a proteoglycan, but is similarly increased in fibrosis. It regulates TGF-β-mediated myofibroblast differentiation and hepatic stellate cell migration [107, 108]. Hyaluronic acid has a high rate of turnover (primarily due to uptake and catabolism by sinusoidal endothelial cells) and is a potential serum marker of fibrosis [109].

The Cellular Source of Matrix Proteins: Myofibroblasts

Multiple cells of the liver contribute in different ways to changes in the ECM in fibrosis and cirrhosis. The major matrix-producing cells in liver as well as other forms of organ fibrosis, however, are myofibroblasts. Myofibroblasts are contractile, fibrogenic cells that express the microfilament α-SMA de novo [110]. In the liver, there is clear evidence that, although myofibroblast populations are heterogeneous, they represent the majority of matrix-producing cells [111–115].

Three factors are required for myofibroblastic differentiation: the growth factor TGF-β, the cellular fibronectin splice variant EDA, and increased local mechanical tension [116]. These act on a variety of precursor cells to yield fibrogenic myofibroblasts. Which cells in the liver serve as these myofibroblast precursors is the subject of considerable ongoing debate. Hepatic stellate cells, however, are without question a key precursor population. Previously called lipocytes, perisinusoidal cells, fat-storing cells, and Ito cells, hepatic stellate cells are vitamin A-storing pericytes located in the space of Disse. A more detailed chapter on stellate cells is also included in this textbook (Chap. 5). They are a heterogeneous population; recent reports suggest that most are derived from mesoderm of the early liver, although in the setting of injury a population of stellate cells may also originate in the bone marrow [117, 118]. Hepatic stellate cells participate in angiogenesis, development, metabolism, and immune regulation, but are best known for their role in fibrosis [119]. Myofibroblasts derived from hepatic stellate cells produce much of the abnormal matrix in fibrosis – especially in nonbiliary disease – including fibrillar collagens, most basement membrane proteins, cellular fibronectin, fibrillin, and proteoglycans [120–122].

Hepatic stellate cells are easily isolated from normal liver in the quiescent, or nonmyofibroblastic, state and undergo spontaneous myofibroblastic differentiation when cultured on stiff substrates such as glass and tissue culture plastic [123, 124]. Cells in culture express α-SMA organized in stress fibers and have contractile, fibrogenic, and migratory capabilities; they are similar, although not identical, to differentiated stellate cells isolated from fibrotic livers and have proven to be useful tools for determining mediators of fibrosis [125–127]. Studying hepatic stellate cells in vivo, particularly in human specimens, is more challenging, primarily because it is difficult to label them definitively. Although vitamin A-containing stellate cells can be identified by gold and silver chloride impregnation methods as well as UV autofluorescence, subsets of quiescent stellate cells lack vitamin A, and vitamin A storage is lost as the cells undergo myofibroblastic differentiation. The intermediate filament desmin reliably marks hepatic stellate cells in rodents, but not in humans [128]. A recent systematic study suggested that vinculin and cellular retinol-binding protein-1 were the most reliable markers of human hepatic stellate cells in both the normal and diseased liver [129].

Portal fibroblasts, cells around the portal tract that share a common precursor with hepatic stellate cells [118, 130], are also significant contributors to the myofibroblast population in fibrosis and cirrhosis, particularly in biliary disease [131]. An independent chapter discussing the role of portal fibroblasts in cirrhosis is also included in the textbook (Chap. 31). There is extensive evidence that nonstellate cell-derived myofibroblasts drive biliary fibrosis, and it has been proposed that portal fibroblasts are the "first responders" in biliary fibrosis, with stellate cells migrating at later times to regions of injury [112, 132–137]. Bile ducts are the primary site of injury in many forms of fibrosis, and it makes intuitive sense that local portal fibroblasts, rather than more distant, perisinusoidal stellate cells, are the major mediators of fibrosis [138]. Portal fibroblasts and the myofibroblasts derived from them are heterogeneous, and a variety of different markers, including fibulin-2, IL-6, elastin, and the ecto-ATPase nucleoside triphosphate diphosphohydrolase-2, have been proposed to label them specifically. Unfortunately, these markers have not been definitively analyzed in human livers [115, 131, 132].

Other cells also serve as myofibroblast precursors in liver fibrosis. These include smooth muscle cells of the vasculature and fibroblasts in Glisson's capsule and around the central veins [114, 115]. Circulating cells derived from the bone marrow, including fibrocytes (mesenchymal cell precursors), also become fibrogenic myofibroblasts in fibrosis [117, 139–142]. The relative contribution of any of these myofibroblast precursors, including hepatic stellate cells and portal fibroblasts, has not been determined for the various etiologies of human liver disease.

The Cellular Source of Matrix Proteins: Nonmyofibroblastic Contributors

Whether or not hepatocytes contribute to matrix deposition in fibrosis is an ongoing debate. While there were many publications in the 1980s and early 1990s suggesting that

hepatocytes were fibrogenic, definitive studies using in situ hybridization of tissue sections and mRNA analysis of freshly isolated rodent cells demonstrated clearly that mature hepatocytes deposit little, if any, of the fibrillar collagens or basement membrane proteins [64, 121, 122, 143]. Although hepatocytes are the source of plasma fibronectin in the body, hepatocytes do not express the cellular fibronectin isoform that is characteristic of fibrosis [39, 144]. Recently, some investigators have suggested that hepatocytes undergo an epithelial to mesenchymal transition (EMT) in fibrosis, becoming fibrogenic [145, 146]. Lineage tracing analyses using transgenic reporter mice with heritably labeled hepatocytes and GFP-tagged type I collagen, however, demonstrate that EMT of hepatocytes does not occur, although the topic continues to be controversial [111].

The role of cholangiocytes in direct matrix protein deposition remains under investigation. Cholangiocytes are discussed elsewhere in this textbook in greater detail (Chap. 4). Cholangiocytes, unlike hepatocytes, have an organized basement membrane and synthesize basement membrane proteins including type IV collagen, type XVIII collagen, laminin, and perlecan, with slight increases in basement membrane synthesis observed in fibrosis [64, 78, 99, 147]. Cholangiocytes do not synthesize the fibrillar collagens or fibronectin [64], although several publications have recently suggested that they undergo EMT and become fully fibrogenic myofibroblasts [148–151]. These studies, based on analyses of cells in culture and tissue immunostaining showing coexpression of epithelial and mesenchymal markers, are preliminary and require confirmation by more definitive techniques to determine whether cholangiocyte derivatives express α-SMA and synthesize collagen.

Sinusoidal endothelial cells have an important role in early fibrosis. They demonstrate morphological changes soon after injury, preceding matrix deposition, leading some authors to suggest therefore that endothelial cells are the major cells responsible for initiating fibrosis [99, 152–154]. Although sinusoidal endothelial cells isolated from fibrotic rodent livers produce small amounts of type I collagen, they are unlikely to produce significant amounts of the fibrillar collagens and other components of the liver scar [64, 121]. They do, however, secrete the basement membrane proteins perlecan, type IV collagen, laminin, and entactin, and thereby play an important role in the capillarization of the sinusoids [64, 99, 100, 121, 155, 156]. One group has suggested that capillarized sinusoidal endothelial cells are permissive for the myofibroblastic activation of hepatic stellate cells [157]. Others believe that the major role of sinusoidal endothelial cells is to produce the cellular fibronectin splice variant EDA [116]. Experiments using hepatic stellate cells and sinusoidal endothelial cells in coculture demonstrated that endothelial cells produce fibronectin EDA in response to either TGF-β, one of the most important soluble regulators of fibrosis, or malondialdehyde-acetaldehyde (a byproduct of ethanol); EDA in turn mediates stellate cell myofibroblastic differentiation [41, 158, 159].

Stem Cells and Progenitor Cells in Fibrosis

The liver is a highly regenerative organ, and hepatocytes have the ability to undergo multiple rounds of proliferation in response to acute liver damage. Liver regeneration is also a key component of the response to chronic liver disease. In the chronic setting, however, cholangiocyte and hepatocyte proliferation is compromised and small bipotential progenitor cells (also called oval cells and intermediate hepatobiliary cells in the rodent) activate and expand as an alternative mechanism of regeneration [3, 160–162]. For additional details on this cell type, please see Chap. 3. In many forms of fibrosis, in particular biliary fibrosis, these cells are in the form of a "ductular reaction," defined as a duct-like proliferation of progenitor cells. The extent of the ductular reaction correlates with the degree to which hepatocyte replication is impaired; in several diseases, including NAFLD, chronic hepatitis C, Alagille syndrome, and biliary atresia, the extent of the ductular reaction correlates with the stage of fibrosis [163–165].

The relationship between progenitor cells and myofibroblasts is poorly understood. Progenitor cells recruit profibrogenic inflammatory cells, and there is also direct crosstalk between progenitor cells and myofibroblast precursors [166–168]. Myofibroblasts are potential constituents of the stem cell niche, and myofibroblasts and their secreted matrix proteins may regulate the expansion of progenitor cells and their exit from the niche [169–173]. Finally, some groups have suggested that progenitor cells from the liver and the bone marrow are a source of hepatic stellate cells and myofibroblasts and, the reverse, that hepatic stellate cells are themselves stem cells [117, 139, 174–177].

Mediators of Fibrosis Progression

The number of potential mediators of fibrosis has increased dramatically in the last two decades and now includes multiple soluble, mechanical, and immunologic factors in addition to the cells and matrix proteins discussed above. The large number of mediators reflects the general disruption in liver homeostasis after injury, and it is important to note that there is significant cross talk among almost all of the mediators described here. Thus, while specific factors are necessarily studied individually, systems biology approaches will be required before a cohesive model of fibrosis pathophysiology can be developed.

Most information on mediators of fibrosis was generated using either isolated cells in two-dimensional culture (in particular hepatic stellate cells) or rodent models of fibrosis. Although both approaches have proven invaluable in advancing knowledge in the field, both have significant limitations. Hepatic stellate cells and other myofibroblast precursors undergo spontaneous myofibroblastic differentiation in culture, without the requirement for an insult. There are significant phenotypic differences between in vivo and in vitro differentiated cells, and as an example, in vitro artifacts have contributed to much of the confusion surrounding EMT in fibrosis [111, 125]. Animal models are also imperfect representations of human pathophysiology. Fibrosis and cirrhosis develop rapidly (often within weeks) in rodents in comparison to the longer time period (typically years) over which pathology develops in the human liver. Additionally, rodent livers undergo nearly complete resolution of pathology after removal of the offending agent, in contrast to the more limited improvement seen in many human liver diseases (see below). There are currently a variety of animal models of fibrosis available including toxic, nutritional, infectious/immunologic, cholestatic, and increasingly, transgenic; all have both strengths and weaknesses [178, 179].

One of the most important soluble mediators of fibrosis is TGF-β, which is both necessary and, at least in part, sufficient for the development of fibrosis [180, 181]. Multiple animal studies using a variety of anti-TGF-β agents have demonstrated efficacy in both preventing fibrosis progression and treating established fibrosis [180, 181]. TGF-β has multiple effects in the injured liver, including the direct stimulation of myofibroblast differentiation, induction of matrix protein production and splicing, and upregulation of modifying proteins such as LOX [182]. Although TGF-β signals through both canonical (Smad) and noncanonical pathways, the downstream signaling molecule Smad3 appears particularly important in mediating tissue fibrosis and is an attractive therapeutic target [183, 184]. Many other mediators of fibrosis act via the upregulation of TGF-β, and this growth factor clearly occupies a central position in the development of fibrosis.

Several other growth factors are central mediators of fibrosis, including platelet-derived growth factor (PDGF), which is the most potent known mitogen for hepatic stellate cells as well as a promotility agent, and vascular endothelial growth factor (VEGF), which is also a mitogen and proangiogenic agent [185–187]. Connective tissue growth factor (CCN2) has potent effects in sensitizing the liver to profibrogenic stimuli and may act both downstream and independently of TGF-β [188, 189]. Chemokines, in particular monocyte chemotactic protein-1 (MCP-1; CCL2) and its family members, are chemoattractants and are produced by hepatic stellate cells and biliary epithelial cells. MCP-1 receptors are found on Kupffer cells and stellate cells. This growth factor may play a particular role in mediating biliary fibrosis by stimulating Kupffer cell TGF-β production [190, 191].

Adipokines, including leptin, adiponectin, and resistin, are polypeptides produced by adipose tissue as well as other cells in the liver [192, 193]. They mediate fibrosis in NAFLD as wells as other forms of chronic liver disease. Numerous studies have demonstrated that leptin is profibrogenic, while adiponectin is antifibrogenic [192]. Interest in adipokines is in part due to the similarities between hepatic stellate cells and adipocytes, including the expression of the key adipocyte regulator PPAR-γ by stellate cells [194, 195].

Other important soluble factors in fibrosis include the vasoactive compounds endothelin and nitric oxide, which regulate stellate cell contractility [196, 197], and lipid peroxidation products and reactive oxygen and nitrogen species, which have both direct and indirect effects on Kupffer cells and hepatic stellate cells and also regulate production of other important mediators including TGF-β and PDGF [198]. Kupffer cell and hepatic stellate cell uptake of apoptotic bodies (from injured hepatocytes) is profibrogenic, in part through production of reactive oxygen species [199, 200]. Endocannabinoids are newly appreciated players in fibrosis; the presence of endocannabinoid receptors on myofibroblasts may explain the observation that fibrosis worsens in patients with chronic hepatitis C who abuse cannabis [201–203]. Endocannabinoid receptor blockers, which have been in clinical use for other purposes, may have antifibrotic activity [204].

Many of the pathways discussed are downstream of renin-angiotensin system (RAS) signaling. Components of RAS pathways are both pro- and antifibrogenic, and regulation of the balance of RAS signaling via angiotensin converting enzyme inhibitors and angiotensin receptor blockers reduces fibrosis in experimental animals and may have potential for therapy of human disease [205, 206].

Soluble factors interact with mechanical factors to drive myofibroblast differentiation, such that local variations in the mechanical milieu of the liver may determine cell phenotype and function in fibrosis [90, 207, 208]. Increases in collagen cross-linking and LOX activity mediate some of these mechanical changes [57]. In certain settings, the generation of active TGF-β from its latent form requires a stiff matrix, integrins, and intracellular tension, suggesting that some of the effects of mechanics on myofibroblasts are upstream of TGF-β [209].

The immune system, both innate and acquired, is central to the development of fibrosis, particularly in its early stages. Th1 and Th2 lymphocytes, natural killer and natural killer T cells, and B cells play a role [210–212], and hepatic stellate cells themselves are increasingly recognized to be antigen-presenting cells [213]. Stellate cells have also been shown to contain components of the inflammasome, a multiprotein complex that senses cellular stress and induces an inflammatory

response [214]. Dendritic cells, acting via tumor necrosis factor-α, regulate the production of many profibrogenic mediators and have downstream effects on other cells of the immune system [215]. The relationship between fibrogenic and inflammatory factors was recently demonstrated elegantly by the finding that endotoxin, acting via toll-like receptor 4 (TLR4), initiates a sequence of events involving hepatic stellate cells, Kupffer cells, and TGF-β that results in stellate cell differentiation and fibrosis [216]. TLRs and their endogenous ligands may have broad relevance to chronic liver disease and serve as attractive potential therapeutic targets [217].

The importance of specific fibrosis mediators varies from individual to individual. Large scale genetic studies, including quantitative trait locus mapping and genome-wide association studies, offer great promise in identifying susceptibility loci that may be important in predicting the clinical course of individual patients. Studies have already identified a polymorphism in Kruppel-like factor 6 associated with development of fibrosis in NAFLD; a TLR4 polymorphism that appears to be protective against fibrosis has corresponding effects on hepatic stellate cell function in vitro [218, 219]. There are multiple other studies in progress or recently reported regarding genetic associations with fibrosis in specific diseases, and these will likely be important in future clinical trials and patient management.

Progression from Fibrosis to Cirrhosis

Cirrhosis is the end-stage of chronic liver disease, but it would be a mistake to regard it only as advanced fibrosis. It was defined by a working group of the World Health Organization in 1977 as: "a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules" [1, 220]. Critical to this definition are the concepts that the process affects the entire liver and that significant architectural disruption occurs. Note that cirrhosis is a morphological definition, and also that regeneration, while a typical feature of the nodules described, is not required for the diagnosis of cirrhosis. Although the separation of cirrhosis into macronodular (>3 mm nodules) and micronodular (<3 mm) forms is not part of scoring schemes, micronodular may convert into macronodular, particularly during fibrosis regression [221, 222].

Vascular changes, in particular the formation of shunts between afferent and efferent vessels, are an essential feature of cirrhosis and are responsible for portal hypertension and end-stage clinical consequences such as ascites and variceal bleeding [223–225]. In addition to shunting and the persistent capillarization of the sinusoids, vascular alterations associated with cirrhosis include thrombosis (of small and large vessels, both hepatic and portal systems) and tissue underperfusion and hypoxia [226, 227]. The etiology of these vascular changes is multifactorial: the fibrotic liver is rich in proangiogenic factors such as VEGF and PDGF, there is significant tissue hypoxia that stimulates angiogenesis, and hepatic stellate cells function as liver-specific pericytes and may have direct proangiogenic activities [223, 228–231]. Although hepatocytes in nodules in the cirrhotic liver demonstrate a loss of the normal metabolic organization and zonation, the overall dysfunction of the liver is likely secondary to vascular shunting and to the impaired perfusion and solute exchange characteristic of cirrhosis [232, 233].

Matrix Degradation

Matrix protein deposition and breakdown are features of normal liver homeostasis, but are in balance such that total matrix content remains stable. In ongoing fibrosis this balance is disrupted resulting in net matrix deposition. Matrix degrading activity may actually be *increased* in the diseased liver, but does not keep pace with the increase in matrix deposition.

Matrix degradation occurs in two contexts in liver injury: as part of the disruption of the normal architecture, and during regression and remodeling of established fibrosis (see section below). Matrix proteins are highly protease-resistant, and those in the fibrotic liver are often heavily cross-linked and even more resistant to degradation; thus, matrix breakdown requires a specialized family of more than 25 proteases, the MMPs. The MMPs can be divided into five subgroups, although there is significant functional overlap. The interstitial collagenases (MMP-1, 8, and 13) are the only proteases able to degrade native type I and type III collagen in the triple-helical domains. The gelatinases (MMP-2 and -9) degrade type IV collagen, laminin, and fibronectin, as well as fibrillar collagens that have been partially unfolded by interstitial collagenases. Stromelysins (MMP-3, -7, -10, -11) degrade a variety of collagens including denatured fibrillar collagens in addition to proteoglycans and fibronectins. The metalloelastase (MMP-12) degrades elastin, although it also has proteolytic activity against fibronectin and proteoglycans. Members of the fifth family, the membrane-type MMPs (MT-MMPs), are particularly important as activators of other MMPs, most notably MMP-2 [234].

The expression and proteolytic activation of the MMPs is complex and highly regulated in order to prevent inappropriate proteolysis and target activity to suitable locations. TGF-β and other growth factors, other MMPs, plasmin (itself regulated by uroplasminogen activator and plasminogen activator inhibitor 1), and interactions with integrins and the matrix itself are important determinants of MMP expression and

activation [235]. The tissue inhibitors of metalloproteinases (TIMPs), which bind reversibly and noncovalently to the active sites of MMPs, are among the most important regulators of MMP activity and the net degree of matrix degradation is a function of TIMP levels and the concentration of activated MMPs. TIMPs 1 and 2, which are produced primarily by hepatic stellate cells, are highly upregulated in fibrosis [236–239]. Notably, mice overexpressing TIMP 1 have increased fibrosis in response to CCl_4 treatment [240].

The disruption of the normal low-density subendothelial matrix is an important early event in fibrosis, facilitating capillarization of the sinusoids and hepatic stellate cell migration. MMP-2 (also referred to as gelatinase A) is increased in parallel with the development of fibrosis and may be particularly important [236, 241]; other MMPs that increase during this phase include MT1-MMP (which activates MMP-2), MMP-9 which like MMP-2 degrades type IV collagen, and MMP-3, which degrades proteoglycans, laminin, and fibronectin in the space of Disse [242].

Regression

Regression of fibrosis and cirrhosis is a controversial topic. Part of the issue stems from terminology, and the failure of some investigators to distinguish between resolution (or reversion), defined as a return to the normal state, and regression, defined as a decrease in matrix content [223, 243]. Although the occurrence of resolution remains debatable, several pivotal studies from the older as well as more recent literature have now clearly demonstrated that significant *regression* of fibrosis occurs in animal models and in human livers, including in such varied human liver diseases as viral hepatitis, alcoholic liver disease, Wilson's disease, hemochromatosis, autoimmune liver disease, NAFLD, and biliary obstruction [222, 226, 243–247].

Cirrhosis regression is more complicated. While regression of fibrosis does occur in the cirrhotic liver, the vascular manifestations of cirrhosis, especially shunting, appear to persist even after significant decreases in liver matrix content [222–224]. Reversing the vascular changes of cirrhosis will require more than antifibrotics, in particular antiangiogenic agents [229, 248, 249]. While a "point-of-no-return" along the spectrum of fibrosis and cirrhosis has not been defined, it may occur in the later stages of cirrhosis and reflect the characteristics of local cell populations, the matrix, and particularly the vasculature.

Regression of fibrosis requires the loss of fibrogenic cells (generally via apoptosis) as well as matrix degradation and architectural remodeling. Mice expressing a collagenase-resistant form of type I collagen had decreased resolution, underscoring the importance of matrix degradation [250]. Differences in the nature of fibrosis may determine how readily matrix is degraded and therefore the likelihood of regression: perisinusoidal and biliary fibrosis regress more readily than septal fibrosis, thin reticular fibers are more easily degraded than thick septae, and a cellular scar, with the potential for MMP secretion, is more likely to regress than the acellular scars of cirrhosis [28, 245]. Additionally, scars with heavily cross-linked collagen and elastin are typically protease-resistant [28]. Matrix degradation requires protease activity, and as would be predicted, TIMP levels decrease and MMP activity increases during regression [246]. Scar-associated macrophages produce MMP-13 in rodent liver, and production of MMPs is likely to explain the requirement for liver macrophages during regression [251, 252].

The number of fibrogenic cells in the liver decreases as fibrosis regresses. This most likely occurs by apoptosis rather than myofibroblast dedifferentiation into nonfibrogenic cells. There is now an extensive body of literature on regulators of liver myofibroblast survival, which include the transcription factors NF-κB and C/EBP-β, natural killer cells, and intact type I collagen [226]. Whether apoptosis plays a causal or bystander role in regression, however, has not been established. Compounds such as gliotoxin which upregulate apoptosis have been shown to enhance regression, but may have relevant side effects on other cells and so such studies do not definitively establish causality [253, 254]. Myofibroblastic human hepatic stellate cells in culture overexpress Bcl-2 and are resistant to apoptosis, calling into question the relevance of apoptosis in human disease [255].

Clinical Features of Fibrosis and Cirrhosis

Fibrosis per se is asymptomatic and its major clinical importance is the potential for progression to cirrhosis. Cirrhosis, in particular the development of portal hypertension, is responsible for the clinical manifestations of chronic liver disease, although it is heterogeneous both histologically and clinically, and one of the flaws of current histological scoring systems is that cirrhosis is represented as one or at most two stages. Clinically, patients with cirrhosis may be asymptomatic or near death. Complications of cirrhosis include portal hypertension, with the development of ascites, varices, and hepatic encephalopathy. Protein synthetic function is decreased, with abnormal clotting. Jaundice and thrombocytopenia are common in late stages, as is the hepatorenal syndrome. Death typically results from progressive liver failure, from complications of portal hypertension (bleeding), or from hepatocellular carcinoma.

Whether or not a patient is symptomatic from cirrhosis depends largely on the HVPG. A normal HVPG is <5, while any HVPG >5 is associated with significant fibrosis. Multiple

Table 30.2 Child-Pugh score

Number of points	1	2	3
Total bilirubin (mg/dl)	<2	2–3	>3
Albumin (g/dl)	>3.5	2.8–3.5	<2.8
INR	<1.7	1.7–2.2	>2.2
Ascites	None	Mild, well-controlled	Moderate/severe, poorly controlled
Encephalopathy	None	Grade I–II, well-controlled	Grade III–IV, poorly controlled

Classification: Grade A 5–6 points; Grade B 7–9 points; Grade C 10–15 points

studies have now made clear, however, that patients do not develop complications of cirrhosis (ascites, varices) until the HVPG is >10, and that HVPG is an accurate predictor of clinical decompensation [256]. Noninvasive tests to categorize cirrhotics include the Child-Pugh score (also called the Child-Turcotte-Pugh score; Table 30.2). It was originally developed as a means to predict survival during surgery, but is now used to predict the clinical course of patients with cirrhosis [257, 258]. The score is determined from serum bilirubin, serum albumin, prothrombin time or INR, the degree of ascites, and the degree of hepatic encephalopathy, and assigns patients to categories A, B, or C, representing well-compensated, severely functionally impaired, and decompensated liver disease, respectively. Patients with Childs A cirrhosis have an excellent 1 year survival, while patients with Childs C have a 1 year survival of <50% with conservative treatment [259]. Once patients have decompensated, the model of end-stage liver disease (MELD) score, an open-ended scoring system with no upper limit, is a more accurate way to predict 6-month mortality.[1] The MELD, which is based on INR, serum bilirubin, and creatinine, is also widely used to prioritize patients on liver transplant waiting lists [261, 262].

Fibrosis and Cirrhosis in the Future

Basic researchers studying the pathophysiology of fibrosis are developing more and more sophisticated models integrating soluble mediators and mechanical changes with an understanding of the immune response and the direct and indirect roles of different cell populations in fibrosis and regression. The promise of basic science in the development of therapeutic targets in fibrosis and cirrhosis may therefore soon be realized.

The best therapy available to afflicted patients is the removal of the primary insult, whether it is alcohol, viral infection, or metal overload. There are no antifibrotics currently approved, although several potential agents will soon be tested in clinical trials. As trials become more of a reality, however, improved means to assess progression and regression (and by extension fibrogenesis and fibrolysis), especially noninvasively, are urgently needed. From the perspective of individual patients, the major issue is the development of cirrhosis, and thus better tests, including genetic tests, to identify and categorize patients at risk for progression will, with the development of antifibrotics, be a major advance in the future.

[1]MELD score (rounded to the nearest whole number), with United Network for Organ Sharing modifications, for patients age 12 and older: [260]
3.78[Ln serum bilirubin (mg/dl)] + 11.2[Ln INR] + 9.57[Ln serum creatinine (mg/dl)] + 6.43.
(Modifications: if patient has been dialyzed twice within previous 7 days, serum creatinine is set at 4.0 mg/dl; any value below 1.0 is rounded up to 1.0.)

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Chapter 31
Biliary Cirrhosis

Jonathan A. Dranoff

Introduction

Cholangiopathies are defined as pathologic conditions in which bile ducts are primary targets of disease. Together, these conditions account for approximately of one fifth adult liver transplants and the majority of pediatric liver transplants worldwide [1, 2]. While there are multiple individual conditions that make up the cholangiopathic spectrum, the end-stage of disease in each of these is biliary cirrhosis.

Biliary cirrhosis has two defining pathological characteristics. The first of these is development of fibrous scar that originates in the peribiliary zone and ultimately extends from portal area to portal area within the liver [3]. The second of these is dysregulated proliferation of bile duct epithelia (BDE) [4], in most cases resulting in marked upregulation in the number of intrahepatic bile ducts. While BDE have been identified and characterized to a large extent in the past 2 decades [5] (see Chap. 4), the identities of fibrogenic cells within the portal area have been more elusive until recent years. However, recent studies from our laboratory and others have led to the identification, isolation, and characterization of portal fibroblasts (PF) and portal myofibroblasts (PMF) [6–11]. Evidence suggests that these cell populations mediate fibrogenesis in biliary cirrhosis.

Although much research into the pathogenesis of liver fibrosis/cirrhosis has treated biliary and nonbiliary cirrhosis as similar, if not identical, entities, this is not the case. It is now becoming clear that biliary cirrhosis is mediated by BDE and PF/myofibroblasts [12–14] (for the sake of simplicity, the abbreviation PF will be used for both), whereas nonbiliary cirrhosis is mediated largely by hepatic stellate cells (HSC) and inflammatory cells, among others [15, 16]. Thus, recent investigations have focused on identifying and understanding phenotypic changes in BDE and PF in biliary cirrhosis [17–21]. Of particular interest to our group are alterations in functional interactions between these two cell types, which we believe are integral to the pathogenesis of biliary cirrhosis. We believe that these heterotypic cell–cell interactions also shed light on epithelial–mesenchymal interactions in organs and tissues outside of the liver, providing novel models for their study.

This chapter will focus on the elucidating relevant signaling pathways in BDE and PF, demonstrating evidence of functional interactions between the two liver cell types, and speculating upon potential new areas of study to advance the study of biliary cirrhosis in upcoming years.

Historical Perspectives

It has long been established that intra- and extrahepatic bile ducts are lined by flattened columnar epithelia ultrastructurally distinct from hepatocytes. Although these cells were initially regarded as passive lining of duct interiors, important advances beginning in the 1980s defined BDE as dynamic contributors to bile secretion [22, 23]. Since then, a variety of stimuli inducing bile ductular secretion were defined [24–26]. In parallel, it has been demonstrated that BDE are the specific cellular targets of certain cholestatic liver diseases. These cholangiopathies include such diverse processes as primary biliary cirrhosis (PBC) (see Chap. 49), primary sclerosing cholangitis (PSC) (see Chap. 50), biliary atresia (see Chap. 51), and cystic fibrosis hepatopathy [27, 28], which all progress to biliary cirrhosis if untreated. Excellent reviews in recent years have highlighted the critical advances made in the understanding of BDE function in health and disease [4, 5].

While the existence of potentially fibrogenic cells within the liver has probably been documented for over 100 years, the idea of distinct fibrogenic cell populations within the portal area was initially established in the early 1960s. Comprehensive electron microscopy studies indicated that the portal area contained fibroblasts with membranous processes that extended to submicron distances from BDE basolateral membranes, “dilated” endoplasmic reticula, and prominent ribosomes [29]. Proliferation of these cells and

J.A. Dranoff (✉)
Department of Internal Medicine/Digestive Diseases and Yale Liver Center, Yale University School of Medicine, New Haven, CT, USA
e-mail: jonathan.dranoff@yale.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_31, © Springer Science+Business Media, LLC 2011

expansion of scar were noted in the regions in which bile ductular proliferation took place in biliary cirrhosis, suggesting that these cells may be involved in both processes [30]. However, the terms "portal fibroblast" and "portal myofibroblast" are not old; they were not introduced in the mid-1980s [8]. While the study of HSC increased markedly after initial descriptions of their isolation [31, 32], PF/PMF have not undergone such extensive study. In fact, despite strong evidence that PF are distinct from HSC [33–35], there are many investigators who remain unconvinced that PF are truly a separate population of liver cells. However, at least two groups have described the isolation of relatively pure preparations of PF/PMF [7, 19]; isolated PF express distinct markers and respond to distinct stimuli from HSC [7, 36]. A recent review further attempts to characterize and define the nature of PF as distinct cells [37].

Evidence That BDE Mediate Biliary Cirrhosis

As noted above, the evolution of the concept that BDE are active participants in bile secretion has heightened interest in the study of these cells. In recent years, a new concept has arisen – the idea that BDE release inflammatory mediators in disease states. The inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1)/CCL2 is implicated in a variety of fibrotic conditions [38–40], yet is of particular interest in biliary fibrosis/cirrhosis. MCP-1 is expressed by BDE and upregulated in cirrhosis [41]; moreover, BDE MCP-1 expression is upregulated in PBC [42], biliary atresia [43], and cystic fibrosis hepatopathy [43]. BDE also release the proinflammatory cytokine interleukin-6 (IL-6), established as an important regulator of bile ductular proliferation [44, 45]. Although release of IL-6 by BDE is low in the normal liver, cultured human BDE markedly upregulate release of IL-6 in respond to lipopolysaccharide [46]. More importantly, bile ductular release of IL-6 is upregulated in response to biliary obstruction [45, 47], and IL-6 transcripts in BDE were detected in patients with cholangiopathies including PBC [48]. Thus, BDE can release inflammatory mediators in the setting of cholangiopathic injury, providing a rationale for the study of these processes as participants in the pathogenesis of biliary cirrhosis.

Evidence That Portal Fibroblasts/ Myofibroblasts Mediate Biliary Cirrhosis

Several lines of evidence have supported the concept that PF/PMF are important mediators of biliary cirrhosis. First, marked upregulation in proliferation immediately after common bile duct ligation is noted in both BDE (as has been noted on multiple occasions) and in PF [10]. Furthermore, proliferating PF rapidly acquire α-smooth muscle actin (α-SMA) stress fibers, indicative of myofibroblastic differentiation [10]. Second, isolated PF undergo myofibroblastic differentiation in response to signals associated with nonbiliary liver fibrosis, including platelet-derived growth factor (PDGF) [19] and MCP-1 [49]. On the other hand, transforming growth factor-β downregulates proliferation of PF but not HSC [36], and tumor necrosis factor-α has no clear effects on PF proliferation or fibrogenesis (unpublished observations), providing functional evidence that PF and HSC are distinct fibrogenic cell types. Finally, biopsy specimens from cirrhotic patients demonstrate that myofibroblastic PF produce Type I and Type IV collagen [33], demonstrating that these cells are not just potentially fibrogenic, but are actually an in vivo source of scar formation.

Secretion of Cytokines and Regulation of Portal Fibroblasts by Bile Duct Epithelia

The above findings lead to an important question – what induces the transcriptional downregulation of NTPD2 as PF undergo myofibroblastic differentiation? Since BDE and PF are so closely linked in space, it is possible that BDE secrete substances that may regulate PF differentiation and/or expression of NTPD2.

Multiple "activators" of PF to PMF have been identified. PDGF upregulates expression of α-SMA by PF, and PDGF inhibition blocks peribiliary expression of α-SMA in bile duct-ligated rats [19]. However, the effects of PDGF on NTPD2 expression have not been determined. Since PDGF blockade does not affect bile ductular proliferation after bile duct ligation, we would predict that PDGF does not alter NTPD2 expression. Similarly, BDE upregulate expression of connective tissue growth factor (CTGF) in rat liver fibrosis induced by CCl_4 [50]; however, the effects of CTGF on PF are not known. Interestingly, CTGF is upregulated in explant specimens in patients with biliary atresia; however, the source of CTGF is thought to be HSC and hepatocytes [51].

As noted above, several lines of evidence suggest that MCP-1 may be important in the regulation of PF as fibrogenic cells by BDE. MCP-1 is markedly upregulated in regenerating BDE in cirrhotic patients, suggesting that BDE may secrete MCP-1 as a paracrine mediator [41]. MCP-1 induces multiple changes in PF, including upregulation and redistribution of α-SMA, downregulation of NTPD2, and upregulation of α(1)-procollagen synthesis (discussed in greater detail below) [49]. Increased MCP-1 expression in BDE is associated with liver fibrosis progression and liver failure in patients with biliary atresia [52],

again suggesting a profibrogenic role. Interestingly, MCP-1 appears to have potent profibrogenic roles outside of the liver as well [53, 54].

Finally, new evidence suggests that IL-6 may be important in the regulation of PF by BDE. As noted above, IL-6 expression is upregulated in biliary cirrhosis, and BDE can synthesize IL-6 [46]. Moreover, BDE synthesis of IL-6 is upregulated in biliary cirrhosis [55]. Finally, PF express IL-6 receptors, and IL-6 downregulates transcription of NTPD2 by PF [56]. However, IL-6 does not induce myofibroblastic differentiation of PF, suggesting that distinct pathways regulate myofibroblastic differentiation and NTPD2 transcription. Interestingly, mice deficient in IL-6 exhibit greater fibrosis after bile duct ligation [45], suggesting that IL-6 may have a protective role in biliary cirrhosis.

Portal Fibroblasts as Regulators of Purinergic Signaling in Bile Duct Epithelia

Extracellular adenosine triphosphate (ATP) and other nucleotides act as cell signaling molecules via specific plasma membrane receptors, inaptly titled "purinergic receptors" [57–59]. The term purinergic receptor is chosen poorly, since we now know that this class of receptors also includes receptors for nucleotides based on uridine, which is a pyrimidine [60]. Still, purinergic receptor remains the term in common scientific use, since "purinergic/pyrimidinergic receptor" is ridiculously complicated. Purinergic receptors are divided as follows: P1 receptors are receptors for nucleosides, with adenosine as the most important endogenous ligand, whereas P2 receptors are receptors for diphosphate or triphosphate nucleotides [59]. P2 receptors are further divided into ligand-gated ion channels known as P2X receptors and G protein-coupled receptors known as P2Y receptors. P2X receptors have important functions in transmission of afferent nerve impulses [61] and immune function [62], but recent studies suggest a role in bile ductular secretion [63]. P2Y receptors are widely expressed in epithelial and nonepithelial cells and have been shown to be important regulators of such diverse activities as fluid/electrolyte secretion, platelet aggregation, and chemotaxis, among many others [64].

Until recently, P2Y receptors were thought to be of primary importance in BDE as regulators of fluid and electrolyte secretion. P2Y receptors link to apical chloride secretion via apical BDE P2Y receptors [65]. P2Y-mediated secretion is a critical regulator of bile ductular cell volume autoregulation, and thus is of great importance in cell homeostasis. However, P2Y receptors are also expressed at the basolateral aspect of BDE. The basolateral bile ductular P2Y receptors are distinct in two ways: they are not as tightly linked to fluid/electrolyte secretion and they are tonically inactivated by hydrolysis of ATP and other nucleotides [65, 66]. The hydrolysis of nucleotides at the basolateral aspect of BDE is mediated by expression of the ecto-nucleotidase nucleoside triphosphate diphosphohydrolase-2 (NTPD2, also known as CD39L1) [67]. NTPD2 is expressed not by BDE, but by neighboring PF, and specifically on membranous projections extending from PF to less than 1 μ(mu)m from the basolateral aspects of bile ducts [67].

We investigated the role of basolateral BDE P2Y receptors as regulators of bile ductular proliferation for several reasons. First, the importance of P2Y receptors as regulators of cell proliferation is increasingly appreciated. P2Y receptors regulate the growth of such diverse cell types as renal mesangial cells[68], endothelia [69], smooth muscle cells [70], and epithelia [71]. In fact, changes in purinergic stimulation of epithelial proliferation have been proposed in the pathogenesis of polycystic kidney diseases [72]. Second, NTPD2 is expressed by PF, but is transcriptionally downregulated as PF undergo myofibroblastic differentiation into PMF [73]. The downregulation of NTPD2 expression in PF is specific to biliary cirrhosis, since it occurs in common bile duct-ligated, but not CCl_4-treated rats [73]. Moreover, patients with cirrhosis due to PBC or PSC, but not those with cirrhosis due to hepatitis C or cardiac cirrhosis, have a similar marked downregulation in NTPD2 expression to the levels seen in rats with biliary cirrhosis [73] (Fig. 31.1). Thus, NTPD2 was proposed as a critical link between biliary fibrosis and bile ductular proliferation.

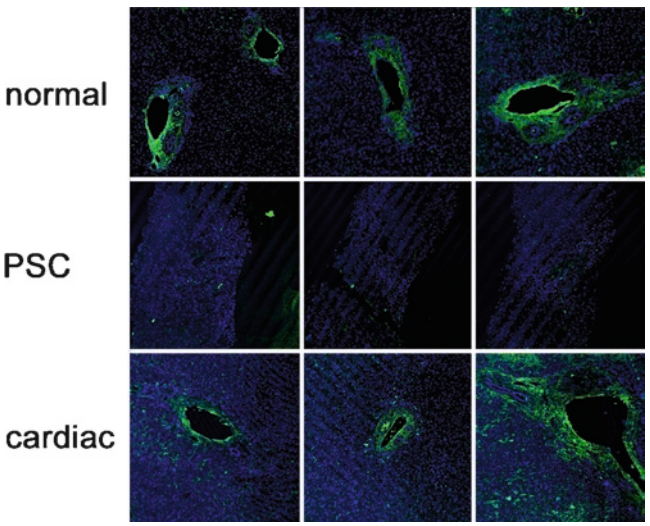

Fig. 31.1 Selective loss of NTPD2 expression in human liver with biliary cirrhosis. Discarded human liver biopsy specimens were obtained from tissue adjacent to liver tumors (normal liver) and explants or autopsy specimens from patients with cirrhosis due to PSC or cardiac cirrhosis and stained for NTPD2 (*green*). Nuclei are *pseudocolored blue*. Images were obtained using confocal microscopy and are seen at 20×. NTPD2 expression is almost completely absent in PSC specimens, but remains present in specimens from cardiac cirrhosis, a form of non-biliary cirrhosis

This hypothesis was tested using a coculture model, in which BDE were cultured with PF, and changes in BDE proliferation were assessed. Coculture of BDE with PF markedly downregulated BDE proliferation; however, when PF NTPD2 expression was attenuated using siRNA transfection, PF had no effect on BDE proliferation [18]. This suggested that PF regulate bile ductular growth in the normal liver via expression of NTPD2. Coculture of BDE with PMF isolated from rats that had undergone bile duct ligation and thus lacked NTPD2 expression had no effect on BDE proliferation; however, when expression of NTPD2 was upregulated in PMF by plasmid transfection, PF downregulated BDE proliferation to the same extent as noted in PF from normal rats. This suggested that PMF lose the ability to regulate bile ductular growth in the biliary cirrhotic liver due to loss of NTPD2. In essence, hydrolysis of extracellular nucleotides by NTPD2 provides a potentially important mechanism for control of bile ductular proliferation, and loss of this control mechanism may be critical in the dysregulation of bile ductular proliferation in biliary cirrhosis.

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The above findings lead to an important question – what induces the transcriptional downregulation of NTPD2 as PF undergo myofibroblastic differentiation? Since BDE and PF are so closely linked in space, it is possible that BDE secrete substances that may regulate PF differentiation and/or expression of NTPD2.

Multiple "activators" of PF to PMF have been identified. PDGF upregulates expression of α-SMA by PF, and PDGF inhibition blocks peribiliary expression of α-SMA in bile duct-ligated rats [19]. However, the effects of PDGF on NTPD2 expression have not been determined. Since PDGF blockade does not affect bile ductular proliferation after bile duct ligation, we would predict that PDGF does not alter NTPD2 expression. Similarly, BDE upregulate expression of CTGF in rat liver fibrosis induced by CCl_4 [50]; however, the effects of CTGF on PF are not known. Interestingly, CTGF is upregulated in explant specimens in patients with biliary atresia; however, the source of CTGF is thought to be HSC and hepatocytes [51].

We directly tested the hypothesis that release of MCP-1 by BDE induces myofibroblastic differentiation of PF [49]. Initial experiments demonstrated that isolated rat PF [7] expressed a functional MCP-1 receptor. MCP-1 induced pertussis toxin-sensitive increases in cytosolic calcium in PF; however, PF did not express the cognate MCP-1 receptor CCR2, suggesting an alternative MCP-1 receptor in these cells. It is noteworthy that HSC also express an MCP-1 receptor distinct from CCR2 [74]. MCP-1 induced several important functional changes in PF, including myofibroblastic differentiation, upregulation of procollagen-1 transcription, and suppression of NTPD2 transcription. To demonstrate directly that bile ductular release of MCP-1 mediated these responses, BDE were isolated from rats that had undergone bile duct ligation and cultured overnight. The media from these cells was collected, and PF were treated with this media in the presence or absence of MCP-1 blocking antibody. Myofibroblastic differentiation of PF induced by BDE media was inhibited by MCP-1 blocking antibody, suggesting that BDE are a potential in vivo source of MCP-1 as a regulator of myofibroblastic differentiation and fibrogenesis mediated by PF.

We have also investigated functional interactions between BDE and PF at the level of IL-6 signaling. Initial experiments focused on the effect of IL-6 on isolated rat PF in vitro [75]. Unlike MCP-1, IL-6 had no effect on PF myofibroblastic differentiation or fibrogenesis. Rather, IL-6 specifically and potently downregulated transcription of NTPD2 by PF. We cloned the NTPD2 promoter and identified three distinct IL-6 response elements, each of which was independently sufficient to mediate IL-6-dependent downregulation of NTPD2 transcription. Since NTPD2 is a critical regulator of purinergic signaling in BDE [18, 67], and purinergic receptors regulate IL-6 release in cells outside of the liver [76, 77], the effects of purinergic signals on BDE IL-6 release were then investigated [78]. A variety of functional and molecular experiments demonstrated that extracellular ATP, likely working through the $P2Y_{11}$ receptor [79, 80] in a manner dependent on cyotosolic calcium and cyclic AMP, regulated IL-6 release by BDE at the transcriptional level. Mutagenesis studies were used to demonstrate that the IL-6 promoter calcium/cyclic AMP response element is critical to this regulation. Since IL-6 is critical for survival of the liver in injury via preservation of hepatic epithelia [45, 81], the crosstalk loop regulating IL-6 and NTPD2 expression in BDE and PF is likely to be important in organ protection in biliary cirrhosis. However, since the "ductular reaction" is associated with poor prognosis in liver disease [82, 83], this loop may act as a double-edged sword.

Lingering Questions and Future Directions

BDE and PF are linked in a bidirectional crosstalk signaling loop. PF regulate the proliferation of BDE via expression of NTPD2, and BDE regulate myofibroblastic differentiation and NTPD2 expression in PF via release of a variety of paracrine mediators, including MCP-1 and IL-6 (Fig. 31.2). However, what has not been established is the origin of the loop. That is, an important question remains as to which is

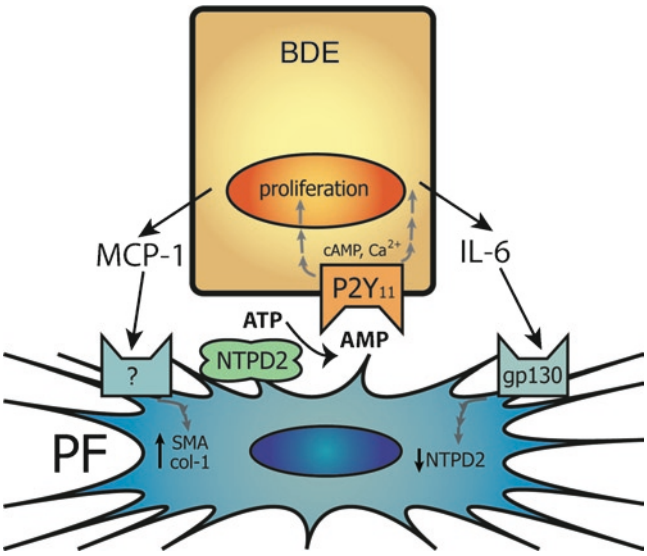

Fig. 31.2 Working model for roles of NTPD2, MCP-1, and IL-6 in BDE-PF crosstalk signaling. The proposed model shows that NTPD2 regulates activation of the BDE $P2Y_{11}$ receptor via hydrolysis of ATP to inactive metabolites. When activated, $P2Y_{11}$, acting via cAMP and cytosolic Ca^{2+} signals, triggers BDE proliferation and release of IL-6. Release of IL-6 signals to PF via the gp130 receptor and results in transcriptional downregulation of NTPD2. BDE also release MCP-1, which induces cytosolic Ca^{2+} signals and upregulates PF myofibroblastic differentiation and procollagen-1 (col-1) transcription in PF; however, the PF MCP-1 receptor is unknown

the cell sensor of injury: BDE, PF, or some other cell? Which cell is the initial detector of early signals in cholangiopathic disease that initiates the crosstalk pathway leading to the clinical manifestations of biliary cirrhosis.

It also remains to be shown whether the crosstalk signaling loop discussed here is generalizable to other tissues and organs. Other organs certainly have clear epithelial–mesenchymal interactions; however, identical signaling pathways linking proliferation and fibrosis have not yet been identified in these systems.

Finally, it will be interesting to discover whether changes in the BDE-PF signaling pathway will be important in liver development and in neoplasia. Development of the liver requires interactions between epithelia and mesenchymal tissues [84], and cholangiocarcinoma is associated with a potent desmoplastic reaction [85]. Future studies should certainly address the possibility that BDE-PF crosstalk is relevant to these critically important processes.

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Chapter 32
Cholestasis

Michael H. Trauner

Introduction

Secretion of bile is an important excretory route for a wide range of endogenous and exogenous compounds, also known as endobiotics (e.g., bile acids, bilirubin, cholesterol, phospholipids) and xenobiotics (e.g., drugs and their metabolites), which may become toxic when accumulating in the liver [1–3]. Bile acids, the major component of bile, are not only essential for the digestion and absorption of lipids from the intestinal lumen, but also have multiple endocrine functions as regulators of hepatic glucose and lipid metabolism, liver regeneration, inflammation, and intestinal bacterial flora [1]. Cholestasis is an impairment of bile secretion which is typically characterized by reduced bile flow and retention of biliary constituents (normally secreted into bile) in blood, liver, as well as extrahepatic organs and tissues [3]. Histopathologically, cholestasis is characterized by bilirubinostasis with bile plugs and cholate-stasis with feathery degeneration of (mainly periportal) hepatocytes [4]. An excellent, generally applicable definition of cholestasis, irrespective of the cause and etiology, has been coined by Serge Erlinger describing this condition as "failure of bile to reach the duodenum in sufficient amounts" [5].

Cholestasis may either result from a functional defect in bile formation at the level of hepatocytes or from impairment in bile secretion and flow at the level of small or large bile ducts (Fig. 32.1) [3–5]. Pathophysiologically and clinically, hepatocellular cholestasis due to "bland" noninflammatory injury (e.g., drugs, hormones, genetic defects) or inflammatory injury (e.g., idiosyncratic drug reactions, alcohol, sepsis) can be distinguished [3–5]. Destruction and disappearance of microscopically small interlobular bile ducts results in a vanishing bile duct syndrome with chronic cholestasis (e.g., primary biliary cirrhosis [PBC]), while mechanical obstruction of large, macroscopically visible bile ducts causes obstructive cholestasis (e.g., gallstones, tumors, primary sclerosing cholangitis [PSC]) [3–5].

Recently, major advances have been made in the molecular identification of membrane transport systems and the regulatory nuclear receptors that control bile formation [2, 6]. Exposure to cholestatic injury (e.g., drugs, hormones, proinflammatory cytokines, biliary obstruction/destruction) or hereditary mutations in transport systems or the combination of both results in reduced expression and function in hepatobiliary transport proteins [2, 3, 6, 7]. In addition to genetic or acquired transporter changes, other mechanisms such as altered-cell polarity, disruption of cell–cell junctions, and cytoskeletal changes may be involved [3, 4]. The recent advances in our understanding of the immunopathogenesis of PBC and PSC have been discussed in separate chapters and also reviewed elsewhere [8, 9]. These molecular advances in our understanding of the molecular pathogenesis of cholestasis may also have major implications for diagnosing and predicting prognosis in cholestatic liver diseases. Moreover, pharmacological interventions in cholestasis may aim at restoring disturbed or stimulating adaptive pathways [6].

Molecular Principles of Bile Formation as Basis for Understanding Cholestasis

The liver comprises a broad range of specific uptake and export systems for various biliary compounds, which are localized to the basolateral (sinusoidal) and canalicular (apical) membrane of hepatocytes (Fig. 32.2) [2, 6]. Bile is primarily formed by canalicular excretion of bile acids and nonbile acid organic anions via ATP-binding cassette (ABC) transporters. These osmotically active compounds induce passive movement of water through the tight junctions [2, 6]. Bile acids are the main solutes in bile and are considered to be the major osmotic driving force in the generation of bile flow ("bile acid-dependent bile flow") [1, 2]. Bile acids then in turn facilitate canalicular phospholipid and cholesterol secretion and form mixed-biliary micelles. Bile acid-independent

M.H. Trauner (✉)
Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria
e-mail: michael.trauner@medunigraz.at

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_32, © Springer Science+Business Media, LLC 2011

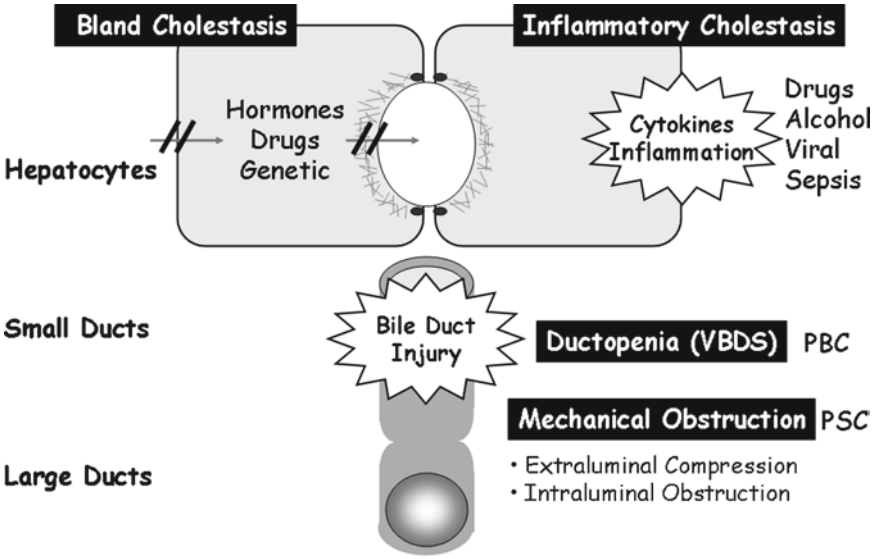

Fig. 32.1 Principal mechanisms and clinical spectrum of cholestasis. Cholestasis may result either from a functional defect in bile formation at the level of hepatocytes, or from an impairment in bile secretion and flow at the bile-duct level. Hepatocellular cholestasis may be noninflammatory (bland; e.g., drugs, hormones, genetic defects) or inflammatory (e.g., drugs, alcohol, sepsis). Destruction and disappearance of microscopically small interlobular bile ducts results in a vanishing bile duct syndrome with chronic cholestasis (e.g., primary biliary cirrhosis, PBC). Mechanical obstruction of large, macroscopically visible bile ducts may also cause cholestasis (e.g., gallstones, tumors, primary sclerosing cholangitis, PSC). Adapted from Trauner et al. [27]. Used with permission

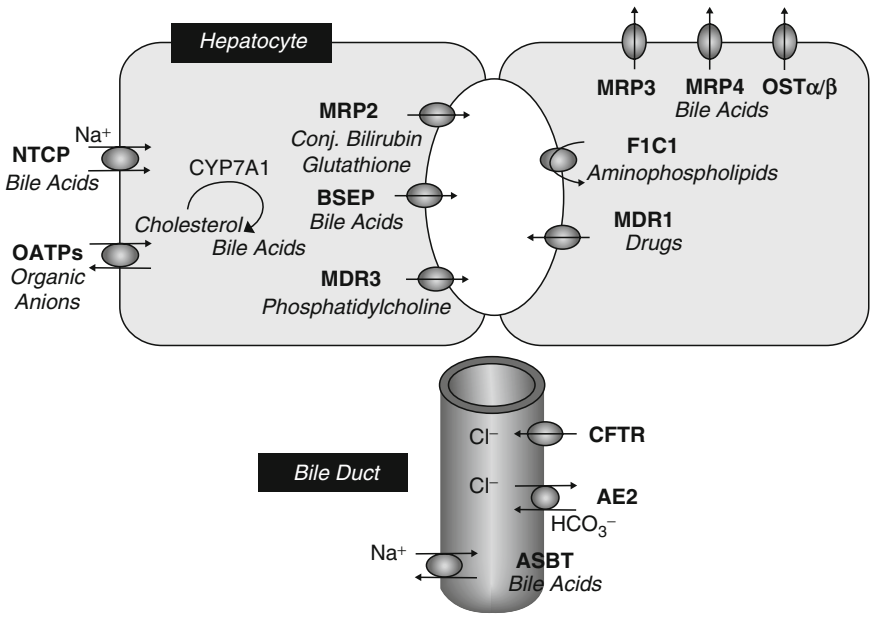

Fig. 32.2 Hepatobiliary transport systems in normal liver. Schematic representation of the most relevant transport systems involved in bile formation. Hepatocellular bile acids are either derived by de novo synthesis from cholesterol via the key enzyme CYP7A1 replacing daily bile acid loss via stool (3–5%) or via hepatocellular uptake from the sinusoidal blood containing bile acids undergoing enterohepatic circulation (*left panel*). Hepatocellular bile acid uptake from the sinusoidal blood is mediated by a high-affinity Na^+/taurocholate cotransporter (NTCP) and a familiy of multispecific organic anion transporters (OATPs). Canalicular excretion of bile constituents via specific ABC transporters represents the rate-limiting step of bile formation (*center panel*). The canalicular membrane contains a bile-salt export pump (BSEP) for monovalent bile acids; a conjugate export pump, MRP2 mediates excretion of various organic anions such as bilirubin and divalent bile acids. The phospholipid-export pump MDR3, flipps phosphatidylcholine that forms mixed micelles together with bile acids and cholesterol. Cationic drugs are excreted by the multidrug export pump MDR1. Moreover, the canalicular membrane contains a P-type ATPase, FIC1, which is a putative aminophospholipid transporter. At the basolateral membrane additional bile acid exports pumps, MRP3, MRP4, and the heterodimeric organic solute transporter OSTα/β are present as back up pumps for alternative sinusoidal bile acid export (*right panel*). Under normal conditions these transport systems are expressed at very low levels, but can be induced under cholestatic conditions or by therapeutic drugs. A chloride–bicarbonate anion exchanger isoform 2 (AE2) mediates biliary bicarbonate excretion in hepatocytes (not shown) and – to a greater extent – cholangiocytes. The cystic fibrosis transmembrane conductance regulator (CFTR) drives bicarbonate excretion by AE2 and is exclusively expressed in cholangiocytes. The biliary epithelium is also involved in the reabsorption of bile acids via an apical Na^+-dependent bile-salt transporter ASBT. Adapted from Wagner et al. [6]. Used with permission

processes also contribute to bile production ("bile acid-independent bile flow"), consisting mainly of the canalicular secretion of reduced glutathione and the excretion of bicarbonate. This canalicular primary bile is further modified by absorptive and secretory processes (mainly bicarbonate) along the biliary tree [2]. Bile acids undergo an enterohepatic circulation which consists of their continuous hepatocellular canalicular secretion, active reabsorption in the terminal ileum and hepatic basolateral reuptake [1]. As such, this bile salt pool circulates 6–10 times per day in humans [1, 2].

Hepatocellular bile acids are either derived from de novo synthesis from cholesterol via the key enzyme CYP7A1 replacing daily bile acid loss via stool (3–5%) or via hepatocellular uptake from the sinusoidal blood containing bile acids undergoing enterohepatic circulation [1, 2, 6]. Hepatocellular bile acid uptake from the sinusoidal blood is mediated by a high-affinity Na^+/taurocholate cotransporter (NTCP) and a familiy of multispecific organic anion transporters (OATPs) (Fig. 32.2) [2]. Canalicular excretion of bile constituents via specific ATP-binding cassette (ABC) transporters represents the rate-limiting step of bile formation [2, 7]. The canalicular membrane contains a bile-salt export pump (BSEP) for monovalent bile acids and a conjugate export pump (MRP2) which mediates excretion of various organic anions such as bilirubin and divalent bile acids. The phospholipid export pump (MDR3) flips phosphatidylcholine, which forms mixed micelles together with bile acids and cholesterol in the biliary lumen. Cationic drugs are excreted by the multidrug export pump (MDR1). Moreover, the canalicular membrane contains a P-type ATPase, FIC1, which is a putative aminophospholipid transporter maintaining the membrane asymmetry required for normal function of embedded enzymes/transporters and vesicle fusion [2, 6, 7]. At the basolateral membrane bile acid exports pumps, MRP3, MRP4, and the heterodimeric organic solute transporter OSTα/β are present as back up pumps for alternative sinusoidal bile-acid export (Fig. 32.2) [2, 6]. The biliary epithelium also actively participates in bile formation though secretory and reabsorptive processes [2]. A chloride–bicarbonate anion exchanger isoform 2 (AE2) mediates biliary–bicarbonate excretion in both hepatocytes, and to a greater extent in cholangiocytes. The cystic fibrosis transmembrane conductance regulator (CFTR) drives bicarbonate excretion by a chloride/bicarbonate anion exchanger 2 (AE2); in contrast to AE2, CFTR is exclusively expressed in cholangiocytes. The biliary epithelium is also involved in the reabsorption of bile acids via an apical Na^+-dependent bile-salt transporter (ASBT) [2, 6].

Coordinated regulation of bile-acid transport, metabolism, and synthesis is essential to maintain bile-acid homeostasis, since bile acids can be cytotoxic when reaching high, pathological concentrations [1, 3, 4]. At a transcriptional level, this is largely achieved through nuclear (hormone) receptors [2, 6, 10]. After binding of their ligands (e.g., bile acids, bilirubin), nuclear receptors undergo a conformational change and allow binding to their specific response elements in the promoters of target genes. To date, three major bile acid-activated receptors have been identified [10]: farnesoid X receptor (FXR) was the first bile-acid receptor to be identified and is activated by a variety of primary and secondary bile acids. Pregnane X receptor (PXR) and vitamin D receptor (VDR) are activated by hydrophobic lithocholic acid and by their natural ligands (xenobiotics including rifampicin, phenobarbital, dexamethasone and statins for PXR, and 1α,25-dihydroxyvitamin D_3 for VDR). The constitutive androstane receptor CAR is activated by xenobiotics and bilirubin, possibly also by bile acids. Thus, cholephiles such as bile acids or bilirubin can regulate their uptake, detoxification, and export by binding to specific nuclear receptors [2, 6, 10]. In addition to transcriptional mechanisms, posttranscriptional events affecting mRNA processing, steady-state mRNA stability, translational efficacy and/or posttranslational changes such as transporter targeting and sorting, transporter redistribution, transporter protein degradation (e.g., via lysosomal or ubiquitin-proteasome pathway), direct protein modifications (e.g., (de-) phosphorylation, (de-) glycosylation), changes in membrane fluidity or cis-/trans-inhibition of transport systems by cholestatic agents (e.g., drugs) also modulate transporter expression and function under normal and pathophysiological conditions [2, 3].

Genetic Causes of Cholestasis

Hereditary defects of hepatobiliary ABC transporters have been linked to a broad spectrum of hepatobiliary disorders ranging from progressive familial intrahepatic cholestasis (PFIC), benign recurrent intrahepatic cholestasis (BRIC) to intrahepatic cholestasis of pregnancy (ICP), drug-induced cholestasis, intrahepatic cholelithiasis, idiopathic adulthood ductopenia, and adult-biliary fibrosis/cirrhosis (Fig. 32.3, Table 32.1) [6, 7]. Generally, more severe (homozygous) defects cause progressive cholestatic syndromes in neonates and children, while milder defects may result in or predispose to cholestatic syndromes which manifest later in juvenile or even adult life stage (Fig. 32.3). Heterozygous transport defects may predispose to acquired cholestatic injury through decompensation of latent/mild defects under challenge with drugs, hormones, and/or inflammatory mediators. Therefore, hereditary transporter mutations may predetermine the individual susceptibility to cholestatic liver injury. In clinical reality, these different conditions represent part of a continuous spectrum [6, 7] rather than individual entities, with homozygous severe defects in neonates and infants being just the "tip of the iceberg" (Fig. 32.3).

Four subtypes (PFIC-1–4) of autosomal recessively inherited disorders in infants and children have been described

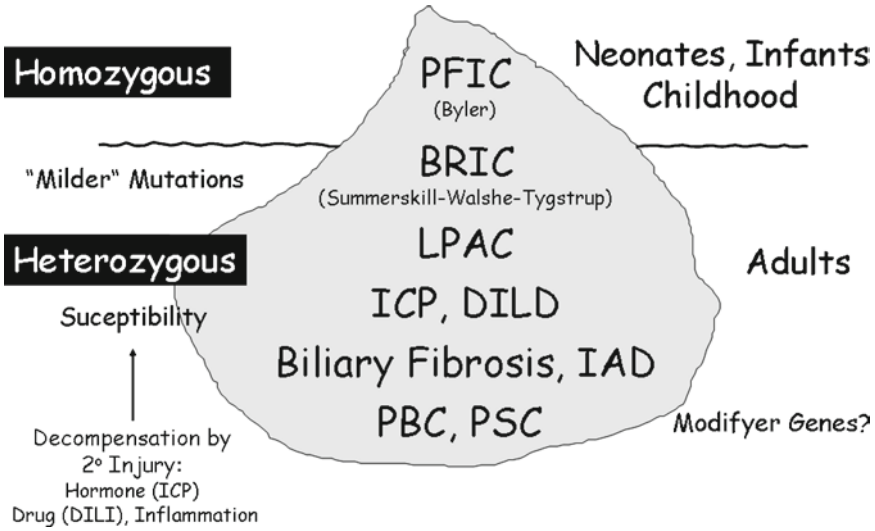

Fig. 32.3 Disease spectrum caused by hereditary transporter defects. Homozygous mutations of transporter genes as tip of the iceberg can result in autosomal recessively inherited syndromes of progressive familial intrahepatic cholestasis (PFIC) (mutations in *FIC1*, *BSEP,* or *MDR3* genes) in early childhood. "Milder defects" in *FIC1* or *BSEP* and MDR3 cause phenotypically milder benign recurrent intrahepatic cholestasis (BRIC) and low-phospholipid associated cholelithiasis (LPAC) in young adults. BRIC patients can progress to more aggressive disease indicating that PFIC and BRIC may belong to a continuous spectrum of pathophysiologically related conditions. Heterozygous *MDR3* and – to a lesser extent – *BSEP* mutations and polymorphisms may increase the susceptibility for acquired cholestatic injury such as intrahepatic cholestasis of pregnancy (ICP), drug induced liver injury (DILI), idiopathic adulthood ductopenia (IAD), biliary fibrosis, or may play a role as modifier genes in more "classic" cholangiopathies (e.g., primary biliary cirrhosis [PBC] and primary sclerosing cholangitis [PSC]). As such, a second hit/injury such as inflammation, drugs, or hormones may be required for fullblown disease manifestation

Table 32.1 Genetic defects causing hereditary cholestatic syndromes and associations with acquired cholestatic diseases (adapted from ref. [3])

Gene	Function	Hereditary cholestasis syndrome	Associated acquired cholestatic diseases (susceptibility gene)
FIC1 (*ATP8B1*)	Aminophospholipid flippase, maintaining phospholipid asymmetry of the canalicular membrane	Progressive familial intrahepatic cholestasis type 1 (PFIC-1, Byler disease)Benign recurrent intrahepatic cholestasis type 1 (BRIC-1, Summerskill syndrome)	Intrahepatic cholestasis of pregnancy
BSEP (*ABCB11*)	Canalicular bile-salt export pump	Progressive familial intrahepatic cholestasis type 2 (PFIC-2, Byler's syndrome)Benign recurrent intrahepatic cholestasis type 2 (BRIC-2)	Intrahepatic cholestasis of pregnancy-Drug-induced cholestasis Potential role for disease progression of primary biliary cirrhosis
MDR3 (*ABCB4*)	Canalicular phospholipid flippase	Progressive familial intrahepatic cholestasis type 3 (PFIC-3)	Intrahepatic cholestasis of pregnancy-Drug-induced cholestasis Low-phospholipid-associated cholelithiasis syndrome Adulthood idiopathic ductopenia and biliary fibrosis Nonanastamotic biliary strictures after liver transplantation Potential role for disease progression of primary biliary cirrhosis and primary sclersoing cholangitis
MRP2 (*ABCC2*)	Canalicular organic anion conjugate export pump	Dubin-Johnson syndrome	Intrahepatic cholestasis of pregnancy
CFTR (*ABCC7*)	Cholangiocyte apical cloride channel	Cystic fibrosis (sclersoing cholangitis as hepatic manifestation of cystic fibrosis)	Potential role for primary sclerosing cholangitis
Tight junction protein 2	Tight junction protein	Hypercholanemia	–
Claudin-1	Tight junction protein	Neonatal sclerosing cholangitis associated with ichthyosis	–

(Table 32.1) [6, 7]. PFIC-1 (also known as Byler disease) is caused by a mutation of the putative aminophospholipid transporter FIC1 and leads to the development of liver cirrhosis in early childhood [6, 7]. This disease is characterized by elevated serum bile acid and low gamma-glutamyl transpeptidase (GGT) levels. The exact pathomechanism of this disease is unknown, since the role for FIC1 in bile secretion is not yet entirely clear. FIC1 might directly contribute to the elimination of hydrophobic secondary bile acids such as toxic lithocholic acid or more likely, may help maintain membrane asymmetry and function of embedded transporters/enzymes as aminophospholipid flippase (Table 32.1). Benign recurrent intrahepatic cholestasis (BRIC-1, Summerskill syndrome) is also caused by mutations of FIC1 and is characterized by recurrent episodes of cholestasis not leading to liver cirrhosis [6, 7]. Both syndromes are associated with extrahepatic manifestations such as diarrhea, bile acid malabsorption, pancreatitis and nephrolithiasis, which can be explained by expression of FIC1 in these tissues. Mutations of the bile salt export pump (BSEP), the major canalicular bile acid export system, cause PFIC-2 [6, 7] (Table 32.1). The clinical course is similar to PFIC-1; however, extrahepatic manifestations are absent since BSEP expression is restricted to the liver. More moderate courses of this disorder cause a variant of benign intrahepatic recurrent cholestasis (BRIC-2). PFIC-3 presents with high levels of GGT and is caused by mutations of multidrug resistance protein MDR3, which is a phospholipid export pump [6, 7] (Table 32.1). Phospholipids in bile are required for the formation of mixed micelles with bile acids and cholesterol to protect the bile duct epithelia from the detergent properties of bile acids. Reduced or absence of phospholipid excretion into bile in PFIC-3 causes bile-duct injury in these patients. *MDR3* mutations causing a phospholipid-deficient, toxic bile have also been suggested to play a role in the pathogenesis of PSC, since mice with targeted deletion of this transporter develop bile-duct injury resembling PSC [11, 12]. Some forms of ICP are associated with *MDR3* mutations and the pathogenesis of cholesterol gallstones is also linked to MDR3 defects [6, 7, 13, 14]. Other transporters such as BSEP and the multidrug-associated protein MRP2 could also play a role in the pathogenesis of ICP, but this remains to be determined. A forth subtype (PFIC-4) is not a transporter defect but caused by hereditary bile-acid synthesis defects [15].

Mutations of *MRP2*, which encodes a gene for a canalicular (bilirubin-)conjugate export pump, cause Dubin-Johnson syndrome (Table 32.1) [6, 7]. Reduced biliary excretion of various endogenous and exogenous compounds (e.g., bilirubin, oral cholecystography agents) is associated with this disorder. Although these patients are only hyperbilirubinemic and not truly cholestatic, Dubin-Johnson Syndrome is yet another important example of how a mutation in a hepatocellular transporter gene can impair bile-excretory function. Other examples of hereditary transporter defects, not causing cholestasis, are mutations of the canalicular copper export pump ATP7B in Wilson's disease and mutations of the sitosterol transporter ABCG5/8 causing sitosterolemia [2, 3]. In addition to these hepatocellular transporter defects, mutations in transporter genes in cholangiocytes can also cause cholestasis (Table 32.1). As such, mutations in the *CFTR* gene, a cAMP-dependent chloride channel, may lead to cholestasis and liver disease, which is a frequent and early complication of cystic fibrosis. Recent data indicate, that mutations and variants of *CFTR* as well as reduced chloride secretion could play a role in the pathogenesis of PSC [2, 3, 6]. However, other studies could not confirm an association of disease-causing *CFTR* mutations with PSC [6].

In addition to transporter gene defects, variations in tight junction genes can also cause cholestasis [6]. As such, missense mutations in tight junction protein 2 have been identified in patients with familial hypercholanemia, an oligogenic disease requiring a mutation in a second gene (bile-acid amino-acid transferase) for full clinical manifestation [6]. Furthermore, mutations of claudin-1, which forms the backbone of tight junctions with occludin and junctional adhesion molecules, causes neonatal sclerosing cholangitis associated with ichthyosis (Table 32.1) [6].Mutations in *JAG-1*, which is involved in notch signaling required for bile duct development, have been identified in patients with Alagille syndrome, an autosomal dominant disorder, which results in cholestasis due to bile duct paucity [6]. Finally, genetic defects in nuclear receptors regulating gene expression of hepatobiliary transporters and enzymes may also be involved in cholestatic liver disease [6]. For example, the nuclear receptor *FXR* variants have been linked to ICP and gallstone disease, while *PXR/SXR* variants appear to modify disease course of PSC and PBC.

Acquired Changes in Hepatobiliary Transport and Metabolism in Cholestasis

Most of our knowledge on acquired alterations of transport systems during cholestasis comes from experimental animal models which mimic to some extent specific clinical conditions. These rodent models of cholestasis have proven very useful to study the role of transport systems in the pathogenesis of cholestasis [3, 6]. In addition to the *Mdr2* knockout mouse model displaying features of (P)SC (see above), examples include rodent models with application of endotoxin (experimental model for inflammatory cholestasis), ethinylestradiol (oral contraceptive-induced cholestasis/cholestasis of pregnancy), alpha-naphthylisocyanate (vanishing bile duct syndrome), and common bile duct ligation (extrahepatic biliary obstruction). While transporter changes in hereditary

cholestasis are primary (i.e., causative), most alterations in acquired cholestasis are secondary (i.e., consequences of retention of cholephiles). Rather, rarely, primary changes in transporter expression or function are the cause of cholestasis. Under such circumstances, cholestatic agents (i.e., drugs, hormones, proinflammatory cytokines) can either reduce transporter gene expression or directly inhibit transport protein function [3, 6]. Typical cholestatic agents/drugs, such as cyclosporin A, rifamycin, glibenclamide, and an estrogen hormone metabolite directly inhibit bile acid secretion by blocking transport activity of the BSEP [16]. More recently, inhibitory antibodies directed against functionally critically domains of the BSEP protein and inhibiting transporter function have been described as novel potential mechanism of cholestasis [17]. It is important to keep in mind that most cholestatic diseases are caused at the bile duct level by obstruction (e.g., extrahepatic biliary obstruction by stones and tumors) or destruction (i.e., vanishing bile duct syndromes (VBDS) such as PSC and PBC) [3, 4]. Decreased expression of transport systems in these disorders may at least in part explain the impairment of transport function resulting in or maintaining cholestasis. However, not all of the encountered changes in transporter expression are "procholestatic" and "negative" from an etiological point of view [3]. While some of these alterations contribute to cholestasis, most of the changes in transporter expression in liver and extrahepatic tissues (e.g., kidney) may represent compensatory ("anticholestatic") mechanisms, which provide alternative excretory routes for accumulating cholephiles in cholestasis [3, 10].

Expression of the basolateral-bile acid uptake systems NTCP and OATP2 in hepatocytes is reduced in acute-inflammation induced cholestasis (caused by alcohol, drugs, or autoimmune hepatitis) and in patients with PBC as prototypic cholestatic syndrome, while expression of canalicular BSEP and MRP2 is relatively well preserved in PBC (Fig. 32.4) [18–21]. Downregulation of basolateral bile acid uptake together with maintained canalicular excretion is regarded a potentially protective mechanism preventing intracellular accumulation of cytotoxic bile acids. Moreover, expression of alternative basolateral-bile acid export systems (i.e., MRP3, MRP4, and organic solute transporter OSTα/β) is induced (Fig. 32.4). After alternative excretion via the basolateral membrane into systemic circulation, bile acids are eliminated via the kidney into urine. In addition to passive glomerular filtration, active tubular excretion of bile acids by MRP2 and MRP4, which are both localized to apical membranes of proximal tubular epithelial cells in kidney, may further assist urinary bile acid elimination (Fig. 32.4). Therefore, transporter changes encountered in cholestasis

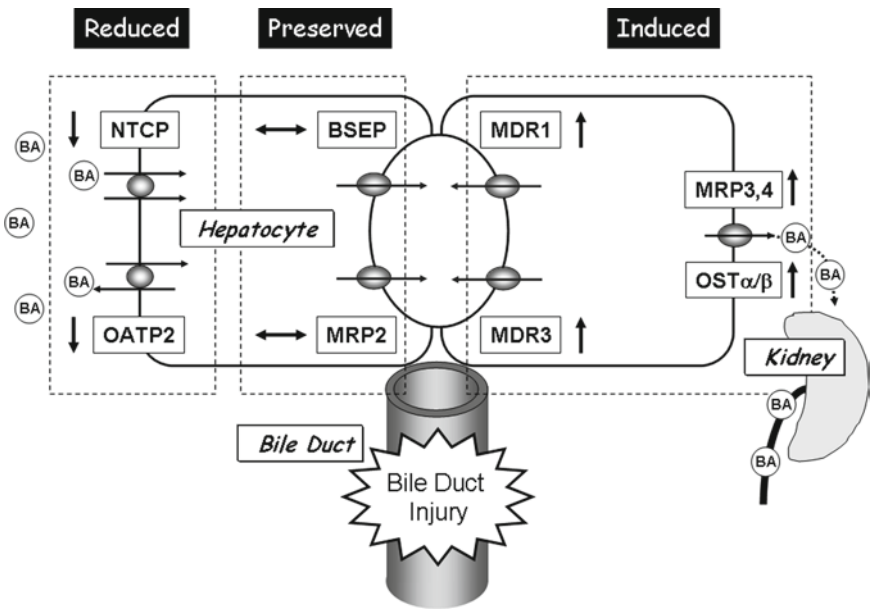

Fig. 32.4 Molecular alterations of transport systems in primary biliary cirrhosis as prototypic cholestatic liver disease. Downregulation of the basolateral NTCP and the organic anion transporting protein (OATP2), together with preserved expression of the canalicular bile salt export systems BSEP and MRP2 reduce the hepatocellular retention of bile acids (BA^-). Furthermore, induction of MRP3, MRP4, and OSTα/β at the basolateral membrane of hepatocytes provides an alternative route for the elimination of bile acids and bilirubin from cholestatic hepatocytes. Decreased basolateral bile acid uptake together with increased basolateral bile acid secretion into the systemic circulation facilitates bile acid delivery to the kidneys. Passive glomerular filtration may be assisted by active tubular secretion of bile acids (via MRP2 and MRP4) and by reduced tubular re-uptake (not shown), facilitating urinary bile acid elimination. In addition, repression of bile acid synthesis reduces bile-acid load and increased phase I (i.e., hydroxylation) and phase II (i.e., sulfation, glucuronidation) renders bile acid less toxic and more amendable for their renal excretion (not shown)

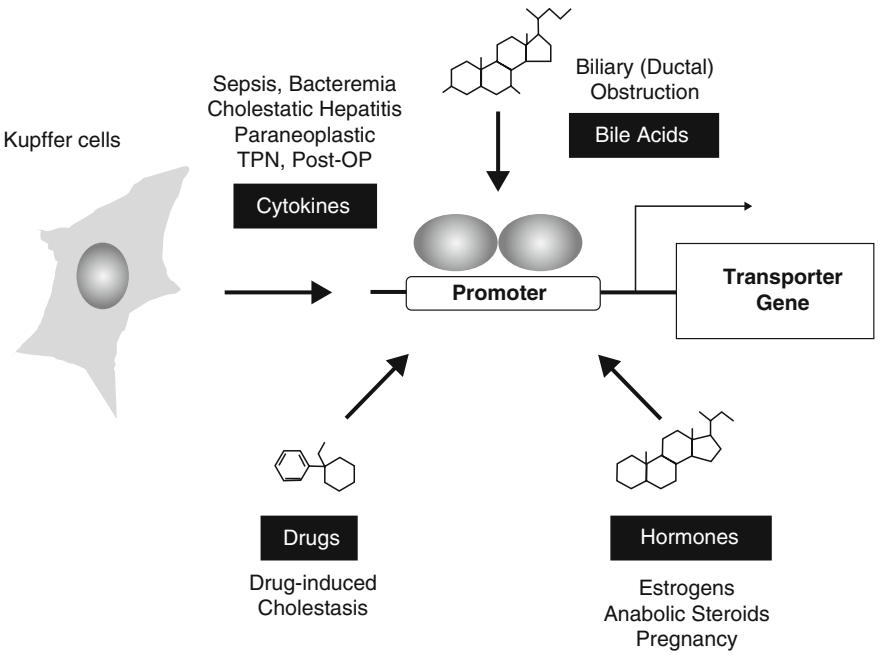

Fig. 32.5 Transcriptional regulation of hepatobiliary transporter genes in cholestasis. Many alterations in bile acid transport and metabolism in cholestasis can be explained at a transcriptional level. Depending on the cause of cholestasis, effects of cytokines, bile acids, hormones, or drugs may dominate the picture. Accumulating bile acids, drugs, and hormones can act as nuclear receptor ligands thus modifying the expression of respective target genes. Inflammatory cytokines in general repress nuclear receptor activity and expression. In clinical practice, often, more than one of these mechanisms may alter transporter gene expression (e.g., bile acid accumulation and cytokine induction in obstructive cholestasis)

represent a mixture of procholestatic and anticholestatic adaptive alterations [3, 6, 21]. These adaptive transporter changes are further assisted by repression of bile-acid synthesis and induction of bile-acid detoxification. Phase I (hydroxylation) and phase II (sulfation, glucuronidation) bile-acid metabolism renders bile acid better water soluble and, thus, less toxic. The functional relevance of these molecular alterations is reflected by the appearance of hydroxylated, sulfated, and glucuronidated bile acids in urine of patients with longstanding cholestasis [3, 10]. Unfortunately, most of these intrinsic adaptive changes apparently are too weak and come too late to fully prevent cholestatic liver injury [3, 6].

Anion exchanger AE2 mediates Cl^-/HCO_3 exchange and regulates intracellular pH and biliary HCO_3 excretion by hepatocytes and cholangiocytes. Expression of AE2 is reduced in patients with PBC and may contribute to reduced bile flow/cholestasis and Sicca syndrome/Sjögren's syndrome, which is frequently associated with PBC [22, 23]. Progression of PBC despite UDCA therapy has been linked to allelic variations in the *AE2* gene [22, 23]. Of note, *Ae2*-knockout mice develop biochemical, serologic, immunologic, and histopathologic features of PBC including development of antimitochondrial antibodies [24]. Ae2 deficiency not only leads to disturbances in intracellular pH homeostasis in cholangiocytes, but also in immunocytes which may explain the combination of immunological and functional changes seen in PBC patients [22–24].

Acquired alterations in hepatobiliary transporter expression in cholestasis are largely mediated by nuclear receptors [10]. Depending on the type of cholestatic injury, the observed changes in transporter expression are not only effects of retained biliary constituents activating nuclear receptors, but are the result of a combination of many factors influencing nuclear receptor activity (e.g., proinflammatory cytokines, hormones, and drugs) (Fig. 32.5) [3, 6, 10]. For example, extrahepatic obstructive cholestasis not only retains bile acids and bilirubin, but also inflammatory cytokines (frequently induced by concomitant cholangitis and bacterial translocation from the gut) and cause deregulation of transporter genes.

Cytoskeletal and Other Hepatocellular Changes in Cholestasis

In addition to transporter changes, other hepatocellular alterations may also interfere with bile secretion. As such, cholestasis is associated with profound alterations of the cytoskeleton of hepatocytes including disruption of the microtubular system, the actin microfilament network, alterations in tight

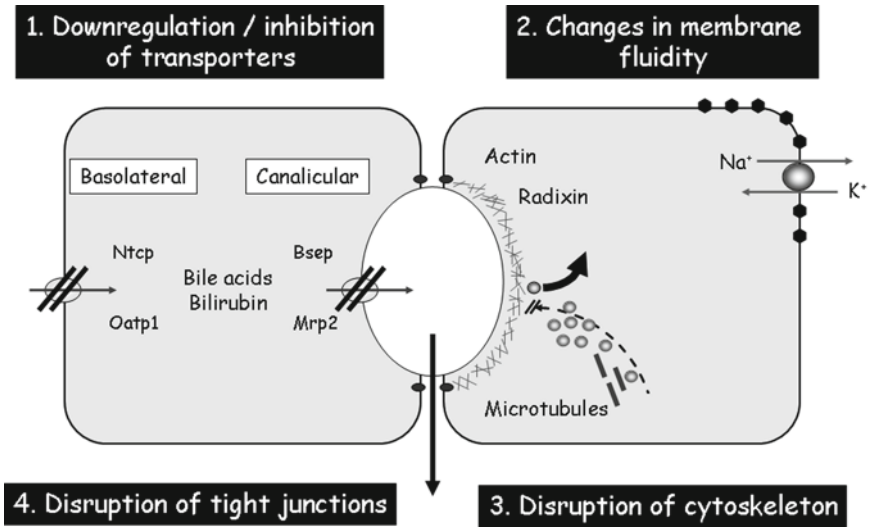

Fig. 32.6 Hepatocellular alterations in cholestasis. In addition to changes in transporter expression and/or function (*1*), cholestasis is associated with profound alterations of membrane fluidity (*2*) with subsequent changes in the function of embedded enzymes/transporters, (*3*) disruption of the cytoskeleton of hepatocytes including alterations of the microtubular system (required for normal vesicular trafficking to/from the canalicular membrane) and pericanalicular actin microfilament network (required for normal canalicular contractility and microperistalsis), and (*4*) tight junction alteration resulting in increased paracellular permeability, regurgitation of biliary constituents into plasma, and finally breakdown of the osmotic gradients in the bile canaliculi that normally constitute the driving force for bile secretion. Tethering proteins such as radixin crosslink actin filaments to integral membrane proteins, and are required for maintenance of structure of the bile canaliculus and for the polarized targeting and retaining of canalicular transport systems

junctions, and increases in cytokeratin intermediate filaments (Fig. 32.6) [25, 26]. Again, many of these cytoskeletal changes are secondary and nonspecific phenomena observed in many different forms of cholestasis. Altered function of different components of the cytoskeleton may lead to cholestasis by interference with transcellular transport processes of biliary constituents or carrier proteins, increased tight junction permeability, and impaired contraction of bile canaliculi.

Alterations in actin microfilaments (caused by drugs such as chlorpromazine) impair bile canalicular contractility and contribute to cholestasis [25, 26]. Reduced canalicular motility is also observed in response to proinflammatory mediators such as nitric oxide, which could contribute to cholestasis of sepsis. Tethering proteins such as radixin, crosslink actin filaments to integral membrane proteins (Fig. 32.6). Radixin is required for maintenance of structure of the bile canaliculus and for the polarized targeting and retaining of canalicular transport systems [25, 26]. Of interest, radixin is reduced and disrupted in late-stage PBC and in various other cholestatic diseases, which might cause altered localization of canalicular transporters such as MRP2 [2, 25, 26].

Drugs and toxins (e.g., colchicin), which disrupt microtubules, may cause cholestasis through impaired vesicular trafficking of transport proteins to the canalicular membrane [2–4]. High concentrations of hydrophobic bile acids impair the function of microtubular motors (e.g., kinesin) [25, 26] suggesting that impaired vesicular targeting may be a universal finding as a result of bile acid accumulation in cholestasis. Furthermore, rapid retrieval of transporters from the canalicular membrane in response to cholestatic injury (e.g., treatment with lipopolysaccharide and sex-hormones, bile acid challenge, hyperosmolarity) may also contribute to cholestasis (Fig. 32.6).

Disruption of tight junctions results in increased paracellular permeability, regurgitation of biliary constituents into plasma, and reduction, or even collapse of the osmotic gradients in the bile canaliculi that normally constitute the driving force for bile secretion (Fig. 32.6) [25, 26]. Moreover, leaky tight junctions between cholangiocytes facilitate periductal inflammation and pericholangitis. The role of tight junctions is further exemplified by mutations in tight junction protein genes that can cause cholestasis and (neonatal) sclerosing cholangitis (see above, Table 32.1).

Summary and Conclusions

In summary, expression of hepatobiliary transport systems and bile-acid metabolizing enzymes undergo marked alterations during cholestasis. While some of these molecular changes, in particular hereditary transporter mutations, can explain reduced transport function in cholestasis, most of the observed acquired changes are consequences but not cause of cholestasis. Such secondary alterations in transporter

expression may be aimed at protecting the hepatocyte from accumulating cytotoxic bile acids. Furthermore, bile acid detoxification (hydroxylation, sulfation, and glucuronidation) is increased and renders bile acids better water soluble and more amenable for alternative urinary excretion. Apart from the pathophysiological implications, targeted stimulation of these adaptive processes via activation of specific nuclear receptors represents an attractive therapeutic approach in the therapy of cholestatic disorders. In addition to changes in transporter expression and function, other hepatocellular events such as alterations in membrane fluidity, cytoskeletal apparatus, vesicular targeting, bile canalicular contractility, and tight junction permeability may also contribute to cholestasis.

Acknowledgments This work was supported by grants P18613-B05, P19118-B05 and F3008-B05 (to M.T.) from the Austrian Science Foundation and a GENAU project grant from the Austrian Ministry of Science.

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Chapter 33
Portal Hypertension

Sumit K. Singla and Vijay H. Shah

Introduction

Portal hypertension is a well-recognized and frequent clinical syndrome defined as a pathological increase in hepatic sinusoidal pressure. The sequelae of portal hypertension, including ascites, variceal hemorrhage, and hepatic encephalopathy, are responsible for much of the disease burden associated with cirrhosis [1]. In fact, these complications are the most frequent indication for liver transplant in cirrhotic patients [2].

The portal pressure gradient is the difference in pressure between the portal vein and the inferior vena cava and reflects the hepatic perfusion pressure. This gradient can be represented as the product of portal blood flow and resistance opposing flow, as depicted by Ohm's law in the following equation:

$$\Delta P = \mathrm{QXR}.$$

In which ΔP represents the portal pressure gradient, Q represents portal blood flow, and R represents resistance. Therefore, portal pressure may be elevated by increased portal blood flow, resistance, or both. Most often, portal hypertension is seen as a result of an increase in both parameters. In this chapter, we will review the pathophysiologic mechanisms behind this increase in portal pressure.

Normal Physiology of Portal Circulation

Anatomy

The arterial blood supply and venous drainage of the entire gastrointestinal tract, from the esophagus to the rectum, is referred to as the splanchnic circulation. The liver receives a dual blood supply, from the oxygen-rich hepatic artery and the nutrient-rich portal vein [3] (see Chap. 1). Approximately 75% of blood reaching the liver is supplied by the portal vein, with the balance delivered via the hepatic artery. In total, hepatic blood flow comprises nearly 30% of total cardiac output.

The liver receives capillary blood via the portal venous system from the esophagus, stomach, small and large intestine, pancreas, and spleen. The inferior mesenteric vein drains into the splenic vein, which combines with the superior mesenteric vein to form the portal vein. The left gastric vein usually drains into the portal vein at the confluence of the superior mesenteric and splenic veins. The portal vein divides into the right and left portal vein branches in the hilum of the liver. The hepatic artery usually arises from the celiac trunk, the first of three major arteries that comprise the splanchnic arterial circulation (in addition to the superior and inferior mesenteric arteries).

Blood from the portal vein and hepatic artery unites in specialized high-compliance channels known as hepatic sinusoids. Primary regulation of hepatic perfusion occurs in this unique microenvironment.

Sinusoidal Microenvironment

Various cell types are found in the perisinusoidal region, including sinusoidal endothelial cells (SEC), hepatic stellate cells (HSC), hepatocytes, and Kupffer cells.

SEC have two unique properties that distinguish themselves from endothelial cells found in other parts of the body. First, SEC contain large intracellular pores known as fenestrae, which permit extravasation of even large macromolecules from the blood. Additionally, SEC lack basement membranes, a property which increases endothelial permeability to solutes [4]. Taken together, these qualities allow for effective transport of molecules from the vascular lumen into the space of Disse, for their uptake into adjacent hepatocytes. Like other endothelial cells, SEC synthesize vasoactive molecules which act on other cell types in the hepatic vascular bed, regulating vascular tone [5].

S.K. Singla (✉)
Department of Internal Medicine, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA
e-mail: singla.sumit@mayo.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_33, © Springer Science+Business Media, LLC 2011

In normal physiologic states, HSC (previously referred to as Ito cells) serve to store vitamin A in its esterified form. Other roles of HSC include synthesis of extracellular matrix components, cytokines, and growth factors. The contribution of quiescent HSC to the regulation of vascular tone in the healthy liver remains controversial. In pathologic states, namely portal hypertension, HSC activation leads to the disruption of microvascular tone regulation [6, 7].

Kupffer cells line the sinusoidal epithelium and function as liver macrophages. Like HSC, Kupffer cells may have additional vasoregulatory properties as well, which can contribute to the development of liver injury.

A unique autoregulatory mechanism exists in order to maintain blood flow at a relatively consistent level in the event of occlusion of either the hepatic artery or the portal vein. In case of hepatic artery occlusion (as seen in hepatic artery embolization, for example), portal vein blood flow experiences a compensatory increase. Similarly, portal vein thrombosis is accompanied by increased hepatic arterial flow. This mechanism is known as the hepatic arterial buffer response.

Causes of Portal Hypertension

Various etiologies for portal hypertension have been identified and a thorough evaluation of each of these is beyond the scope of this chapter. Some of the more prominent causes are briefly outlined below.

The various etiologies underlying portal hypertension have traditionally been classified according to the anatomic location of derangement, i.e., prehepatic, intrahepatic, or posthepatic. Within the liver, these have been further subdivided into presinusoidal, sinusoidal, or postsinusoidal. Unfortunately, this classification does not strictly apply to conditions which cause increased resistance at more than one of these subdivisions. For example, alcoholic cirrhosis can cause increased resistance at the pre-, intra-, and extrasinusoidal levels. Alternatively, a more useful classification system is based on the clinical prevalence of causes of portal hypertension.

Common Causes

Cirrhosis

End-stage liver disease secondary to cirrhosis is by far the most common cause of portal hypertension. The many etiologies of cirrhosis all result in portal hypertension, but there are certain disease-specific features. For example, in both alcoholic liver disease and autoimmune hepatitis, portal hypertension can occur in the absence of cirrhosis. Both mechanical and vascular factors are implicated in the pathophysiology of portal hypertension secondary to cirrhosis.

Extrahepatic Portal Vein Thrombosis

Extrahepatic portal vein thrombosis is a common cause of portal hypertension in children. The most common etiologies of this prehepatic abnormality include polycythemia vera and essential thrombocytosis, although no apparent cause is identified in up to 1/3 of patients. Various other conditions predispose to portal vein thrombosis, including prothrombotic states (antithrombin, protein C, protein S deficiency), paroxysmal nocturnal hemoglobinuria, oral contraceptive use, intraabdominal malignancy, pancreatitis, and pregnancy, among others. Interestingly, cirrhosis itself can lead to portal vein thrombosis [8]. Previously, this was thought to occur primarily in patients with concomitant cirrhosis and hepatocellular carcinoma (HCC) [9]. More recently, portal vein thrombosis has been demonstrated as a common sequela of cirrhosis, and the association with HCC may not be as robust as once believed.

Schistosomiasis

Although rare in the United States, schistosomiasis may be the leading cause of portal hypertension worldwide. Eggs are deposited in the presinusoidal portal venules by *Schistosoma mansoni* and *Schistosoma japonicum*, causing presinusoidal obstruction. The extensive host granulomatous response leads to fibrosis, which is often referred to as "pipestream" [10]. Bleeding from esophageal varices is a major cause of morbidity and mortality in these patients.

Idiopathic Portal Hypertension

While rare in the United States, idiopathic portal hypertension is a common cause of portal hypertension in parts of Asia. This disorder is characterized by signs of portal hypertension in the absence of hepatic lesion on light microscopy [11]. In some cases, increased levels of endothelin-1 (ET-1) have been noted in the pathology specimens, which may be a reflection of the underlying pathophysiology (see below for a discussion of ET-1) [12]. As the name suggests, the cause of idiopathic portal hypertension is generally unclear,

although chronic arsenic intoxication, exposure to vinyl chloride, and hypervitaminosis A have been implicated as potential etiologic factors.

Less Common Causes

Nodular Regenerative Hyperplasia

Nodular regenerative hyperplasia (NRH) is a histopathologic condition characterized by selective hyperplasia of zone 1 hepatocytes in conjunction with atrophy of zone 3 hepatocytes. Lack of fibrosis on biopsy is characteristic of this disorder [13]. The nodular hyperplasia centered around the portal triad results from local hyperperfusion, while the peripheral atrophy results from hypoperfusion. Autoimmune, hematologic, and toxic etiologic factors have been implicated in the pathogenesis of NRH.

Partial Nodular Transformation of the Liver

This uncommon disorder is characterized by large nodules in the perihilar region, which may be visible on imaging studies [14]. The remainder of the liver is grossly normal or may exhibit changes consistent with NRH. Like NRH, the pathogenesis of partial nodular transformation (PNT) is related to perfusion imbalances. However, the perfusion defects are localized to the hilar region in PNT, compared to the widespread abnormalities of NRH.

Sarcoidosis

This systemic, noncaseating granulomatous disorder more often involves the liver than any other gastrointestinal tract organ. Most patients with hepatic sarcoidosis are asymptomatic. Those who do manifest signs of portal hypertension demonstrate increased resistance initially at the presinusoidal level. Later in the course of disease, postsinusoidal resistance is more commonly elevated.

Splanchnic Arteriovenous Fistula

Acute portal hypertension may be due to rupture of a splanchnic arterial vessel into the lumen of a portal vein tributary [15]. Over time, perisinusoidal fibrosis due to increased inflow will occur. Timely embolization or ligation can correct the portal hypertension, but once fibrosis is widespread, the changes are most likely irreversible.

Other less common causes of portal hypertension include congenital hepatic fibrosis, polycystic liver disease, hereditary hemorrhagic telangiectasia, myeloproliferative disorders, and malignancy.

Mechanical Factors in Pathophysiology of Portal Hypertension

In cirrhosis, portal hypertension largely results from the mechanical process of vessel diameter reduction. Resistance is inversely proportional to vessel radius to the fourth power, as represented by Poiseuille's Law in the following equation:

$$R = 8\eta L / r^4.$$

Progressive extracellular matrix deposition primarily occurs in the space of Disse and causes narrowing of the sinusoidal lumen and increased resistance to portal flow (Fig. 33.1). The role of HSC in this process is examined in more detail in Chap. 30. Collagen is subsequently converted into fibrotic tissue, a step signaling the irreversible nature of mechanical obstruction.

Hepatic fibrosis results from acute or chronic liver insult and refers to the formation of scar-like septum surrounding

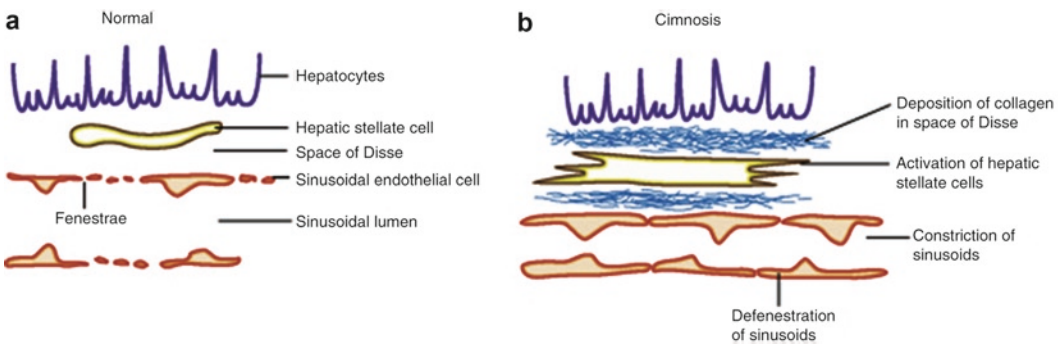

Fig. 33.1 Anatomy of the hepatic microvasculature

nodules of hepatocytes, resulting in mechanical obstruction to flow. The regenerative capacity of the liver in response to injury of various etiology leads to bands of fibrous tissue and nodules, with resultant architectural distortion [16].

In conjunction with collagen deposition, hepatic resistance is further increased by compression of centrilobular venules by regenerating nodules of hepatocytes and inflammation. Other well-characterized mechanisms of mechanical resistance include capillarization of the space of Disse, which involves the development of a pathologic subendothelial basement membrane. The sinusoidal endothelium undergoes defenestration in cirrhosis, thus further impairing bidirectional communication between sinusoidal blood and liver parenchyma.

Vascular Basis of Portal Hypertension

Vascular factors play a central role in the pathogenesis of portal hypertension. These include intrahepatic vasoconstriction, impaired vasodilation, and angiogenesis, which together contribute to increased intrahepatic resistance. Additionally, splanchnic vasodilation leads to a hyperdynamic circulatory state, which increased portal inflow and thereby resistance. In an ultimately unsuccessful attempt to shunt blood away from the portal circulation, collateral vessels to the systemic venous circulation develop (Fig. 33.2).

In recent years, more interest has centered upon the vascular, rather than mechanical, etiology of portal hypertension. This is largely due to the multitude of potential therapeutic targets available in this dynamic, reversible area, in contrast to the lack of potential modification of static, fixed mechanical scarring. Once thought to contribute only a small fraction, vascular changes are now estimated to account for approximately 30% of the increased resistance seen in portal hypertension [17, 18].

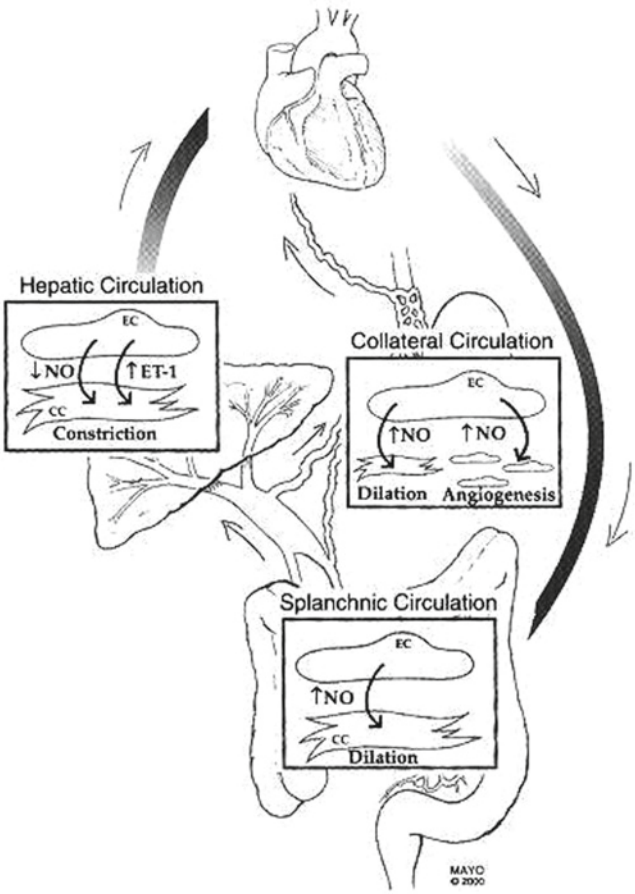

Fig. 33.2 Mechanisms of endothelial cell regulation of portal hypertension. By permission of Mayo Foundation for Medical Education and Research. All rights reserved

Intrahepatic Vasoconstriction/Impaired Responsiveness to Vasodilation

Increased Vascular Tone of Sinusoids

The finely regulated balance of the hepatic microcirculation is due to the delicate interplay among SEC, HSC, and Kupffer cells. In pathologic states, this homeostasis is disrupted, leading to vasoconstriction, hyporesponsiveness to vasodilation, and increased resistance to flow. The crucial pathophysiologic mechanism contributing to increased intrahepatic resistance is endothelial dysfunction.

SEC produce and release a variety of vasoactive substances, which diffuse and exert paracrine effects on HSC [19–21]. In portal hypertension, the balance between vasoconstrictors and vasodilators shifts heavily towards the former. In addition to SEC dysfunction, HSC become activated in pathologic states, exhibiting structural and functional homology with vascular smooth muscle cells.

Endothelial Dysfunction

Endothelial cell dysfunction is caused by long-term exposure to noxious stimuli, which may be physical or chemical [22]. In other disease processes, namely atherosclerosis and diabetes, endothelial dysfunction is known as an early pathogenic mechanism of disease propagation. Factors implicated as causative of SEC dysfunction include oxidative stress and inflammation.

Oxidative Stress

Oxidative stress causes endothelial dysfunction by several mechanisms. First, reactive oxygen species interact directly

with intracellular nitric oxide (NO), decreasing the bioavailability of NO within endothelial cells [23]. In addition, oxidative stress has been shown to impair eNOS activity by increasing the interaction between eNOS and caveolin-1, a potent eNOS inhibitor [24]. These two processes lead to diminished NO function.

Inflammation

Inflammation is a cardinal feature of cirrhosis and contributes to endothelial dysfunction by decreasing eNOS-driven NO production. Endotoxin increases production of caveolin-1 as well as its inhibitory interaction with eNOS. It also inhibits eNOS activity by decreasing ET-1-mediated activation of eNOS [25, 26].

Hepatic Stellate Cell

In portal hypertension and other models of liver injury, normally quiescent HSC transform into myofibroblast-like cells and contribute to increased intrahepatic resistance. HSC are located in the space of Disse, adjacent to sinusoids (Fig. 33.3). Morphologically, activated HSC are characterized by loss of vitamin A droplets and expression of proteins characteristic of contractile cells. Functionally, activated HSC exhibit increased proliferation, migration, extracellular matrix production, and release of cytokines leading to inflammation, fibrosis, and mitogenesis [27].

Increased extracellular matrix synthesis is one of the most well-recognized and prominent functions of activated HSC [28]. This contributes to mechanical resistance to flow. In the healthy liver, HSC make up 13% of the volume of sinusoidal cells. Proliferation, largely in response to platelet-derived growth factor (PDGF), increases this percentage substantially [29–31]. Mitogens contributing to HSC proliferation include PDGF, fibroblast growth factor, ET-1, and transforming growth factor alpha (TGF-α) [32–34]. Among these, PDGF appears to play the strongest role [35].

Activated HSC demonstrate up-regulation and overexpression of smooth muscle markers, including α-actin, desmin, NG2, and myosin, leading to increased contractility [36, 37]. HSC-induced contraction occurs most strongly in response to ET ligand activity and is inhibited by NO [38, 39]. HSC migration and proliferation enhance their coverage of sinusoidal vessels. In conjunction with their capacity to act as smooth muscle cells, this leads to vasoconstriction.

In addition to responding to SEC-produced vascular factors, HSC produce cytokines and other active peptides that work in a paracrine and autocrine fashion to influence sinusoidal vascular tone. TGF-β1 production leads to fibrogenesis, for example [40]. Interestingly, PDGF production leads to autocrine and paracrine signaling that culminate in cell migration [41].

Multiple signaling pathways have been implicated in HSC mobility and migration. The roles of NO and ET will be discussed in further detail below. TGF-β is well characterized in HSC-mediated collagen deposition, but also appears to contribute to HSC migration. PDGF-dependent signaling also plays a key role, and studies have shown that PDGF and TGF signaling may converge at the level of the c-abl tyrosine kinase [42, 43]. Recent pulmonary studies have demonstrated that imatinib, a small molecule tyrosine kinase inhibitor, inhibits this intracellular common pathway, providing an exciting future horizon for treatment of intrahepatic resistance [43]. Other mechanisms leading to HSC apoptosis are currently under investigation as well.

In summary, HSC activation is a central mechanism of intrahepatic resistance in models of liver injury, including portal hypertension. Specifically, the role of HSC as a liver-specific pericyte contributing to sinusoidal remodeling through angiogenesis pathways is an area of active investigation.

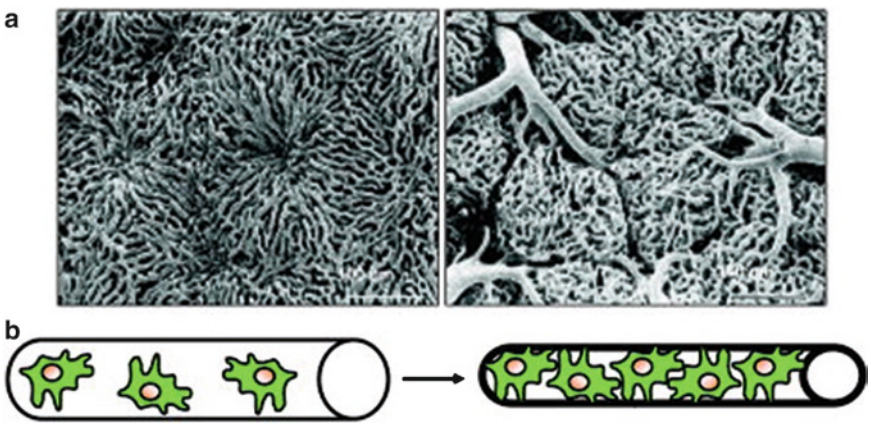

Fig. 33.3 HSC contribute to vascular remodeling in chronic liver disease. From Lee et al. [114]. Used with permission

Decreased Production of Nitric Oxide

Background

Nitric oxide's role as endothelium-derived relaxing factor was first uncovered in 1987. Since then, it has garnered much attention for its central role in intrahepatic vasodilation. Nitric oxide synthases (NOS) generate NO in response to specific stimuli. Close homology has been established between NOS and cytochrome P450 reductase. Three isoforms of NOS have been identified: inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and endothelial nitric oxide synthase (eNOS).

Although nNOS-containing neurons have been detected in the hepatic perivascular arterioles and venules, their activity and regulation appear less important than that of iNOS and eNOS.

iNOS synthesis is induced by lipopolysaccharides and inflammatory cytokines and occurs in hepatocytes, vascular smooth muscle cells, and HSC. iNOS production of NO is regulated predominantly at the transcriptional level.

eNOS production in the liver occurs exclusively in endothelial cells, although mesangial cells, glial cells, and neurons, among other cell types, have been shown to express this protein as well. In contrast to the single level of iNOS regulation, eNOS-derived NO production can be modulated at multiple steps [44]. This gives researchers multiple potential targets to upregulate eNOS function, and thereby manipulate hepatic microcirculation perfusion.

The two major regulators of eNOS-driven conversion of L-arginine into NO are caveolin-1 (Cav-1) and activating kinase (Akt1). Cav-1 inhibits this process, while Akt-1, as its name suggests, positively regulates production of NO. Indeed, mice lacking the Akt-1 isoform exhibit severe deficits in angiogenesis, secondary to defects in eNOS phosphorylation and NO production [45]. Similarly, mice deficient in the negative regulator Cav-1 demonstrate elevated NO production, in addition to defects in prostacyclin production and calcium signaling [46].

Shear stress within vessels has been recognized as the most potent physiologic regulator of eNOS activation in SEC [47]. The increased perfusion pressure and flow associated with shear stress leads to increased NO production, which, in physiologic states, should effect vasodilation and blunt the primary rise in perfusion pressure [48].

Mechanism of Action

NO is able to diffuse freely across cell membranes, secondary to its hydrophobic nature and neutral charge. Once inside effector cells, NO binds soluble guanylate cyclase, leading to conversion of guanosine 5′triphosphate (GTP) to cyclic guanosine 3′-5′-monophosphate (cGMP) [49]. A cGMP dependent-kinase subsequently phosphorylates key proteins involved in calcium homeostasis, leading to decreased intracellular calcium. This culminates in smooth muscle relaxation and vasodilation.

Inadequate Nitric Oxide

Deficient NO-induced vasodilation of HSC has been well described as one of – if not the – primary pathogenic mechanisms of increased vascular tone in the cirrhotic liver, increasing intrahepatic resistance and leading to portal hypertension. Much experimental evidence has shown, indeed, that NO production by liver endothelial cells (therefore, via eNOS) is significantly decreased compared to that by the healthy liver [48, 50–52]. Interestingly, decreased eNOS protein levels do not appear to account for the inadequate production of NO. Studies have demonstrated that comparable levels of eNOS mRNA and protein levels are found in cirrhotic and healthy endothelial cells [48, 51]. Instead, multiple posttranslational defects appear to result in deficient NO production. First, cirrhotic SEC display increased eNOS binding to the inhibitory protein caveolin, in combination with decreased calmodulin binding [48, 53]. Studies utilizing bile duct ligation (BDL) as a model of induced portal hypertension have demonstrated rescued eNOS activity in liver lysates after calmodulin administration [52]. Additionally, impaired phosphorylation (and thus, activation) of eNOS by Akt appears to be an important causative factor [54].

HSC Resistance to NO-Mediated Relaxation

In addition to deficient eNOS-mediated NO production in portal hypertension, activated HSC are more resistant to the vasodilatory effects of NO [55]. Studies have demonstrated that this may be secondary to defects in the guanylate cyclase signaling pathway [56–59]. As targeted NO delivery to the intrahepatic circulation has become an area of investigation, the intrinsic resistance of HSC to vasodilation will have increased clinical significance.

Potential NO Therapy

Studies demonstrating a reduction in portal hypertension in cirrhotic rats after activation of eNOS underline the central role of NO deficiency as a causative pathophysiologic mechanism of portal hypertension [18, 60]. Transduction with adenovirus constitutively expressing Akt, a well-described activator of eNOS function, led to reduced portal pressure, via increased eNOS phosphorylation and cyclic GMP levels

[18]. Confirming the observation that decreased eNOS protein levels are not responsible for deficient NO production, adenovirus with overexpression of eNOS did not significantly decrease portal pressure [56].

Statins have been shown to clinically improve hemodynamics in portal hypertension. The mechanism for this appears to be regulation of eNOS activity. Simvastatin was shown to enhance NO production and decrease hepatic vascular tone, via Akt phosphorylation and caveolin eNOS dissociation [61]. A similar paradigm has been recently demonstrated in humans [62].

Increased Vasoconstrictor Activity

In addition to deficient NO-induced dilation, overproduction of vasoconstrictor substances contributes to increased intrahepatic vascular tone.

Increased Endothelin Activity

Endothelin (ET) is a potent vasoconstrictor produced and secreted by SEC. ET has been shown to have a significant effect on the pathogenesis of increased intrahepatic pressure.

There are three isoforms of endothelin, with ET-1 being the most important in liver pathology [33]. ET acts through G-protein-coupled membrane receptors on various target cells, including hepatocytes, HSC, endothelial cells, Kupffer cells, and cholangiocytes [63–65].

Endothelin acts primarily through two receptor subtypes, ET-A and ET-B. Ligand binding to ET-A initiates a signaling cascade culminating in cell contraction, via mobilization of intracellular calcium and protein kinase C activation [63]. ET-B receptor activation also leads to effector cell contraction. Interestingly, ET-B receptors located on endothelial cells are linked to eNOS activation and NO production, which would seem to at least partially counteract ET-induced vasoconstriction [66]. In HSC, however, ligand binding does lead to contraction [67].

In the diseased liver, the above mechanisms become overactive, leading to the excessive intrahepatic vasoconstriction associated with portal hypertension. Current evidence suggests that overactivation of the ET-1 system is induced at least in part by TGF-β, which is also involved with the development of cirrhosis. Other mechanisms for activation have yet to be elucidated.

ET-1 has been shown to be present in higher concentrations in liver tissue isolated from individuals with portal hypertension as compared to control [68, 69]. This finding was confirmed by demonstrating increased ET-1 expression in HSC and SEC in animal models utilizing BDL and CCl_4-induced fibrosis [7, 33, 70]. Further, an ET-A receptor antagonist, BQ-123, has been shown to counteract the increased intrahepatic resistance demonstrated in the BDL rat model of portal hypertension [70].

ET-1 primarily exerts its effects via action on HSC, via ET-A receptors. As expected, HSC show increased ET-A receptor expression in animal models of portal hypertension [71]. ET-1 mRNA expression is also increased in HSC, suggesting a possible autocrine mechanism for HSC constriction, in addition to stimulation by endothelial cell-derived ET-1 [6, 7, 70].

Further understanding of ET's diverse effects upon HSC, especially in regard to the yin-and-yang nature of ET-A and ET-B, may result in effective inhibition of the excessive vasoconstriction associated with portal hypertension.

Splanchic Vasodilation

In addition to enhanced intrahepatic resistance, prehepatic vascular changes are crucial to the development and propagation of portal hypertension. Specifically, vasodilation occurs at the level of the splanchnic arteriole and is primarily mediated by endothelial cells. In portal hypertension, the splanchnic circulation becomes less responsive to vasoconstrictor action. Removal of endothelial cells has been demonstrated to facilitate vasoconstriction in response to methoxamine, confirming the central role of the endothelium in regulation of splanchnic vascular tone [72].

Multiple cellular mediators appear to be relevant to splanchnic endothelial cell activation. In particular, NO has surfaced as the main mediator of the portal hypertensive mesenteric vasculature.

Role of NO

In contradistinction to the deficient amount of NO in the intrahepatic circulation, current evidence suggests excess NO in the splanchnic circulation as contributing to vasodilation and subsequent hyperdynamic circulation [20]. Pharmacologic inhibition of NO production has been shown to reduce portal venous inflow in vivo. In addition to increased eNOS-driven NO production, iNOS and nNOS mechanisms have been implicated in generating this excess amounts of NO [73–76].

Cytokine-mediated iNOS induction has been postulated as responsible for increased NO production [77]. Studies have demonstrated that inhibition of TNF, which is implicated in iNOS activation, leads to reversal of NO-induced vasodilation [78, 79]. However, an increase in iNOS protein expression has not yet been consistently identified in the portal hypertensive mesenteric vasculature [72, 80, 81].

An increase in eNOS mRNA or protein levels in the portal hypertensive vasculature has been demonstrated with more regularity [82, 83]. It was initially unclear whether this increase was causative to or reflective of splanchnic vasodilation, as the increased flow and shear stress typical of hypertensive prehepatic flow have been shown to be potent activators of eNOS [84]. Recent evidence, however, suggests augmented eNOS function (whether secondary to eNOS activation, gene induction, or both) precedes development of the hyperdynamic circulation [85]. This evidence supports the hypothesis of eNOS as a causative factor in the hemodynamic alterations characteristic of portal hypertension.

Multiple mechanisms lead to increased eNOS production of NO in the splanchnic circulation. Shear stress induced by initial portal hypertensive changes (i.e., intrahepatic resistance) has been shown to be a potent activator of eNOS activity, via increased transcription, Akt phosphorylation, binding to heat-shock protein 90, and increased calmodulin binding [59, 86, 87]. Increased NO production may also result from some element of bacterial translocation, leading to increased tetrahydrobiopterin (BH4) generation or Akt phosphorylation [88, 89].

Recently, studies have focused on the role of vascular endothelial growth factor (VEGF) as a potential initiator of eNOS activation, via Akt activation [90]. Significant reductions in collateral formation and portal pressure have been demonstrated by the administration of a VEGF receptor blocker (SU5416) [91]. Additionally, mild increases in portal pressure increase VEGF expression and subsequent eNOS activity. The VEGF receptor blocker inhibits this enhanced eNOS activity, suggesting a role for VEGF blockade in decreasing the arteriolar vasodilation characteristic of portal hypertension [59]. Indeed, modulation of VEGF signaling is currently an exciting topic with clinical relevance for cancer, portal hypertension, and other pathologic states involving angiogenesis.

In addition to mediators increasing the activity of eNOS, the intracellular location of the enzyme appears to regulate its function. Normally, eNOS is located at the plasma membrane and the Golgi complex [92–94]. However, in endothelial cells isolated from portal hypertensive vasculature of cirrhotic rats, eNOS appears to lose its Golgi localization and diffuses freely throughout the cell. This alteration in location substantially alters the function of eNOS and may contribute to the excess NO in arterial splanchnic circulation [95].

NO-Independent Mechanisms of Splanchnic Arteriolar Relaxation

In addition to NO-driven relaxation, other mechanisms of vasodilation contribute to increased portal inflow. This may be due to dilatory peptides, a primary defect in portal hypertensive smooth muscle cells, or both [96].

Carbon monoxide (CO) plays a role in vascular regulation by causing vasodilatation via activation of guanylate cyclase of smooth muscle cells [97]. CO is an end product of the heme oxygenase (HO) pathway, which catabolizes hemoglobin and other heme-moiety containing proteins into biliverdin, and finally to bilirubin and CO [98]. Elevated levels of an inducible isoform of HO, known as HO-1 or heat-shock protein 32, have been demonstrated in diseased systemic and portal hypertensive vasculature [99]. The excess CO produced by this pathway has been postulated to contribute to arterial vasodilation [100, 101].

Elevated levels of prostacyclin, a known vasodilatory peptide synthesized by cyclooxygenase-1 (COX), have been demonstrated in portal hypertensive vasculature [102]. Anandamide is a lipid cannabinoid which causes vasodilation and hypotension via binding to the CB1 receptor. Activation of this receptor in cirrhosis leads to splanchnic vasodilation, and inhibition of this process has been shown to constrict the mesenteric vasculature [103, 104].

Collateral Vessel Flow

As portal pressure rises, collateral vessel formation between the high-pressure portal system and the relatively low-pressure systemic venous system develops. Portal blood flow is lowered according to the following equation:

$$1 / R_{\text{portal}} = 1 / R_{\text{hepatic}} + 1 / R_{\text{collaterals}}.$$

Not only does this incompletely decompress the hepatic microcirculation, but leads to much of the morbidity associated with portal hypertension. Esophageal varices and portal hypertensive gastropathy, for example, are two life-threatening complications of collateral development.

Vascular communication between the portal and systemic circulation arises from a combination of increased flow through existing vessels and development of new vessels (angiogenesis). Inhibition of NO signaling has been shown to inhibit both of these processes, suggesting that collateralization is an NO-dependent process. Studies are ongoing to delineate the regulation of, and molecular mediators involved in, angiogenesis.

NO-induced vasodilation of existing collaterals between the portal and systemic circulation appears to be mediated by mechanical forces and growth factors and can occur shortly after development of portal hypertension. Portal hypertensive rats administered with NOS inhibitors demonstrated decreased shunting [105–107].

Vascular remodeling generally occurs chronically as a result of increased flow. Characteristic changes involve decreased wall thickness and increased luminal diameter, findings that have been reversed with NOS inhibition [108].

Angiogenesis has been increasingly recognized for its role in development and perpetuation of collateral circulation. Endothelial progenitor cell recruitment leads to de novo synthesis of vessels, a process known as vasculogenesis [109]. This process contributes to endothelial regeneration in mice after hepatectomy and is dependent upon eNOS. Angiogenesis also results from the proliferation of existing endothelial and vascular smooth muscle cells [110].

VEGF plays a central role in vasodilation, remodeling, and angiogenesis, through a combination of NO-dependent and NO-independent mechanisms [111, 112]. Indeed, animal studies utilizing an inhibitor to VEGF receptor 2 led to decreased portosystemic shunting [90]. Inhibition of collateral vessel formation and propagation through targeting of NO and VEGF pathways appears to be a promising avenue of research to decrease morbidity associated with portal hypertension.

Conclusions

Discussions regarding the pathophysiology of portal hypertension have traditionally focused on morphologic changes within the liver leading to resistance to blood flow. More recently, attention has shifted to the role of dynamic vascular abnormalities in the hepatic, splanchnic, and collateral circulations in the development and perpetuation of portal hypertension.

Abnormalities in NO function seem to link these heterogeneous vascular systems at the molecular level. Particularly, diminished NO-induced vasodilation in the intrahepatic circulation leads to elevated vascular resistance, while excessive NO production in the splanchnic circulation increases portal blood flow. Any systemic administration of NO to counteract the intrahepatic deficiency would only worsen systemic and splanchnic vasodilation. Targeted delivery of NO to the hepatic circulation, potentially as bound to nanoparticles or other materials, could potentially selectively correct this anomaly and is therefore an area of active ongoing investigation.

Growth factors that regulate angiogenesis in the intrahepatic, splanchnic, and collateral circulations, including VEGF and PDGF, may provide another potential target in the management of portal hypertension [113]. Sorafenib and Imatinib, two tyrosine kinase inhibitors actively utilized in cancer research and treatment, have been shown to have beneficial effects on portal hypertensive vasculature through regulation of VEGF, PDGF, and NO [114].

In summary, portal hypertension results from increased intrahepatic resistance coupled with increased splanchnic blood flow, resulting in the pathologic development and perpetuation of collateral blood flow.

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Part V
Molecular Pathobiology of Non-Neoplastic Hepatobiliary Disorders

Chapter 34
Nonalcoholic Fatty Liver Disease

Onpan Cheung and Arun J. Sanyal

Introduction

Overview of Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in North America, affecting approximately 30% of the population [1]. It is the hepatic manifestation of the metabolic syndrome, and is directly linked to the escalating prevalence of obesity and the associated insulin resistance. Histologically, NAFLD is characterized by a spectrum that ranges from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) which can progress to cirrhosis in 15–20% of subjects [2]. NASH is further distinguished from NAFL by the presence of inflammation and cytologic ballooning with or without Mallory hyaline or pericellular fibrosis in addition to steatosis [3]. Recently, studies have reported that NAFLD may predispose patients to hepatocellular carcinoma (HCC) in the absence of significant fibrosis or cirrhosis, and these subjects also seem to have more features of the metabolic syndrome [4].

Patients with NAFLD are often asymptomatic although some may present with vague abdominal pain, fatigue, and anorexia. It is often diagnosed when mildly elevated aminotransferases are incidentally reported. NAFLD is characterized by a hyperechoic appearance of the liver on ultrasound and low density hepatic parenchyma on computed tomography imaging. Currently, there is no proven non-invasive marker to identify those with NASH or significant fibrosis. Liver biopsy remains the gold standard to distinguish between NAFLD and NASH, and to assess the severity of fibrosis.

With the increase in the frequency of obesity in the USA, the overall negative impacts of NAFLD on both the health status of an individual at risk, and the socioeconomic aspect of the health-care delivery remain a significant concern. While risk factors for NAFLD are well defined, recent advances in molecular research have continued to contribute to our current understanding of this debilitating disease process. However, proven therapies are still lacking. Over the years, research efforts have primarily focused on the "two hits" theory for the development of NAFLD and NASH. The first "hit" is purported to be abnormal lipid metabolism, in association with obesity, insulin resistance and the metabolic syndrome, as defined by dyslipidemia, hypertension, and diabetes mellitus that lead to the development of steatosis. The second "hit" includes mechanisms contributing to the development of necroinflammation and fibrosis. These include processes such as oxidative stress and mitochondrial dysfunction. This "two hits" theory expands into a complex network of interactions between multiple signaling and regulatory pathways that ultimately contribute to the development of NAFLD and NASH.

A.J. Sanyal (✉)
Department of Medicine, Division of Gastroenterology and Hepatology, Virginia Commonwealth University, Richmond, VA, USA
e-mail: ajsanyal@vcu.edu

Development of Hepatic Steatosis

Abnormal Lipid Metabolism

To read about the general molecular basis of fatty liver, please see Chap. 29. Hepatic steatosis is associated with increased plasma level and hepatic accumulation of triacylglycerol (TAG) [5, 6], which can be due to: (1) dietary excess, (2) increased synthesis in the liver from fatty acids formed during *de novo* lipogenesis (DNL), (3) increased fatty acid influx in to the liver from lipolysis of adipose tissue and subsequent conversion to TAG, (4) diminished export of lipids from the liver in very-low-density-lipoprotein (VLDL), and (5) decreased fatty acids oxidation.

Increased DNL in the Liver

Liver is one of the major organs playing a role in lipid metabolism (see Chap. 10). DNL is mediated by transcription factors sterol responsive element binding protein-1c (SREBP-1c),

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_34, © Springer Science+Business Media, LLC 2011

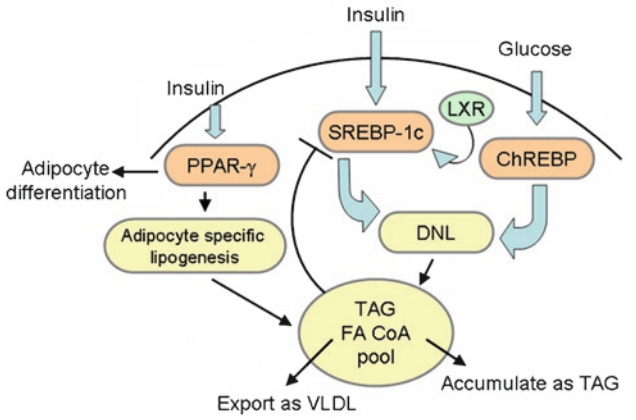

Fig. 34.1 Hepatic *de novo* lipogenesis. Fatty acid synthesis is induced by insulin and glucose via transcription factors SREBP-1C and CHREBP, respectively. LXR also promotes fatty acid synthesis by inducing SREBP-1C. Lipogenic genes including ACC, FASN, and SCD-1 are transcriptionally activated by both SREBP-1C and CHREBP. Insulin enhances the transcriptional activity of PPAR-Γ(gamma) that contributes to lipogenesis via increased expression of adipocyte specific genes including adipsin and adiponectin. PPAR-Γ also plays a role in adipocyte differentiation

peroxisome proliferators activated receptor (PPAR)-γ(gamma) and carbohydrate response element binding protein (ChREBP) [7, 8] (Fig. 34.1). In an insulin resistant state, high circulating level of insulin stimulates DNL by activating lipogenic genes i.e. acetyl CoA carboxylase (ACC), fatty acid synthase (FASN), and stearoyl-CoA desaturase-1 (SCD1); all are transcriptionally activated by SREBP-1c. SREBP-1c also increases the expression of acetyl CoA synthetase, which leads to increased acetyl CoA, the starting material in fatty acids synthesis [9]. The role of PPAR-γ in steatosis is demonstrated in animal studies that have shown that hepatic PPAR-γ mRNA expression of animals with insulin resistance, dyslipidemia, and fatty liver was significantly elevated [10]. Specifically, isoform PPAR-γ2 regulate adipocyte differentiation, lipid storage, and lipogenesis by promoting lipoprotein lipase activity, thus making triglyceride-derived fatty acids available for uptake [11]. PPAR-γ1 on the other hand is mainly expressed in the liver and is postulated to induce adipogenic transformation of hepatocytes with subsequent expression of adipocyte-specific genes i.e. adipsin and adiponectin, and accumulation of lipid. The development of steatosis secondary to PPAR-γ over-expression is therefore related to the activation of these adipocyte-specific lipogenic genes (adipogenic hepatic steatosis) [12]. ChREBP is required for basal and carbohydrate-induced expression of liver enzymes involved in the control of glucose metabolism and synthesis of fatty acids and triglycerides. In animal studies, ChREBP expression has been shown to be significantly increased in livers of ob/ob mice, and improvement in hepatic steatosis secondary to decreased lipogenesis in these animals was observed after liver-specific inhibition of ChREBP. ChREBP also induces pyruvate kinase to provide precursors for DNL. It is also a direct target of nuclear receptors, Liver X receptors (LXRs) [13], which are implicated in the lipogenic pathway through direct transcriptional activation of ACC and FASN, and indirectly via SREBP-1c.

Increased Lipolysis of Adipose Tissue

Obesity is highly prevalent in NAFLD and is characterized by increased white adipose tissue mass, which is tightly linked to insulin resistance [14]. The expansion of adipose tissue mass requires adipocyte-precursor cells, mesenchymal cells, vascular endothelial cells and macrophages [15], which are well known to express high levels of chemokines and adipokines i.e. resistin and visfatin [16]. These peptides are particularly essential for inflammation and energy homeostasis. Resistin induces lipolysis of TAG stores in macrophages, increases expression of FASN in adipocytes with subsequent increase in cellular TAG and free-fatty acids (FFA), and stimulates adipocyte triglyceride lipolysis [17, 18]. The excess influx of FFA in to the liver is then converted to TAG, thus contributing to hepatic steatosis.

Impaired Fatty-Acid Metabolism

Disturbance in fatty-acid metabolism results in excess lipid storage in the liver and contributes to hepatic steatosis. β(beta)-oxidation is confined to the mitochondria and peroxisomes, whereas CYP4A (cytochrome p450 enzyme 4A) catalyzed ω(omega)-oxidation occurs in the endoplasmic reticulum [19]. Mitochondrial dysfunction is therefore associated with NAFLD via impairment of fat homeostasis and overproduction of reactive oxygen species (ROS) [20]. In general, mitochondrial β-oxidation is required for the oxidation of short, medium, and long chain fatty acids, and key enzymes involved in this process are encompassed in the mitochondrial trifunctional protein (MTP), which is recessively inherited and associated with microvesicular steatosis in infants with the genetic defect. The less abundant and more toxic very-long-chain-fatty acids (VLCFA) and dicarboxylic acids require the peroxisomal β-oxidation for shortening before being completely oxidized in the mitochondria. An effective peroxisomal oxidation system is therefore crucial to minimize the toxic effects of these fatty acids on the mitochondria. Studies have shown that defects in key enzymes involved in peroxisomal β-oxidation led to severe steatosis and steatohepatitis in mice, which also exhibited very high serum levels of VLCFA [21]. Microsomal ω-oxidation is involved in the metabolism of saturated and unsaturated fatty acids by CYP4A enzymes. Although this is only a minor pathway of fatty acid metabolism, this oxidation system

remains crucial in conditions where there is over abundance of fatty acids in the liver (i.e. obesity and diabetes) and a significant amount of dicarboxylic acids are formed, and they can also serve as PPAR-α(alpha) ligands to activate all three oxidation systems [7, 19, 22]. In animal studies, PPAR-α knockout mice failed to induce fatty acid oxidation in the liver and subsequently developed severe hepatic steatosis. Treatment with exogenous PPAR-α agonists subsequently prevented and improved steatosis in these animals [23, 24].

Altered Insulin Signaling

Insulin resistance is associated with a complex set of changes that affect virtually every aspect of cellular and bodily function. Most people who develop NAFLD have an insulin resistance state, which is characterized by impaired metabolic clearance of glucose, thus resulting in diminished energy production. In these patients, hepatic glucose production is normal or high normal in the fasting state, despite basal hyperinsulinemia, indicating the presence of hepatic insulin resistance, in addition to whole body and adipose tissue insulin resistance. Based on our current understanding of the relationship between obesity, NAFLD, and insulin resistance (Fig. 34.2), it is well recognized that DNL in the liver is under the transcriptional control of SREBP-1c, which is upregulated by hyperinsulinemia. Insulin resistance also increases lipolysis and allows more FFA to be delivered to the liver, where they can either undergo oxidation or be re-esterified to triglycerides. A recent review interestingly has emphasized the hypothesis that specific lipid intermediates can indeed initiate pathways that alter insulin signaling in the first place [25]. Future studies to investigate these individual signaling molecules now await to be independently performed.

The mechanism by which obesity is associated with insulin resistance is unclear, but circulating hormones and adipocytokines secreted from adipose tissue have been implicated in modulating insulin sensitivity. Studies have shown that patients with abdominal obesity have very low levels of adiponectin and high levels of tumor necrosis factor-α (TNF-α) [26]. The secretion of TNF-α from adipose tissue is strongly associated with obesity-related insulin resistance, which suggests that TNF-α may function in a paracrine fashion in adipose tissue; in contrast, expression of adiponectin from adipose tissue is associated with higher degrees of insulin sensitivity and lower TNF-α expression [27]. In addition, TNF-α has been shown to decrease levels of adiponectin [28]. Thus, a combination of increased TNF-α and decreased adiponectin leads to severe insulin resistance, which in turn leads to NAFLD.

Macrophages within the adipose tissue are a major source of TNF-α, and adipocytes secrete most of the leptin and other pro-inflammatory cytokines i.e. interleukin (IL)-6 in adipose tissue [29]. Various experimental models have demonstrated that both TNF-α and IL-6 contribute to the development of insulin resistance, primarily via inhibiting the activation of insulin receptor substrate (IRS)-1 [30], competing for docking sites by suppressors of cytokine signaling (SOCS), and by elevating plasma FFA via stimulation of

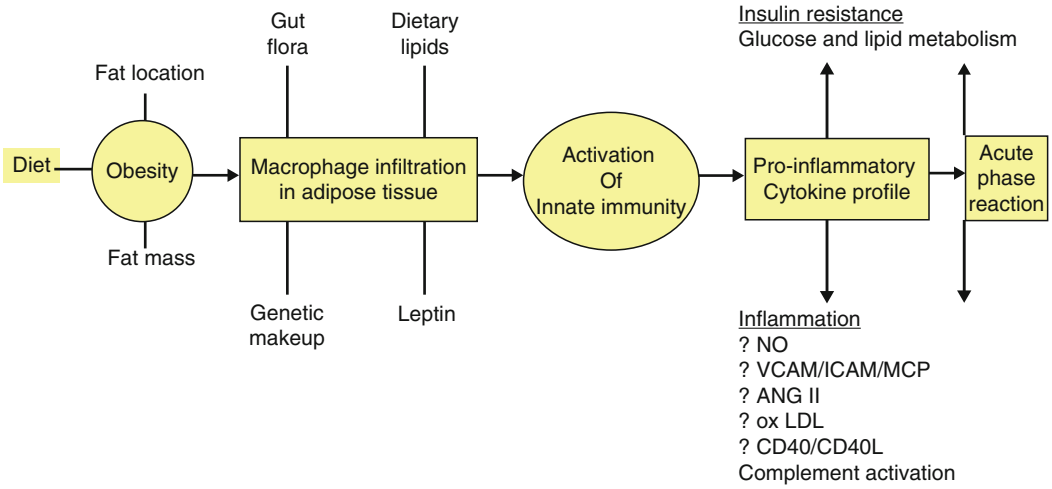

Fig. 34.2 Relationship between obesity, inflammation, and insulin resistance. In the setting of obesity, multiple factors such as gut flora, tissue hypoxia, diet, leptin and genetic factors lead to macrophage infiltration of adipose tissue. This results in activation of the innate immune system, proinflammatory cytokine signaling, and activation of the acute phase response that exacerbates insulin resistance and perpetuates inflammation. *ANG II,* angiotensin II; *ICAM,* intercellular adhesion molecule; *MCP,* monocyte chemoattractant protein; *NO,* nitric oxide; *VCAM,* vascular cell adhesion molecule

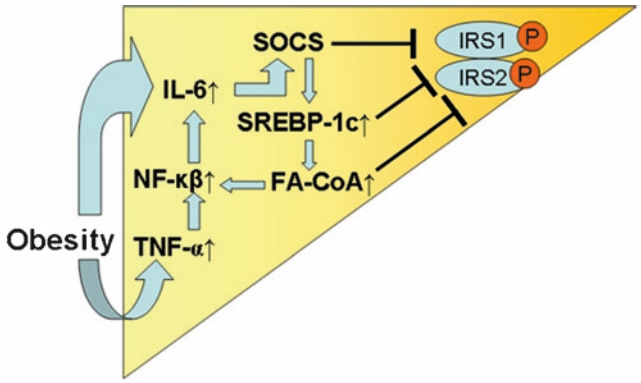

Fig. 34.3 Causes of hepatic insulin resistance. Obesity mediates an overall proinflammatory response that promotes insulin resistance by increasing hepatic SOCS expression via IL-6. SOCS inhibits insulin signaling by directly competing for the phosphorylation sites available on the insulin receptor, and indirectly by activating SREBP-1c. SREBP-1c decreases the synthesis of insulin receptor substrate 2 (IRS-2) and increases fatty-acid synthesis. This increase in fatty acyl CoA (FA-CoA), which also comes from dietary intake, DNL, and impaired β(beta)-oxidation, then activates the NF-κ(kappa)β(beta) pathway that leads to the release of IL-6 that augments the induction of SOCS

adipose-tissue lipolysis [31, 32] (Fig. 34.3). FFA impairs insulin signaling in striated muscle and decreases the metabolic clearance of glucose. A recent study has suggested that TNF-α can induce insulin resistance and whole body glucose uptake via IL-18 in muscle tissue. Studies have demonstrated that increased circulating FFA levels heightens the production of TNF-α from adipose tissue and raises their circulating levels, these in turn contribute to increased systemic inflammation and insulin resistance observed in obesity [33].

Endocannabinoids Signaling

Endocannabinoids are endogenous lipid compounds that bind and activate the cannabinoid receptors (CB1 and CB2) located both centrally and peripherally. Both CB1 and CB2 receptors belong to the family of G protein-coupled receptors and show a wide tissue distribution with distinct specificities. CB1 receptor is most abundant in the brain; however, it is also widely distributed at significant levels, in organs that control energy balance, i.e. adipose tissue and liver. CB2 receptors are expressed at lower levels than CB1 receptors and predominate in cells of the immune system. In addition to their anticipated CB1-dependent central effects, cannabinoids also display a wide variety of CB1- or CB2-mediated peripheral functions, including regulation of energy balance, immune and inflammatory responses, vasoregulatory and lipogenic effects [34]. Anandamide is an endogenous cannabinoid that activates the CB1 receptor in hepatocytes, causing overexpression of the lipogenic transcription factor SREBP-1c and its targets ACC and FASN, thus contributing to *de novo* fatty acid synthesis in the liver [35]. High fat diet increases hepatic levels of anandamide, CB1 density, and basal rates of fatty acid synthesis. In vivo studies have shown that CB1-knockout mice were resistant to diet-induced fatty liver, and wild type mice with diet induced fatty liver exhibited a significantly higher hepatic anandamide concentration than lean controls [35]. Aside from lipogenic effects in the liver, activation of CB1 receptors also affects lipolysis in the adipose tissue, thereby enhancing the influx of triglycerides in the liver. CB2 receptor antagonism has been shown to blunt hepatic steatosis and therefore unravels its potential role in the control of disorders associated with the metabolic syndrome. Currently, data on the activation of the endocannabinoid system in relation to altered lipid metabolism in human NAFLD is lacking. Additional studies to further explore this area of interest are warranted.

MicroRNA Signaling and Its Emerging Role in Lipid Metabolism

microRNAs (miRNA) are small naturally occurring single-stranded RNA that are generated from endogenous transcripts encoded in the genomes of humans, animals, viruses and plants. Since its first discovery in 1993 by Ambros et al. [36], more miRNAs in various organisms have been identified. Currently, a total of 873 miRNAs have been reported in human (miRBase 11.0, April 2008). The expression of miRNA is both organ-specific and dependent on the stage of development [37, 38]. They have diverse functions including regulation of important cellular processes e.g. cancer, cell metabolism, immune function, cell proliferation, apoptosis, and tissue development and differentiation [39, 40].

As shown in Fig. 34.4, miRNAs in animals are first transcribed as long primary transcripts (pri-miRNAs) by RNA polymerase II enzyme. After additional processing by the nuclear RNase III enzyme Drosha, the pre-miRNA intermediates are exported out of the nucleus by exportin-5 and are subsequently cleaved by the cytoplasmic RNase III Dicer into ~22-nucleotides miRNA duplexes [29, 41, 42]. The mature miRNA is then incorporated into a ribonucleoprotein complex, miRNP that is similar to the argonaute containing RNA-Induced Silencing Complex (RISC). The RISC–miRNA combination mediates downregulation of the target mRNA activity by translation inhibition; its mechanism of action remains controversial, with only limited in vivo functions of miRNAs that have been demonstrated in any organism.

A recent study has demonstrated the differential expression of miRNAs in subjects with NASH [43]. The potential targets of these differentially expressed miRNAs are known

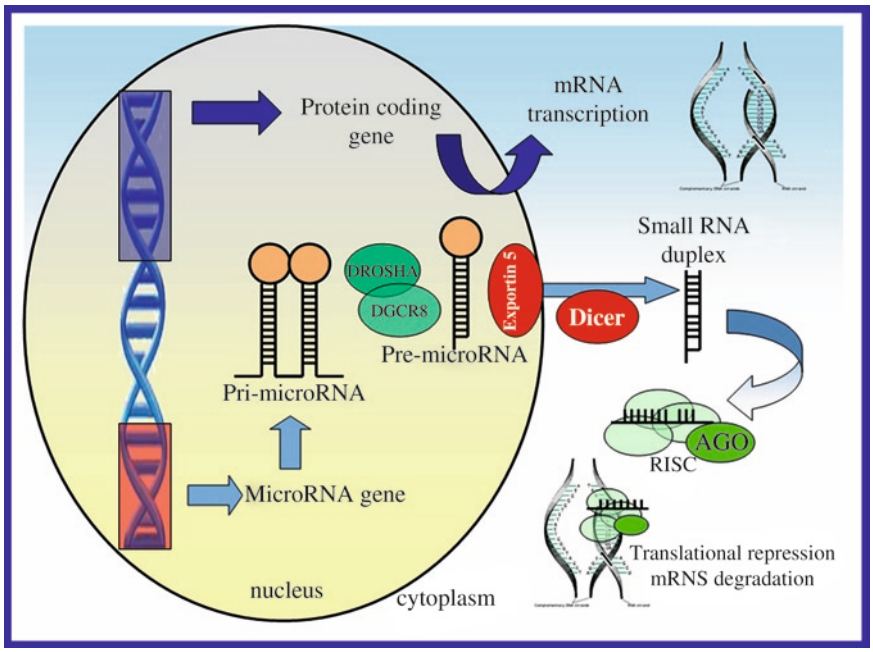

Fig. 34.4 A model for the microRNA biogenesis pathway. See text for details

to play a role in lipid metabolism, cell growth and differentiation, apoptosis, and inflammation. The potential consequences of these changes can affect insulin signaling, lipid metabolism, cellular responses to stress and apoptosis, inflammation and response to tissue injury (Table 34.1). Specifically, miR-122 is of particular interest and was found to be under-expressed in subjects with NASH when compared to matched control [43]. miR-122 is the most abundantly expressed miRNA in the human liver where it accounts for almost 70% of all miRNAs. It has also been shown to play an important role in lipid and cholesterol metabolism, and adipocyte differentiation [39, 44, 45], which are at the core of fatty liver disease. Several studies have shown that the inhibition of miR-122 in a diet-induced obesity mouse model resulted in decreased mRNA expression of ACC2, FASN, SCD-1, and HMG CoA reductase, and also significant reduction in plasma-cholesterol levels [39]. Histologically, these animals showed substantial improvement in liver steatosis after 2 weeks of miR-122 silencing treatment. In another recent study by Krutzfeldt et al, HMG CoA reductase and plasma-cholesterol levels in mice were significantly reduced after silencing of miR-122 [44]. These findings strongly suggest the significance of microRNA in the regulation of lipid metabolism and their contribution to the development of hepatic steatosis.

While the regulation of miRNA function and its mechanism of actions on translational control of target mRNA expression remain unknown, advances in miRNA research allow identification and biochemical characterization of events that limit protein expression. It is hoped that by having a complete and accurate understanding of the functions of miRNAs in the regulation of lipid metabolism, potential therapeutics and preventive measures can be developed to modify and alter the NAFLD disease progression.

Mechanism of Cell Injury

FFA-Induced Cellular Lipotoxicity: A Manifestation of Oxidative Stress and Mitochondrial Dysfunction

FFAs are important mediators of cellular lipotoxicity. The exact mechanisms of their cytotoxic effects are unclear, but can involve multiple independent yet closely linked regulatory and signaling pathways. Among these is mitochondrial dysfunction secondary to ROS overproduction, lipid peroxidation, and oxidative stress [46]. These processes subsequently result in increased CYP2E1 expression [47, 48], which enhances NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) activity and results in further increased in ROS production. Chronic oxidative stress also leads to lysosomal dysfunction and abnormal cytokine secretion particularly that of inflammatory cytokines (i.e. TNF-α and IL-6). It can also lead to dysregulated adipocytokine production in adipose tissue [49]. In the setting of insulin resistance and abnormal lipid metabolism, their effects are predominantly pro-inflammatory. Since the principal metabolic effect of these cytokines is a relatively greater activity of hormone sensitive lipase at any given level of plasma insulin, this

Table 34.1 Examples of microRNA and their potential gene targets

Functional class	Gene target	Examples of microRNA
Transcription factors	Homeobox protein (HOX)	146b, 199a*, 455, 128b, 128a, 145, 92b, 122
	Hepatocyte nuclear factors (HNF)	146b, 214, 23b, 617, 375, 92b, 26b
Protein synthesis and processing	Eukaryotic translation initiation factor (eIF)	23a, 23b, 146b, 128a, 145, 139, 125b
	Ubiquitin conjugating enzymes (UBE)	23b, 146b, 27b, 199a*, 16, 128a, 128b, 122
	X-box binding protein 1 (XBP1), EDEM	34a, 125b, 200a, 214
Oxidative-stress response	Cytochrome C-oxidase	99b, 423, 27b, 127, 128a, 128b, 601, 198, 361
	Biliverdin reductase	99b, 100, 221
Cell growth and cell cycle	Interferon regulatory factor (IRF)	16, 24, 23a, 23b, 125b, 128a, 128b, 214, 145
Cell differentiation	Cyclin dependent kinases (CDK)	34a, 199a*, 99b, 26, 122
	Phosphatidylinositol kinases (PIK)	23b, 27b, 26b, 145
	FAD104, AEBP2, MMD	23b, 125b, 181a, 122, 146b, 23b, 146b, 26b
Anti-apoptosis	BCL2L (1, 2)	16, 24, 214, 200a, 145, 92b
	BCL2L (7, 11)	27b, 125b, 16, 24, 181, 199a*, 224, 24, 26b
Pro-apoptosis	PDCD4, GAS1	16, 21, 199a*, 200a, 34a, 122, 145
Anti-inflammatory	Suppressor of cytokine signaling (SOCS)	16, 199a*, 214, 23a, 23b, 27b, 125b
	Mitogen activated protein kinases (MAPK)	23b, 125b, 199a*, 214, 27b, 128a, 128b, 145
Pro-inflammatory	C-jun kinase	27b, 128a, 128b, 214
	Interleukin precursors	125, 214, 16, 128a, 128b, 23b, 122, 188, 191*
Cholesterol metabolism	Oxysterol binding proteins	128b, 146b, 214, 181b, 23a, 23b, 199a*, 145
Fatty acid metabolism	HMGCR, ACAT	125b, 224, 122, 145, 188, 375
Carbohydrate metabolism	Oxysterol receptor LXR	423, 145, 191*, 375, 574, 92b, 139
Insulin signaling	PPAR, ACSL, ACC, DGAT, SREBP, ACLY, FAS	128a, 128b, 146, 122, 125b, 214, 23b, 16, 188
	AMPK, SCD, FABP, LDLR, VLDLR, ELOV	30d, 199a*, 23a, 181b, 200a, 203
	ALDOA, GYS, glycogenin, PRKC, PYGL	23b, 27b, 34a, 221, 21, 125b, 128b, 181b, 122
	IRS1, myotrophin, Islet1	23a, 16, 27b, 128a, 128b, 375

Gene abbreviations were adopted from Ensembl and NCBI

results in a net increase in peripheral lipolysis and release of FFAs in to the circulation. FFAs themselves are pro-inflammatory, specifically the n6 polyunsaturated fatty acids (PUFAs) class. In fact, the most studied of these is arachidonic acid which is oxidized to eicosanoids (i.e. prostaglandins, leukotrienes, and thromboxanes), which are potent mediators of inflammation. These oxidized PUFAs can cause lipid peroxidation and alteration of phospholipids composition, resulting in changes in cell-membrane fluidity [50, 51]. Alteration in eicosanoid production, along with the direct effects of lipids on gene expression further modulate the activity of transcriptional factors and expression of cell-surface molecules e.g., histocompatibility antigens [52]. This subsequently interferes with antigen presentation by macrophages and modulates phagocytosis. FFAs also activate the proapoptotic protein Bax, in a c-jun N-terminal kinase (JNK)-dependent manner, and the lysosomal pathway of cell death and regulate death receptor-gene expression [53]. Delivery of FFAs may also render hepatocytes more vulnerable to bile acid-induced apoptosis by means of increased caspase 3/7 activity and transcription of IL-8 and IL-22 [54]. Polyunsaturated fatty acids (PUFA) of the n3 family on the other hand, exhibit anti-inflammatory properties by inhibiting lymphocyte proliferation, antibody and cytokine production (i.e. TNF-α, IL-1), adhesion molecule expression, natural killer-cell activity, and triggering cell death.

Endoplasmic Reticulum Stress and the Unfolded Protein Response

Another potential mechanism of FFAs induced-hepatocellular injury involves activation of the endoplasmic reticulum (ER) stress and dysregulation of the unfolded protein response (UPR). This was clearly demonstrated in a recent study that the UPR was dysregulated in patients with NAFLD [55]. Dysregulation of the UPR is specifically characterized by the translational failure to generate the X-box binding protein (XBP), thus inhibiting the expression of its downstream target EDEM (ER degradation enhancing mannosidase) that mediates the proteosomal degradation of the unfolded protein [56]. The failure to restore protein homeostasis within the cell due to an imbalance between decreased protein synthesis and increased protein degradation results in activation of the alarm pathways, i.e. JNK, thus promoting cellular injury with increased inflammation and apoptosis. A comprehensive account on the molecular mechanisms of liver-cell death is also available elsewhere in the text (see Chap. 24). This observation was also corroborated by a recent study that demonstrated the significance of JNK dependent lipoapoptosis in FFAs induced cellular injury [57]. Furthermore, it has recently been shown in a human study that NASH is associated with

differential expression of miRNAs. Specifically, miR34a and 451 were both found to be overexpressed in subjects with NASH, and they both are predicted to target XBP translation [43].

Mechanism of Cell Injury: Interactions Between Macrophages and Adipocytes

Macrophages have important roles in the complex relationship between obesity, inflammation, and insulin resistance that are determined by whether they are classically or alternatively activated by T-helper-1 (Th1) or T-helper-2 (Th2) cytokines, respectively. To learn about the role of inflammation in liver injury, please see Chap. 27. Th1 leads to insulin resistance and Th2 to protection against this condition. However, the mechanisms by which macrophage infiltration occurs and its interaction with adipocyte within the white adipose tissue remain to be fully defined. Ferrante et al. has recently reported that the C–C motif chemokine receptor–2 (CCR2) is responsible for the regulation of macrophage recruitment and is necessary for macrophage-dependent inflammatory responses. The study has shown that in obese mice, both genetic CCR2 deficiency and pharmacological inhibition of CCR2 reduced macrophage content and the inflammatory profile of adipose tissue, as well as improving systemic glucose homeostasis and insulin sensitivity [58]. It has been suggested in a recent study that macrophage infiltration into adipose tissue, during immune responses, is dependent on the expression of osteoponin, an extracellular matrix protein and proinflammatory cytokine that promotes monocyte chemotaxis and cell motility [59]. In this study, mice exposed to high-fat diet exhibited increased plasma osteoponin levels, and increased macrophage infiltration into adipose tissue. These animals were also found to be insulin resistant and have elevated levels of inflammatory markers. Another recent study that is noteworthy to mention demonstrated the potential anti-inflammatory effect of PPAR-gamma activation on a mouse model of obesity-induced inflammation [60]. The study corroborated with the finding that during diet-induced obesity, the phenotype of adipose-resident macrophages changes from alternatively activated form to the more classical and pro-inflammatory phenotype. PPAR-gamma treated mice increased adipose mass, reduced liver triglycerides, and changed adipose tissue morphology towards smaller adipocytes that are generally less metabolically active compared to larger adipocytes [61]. PPAR-gamma treatment also markedly increased the number of alternatively activated macrophages in adipose tissue, as demonstrated by upregulation of anti-inflammatory marker IL-10 in adipose tissue from this group of animals.

Innate Immunity, Role of Cytokines and Adipokines

Activation of the innate immune system plays an important role in both the development of the metabolic syndrome and in the sustenance of a systemic pro-inflammatory and profibrogenic state. The necro-inflammatory component of NASH has indeed been shown to be modulated by interactions among various factors such as cytokines and adipokines that regulate the biological activity of TNF-α and other proinflammatory (Th-1) cytokines. An emerging number of cytokines and adipokines have been identified and they support a complex interaction between each other and the pathogenesis of NAFLD (Table 34.2) [62, 63]. Adipokines are believed to act through their effects on insulin sensitivity. For example, adiponectin and leptin have been shown to decrease insulin resistance, while others, including TNF-α, IL-6, and resistin enhance insulin resistance. Indeed, NASH has been shown to be associated with lower adiponectin levels and higher HOMA–IR (Homeostatic model assessment–insulin resistance). HOMA–IR and low serum adiponectin are independently associated with increased grades of hepatic necroinflammation. Interestingly, hypoadiponectinemia has been shown to present before overt diabetes and obesity appear, thus indicating its pathogenic role in pancreatic beta-cell dysfunction

Table 34.2 Adipocytokines and their known effects on inflammation

Adipocytokines	Effect in inflammation
TNF-α(alpha)	↑ ICAM-1, VCAM-1, MCP-1
	↑ E selectin
	↑ EC apoptosis
	↑ NF-kB
Leptin	↑ NO and ET-1
	↑ EC and VSMC proliferation
	↑ Macrophage survival/proliferation
	↑ Angiogenesis
IL-6	↑ ICAM-1, VCAM-1, MCP-1
	↑ Acute phase reactants
ANG II	↑ ICAM-1, VCAM-1, MCP-1
	↓ NO and ↑ NF-kB
	↑ Fibrosis
Resistin	↑ ET-1 release
	↑ ICAM-1, VCAM-1, MCP-1
	↓ TRAF-3
PAI-1	↓ Fibrinolysis
Adipsin	Complement activation
Adiponectin	↓ ICAM-1, VCAM-1, selectins
	↓ NF-κ(kappa)β(beta)
Visfatin	↑ Susceptibility to endotoxin
	Mediated EC injury in lungs

ANG II, angiotensin II; *EC,* endothelial cell; *ET,* endothelin; *ICAM,* intercellular adhesion molecule; *IL,* interleukin; *MCP-1,* monocyte chemoattractant protein; *NF,* nuclear factor; *NO,* nitric oxide; *TRAF,* tumor necrosis factor associated factor; *VCAM,* vascular cell adhesion molecule

and hepatic necro-inflammation and fibrosis, independent of insulin resistance [64]. While it is widely accepted that TNF-α expression increases in patients with NASH, the mechanisms driving chronic overproduction of this specific cytokine remain unknown, though recent studies have speculated an increased prevalence of TNF-α polymorphism in these subjects [65]. Animal studies have clearly demonstrated the role of TNF-α in hepatic insulin resistance. Ob/ob mice treated with neutralizing anti-TNF antibodies have decreased activities of JNK and IKKb (inhibitor of K-Kinase b); both of these kinases have been found to cause cellular-insulin resistance, and both are targets of TNF-α initiated activation. Noteworthy to mention is another class of molecule, sterols that can modulate the innate immune system particularly via the orphan nuclear receptor LXR, which in turn mediates the expression of cytokines, i.e. vascular endothelial growth factor, and genes involved in macrophage survival [66–68]. The role of dietary cholesterol and its processing into various forms of modified plasma lipoproteins has been implicated as an important risk factor for the progression to hepatic inflammation in diet-induced mouse model of NASH. Hyperlipidemic (low-density lipoprotein receptor-deficient and apolipoprotein E2 knock-in) mice fed a high fat, high cholesterol diet developed severe inflammation characterized by hepatic infiltration of macrophages and increased nuclear factor kappaB (NF-κ(kappa)β(beta)) signaling compared with livers of normolipidemic wild type animals. These results suggested that dietary cholesterol in the form of modified plasma lipoprotein plays an important role in inflammation and the progression to NASH [69].

Within the liver, the products of hepatocyte injury and cytokine milieu combine to promote inflammation. However, although sustained exposure to these inflammatory mediators promotes the generation of various pro-fibrogenic and apoptotic factors, progression from NASH to end stage liver disease, i.e. cirrhosis is relatively uncommon. This suggests that other factors, perhaps genetics may be involved in subpopulations of individuals who may have hepatic innate immune system defects. Furthermore, the precise mechanisms of various cytokines and adipokines in the link between insulin resistance, obesity, and NAFLD remain to be clarified.

Molecular Basis of Fibrosis

Hepatic Stellate Cell Activation

Fibrosis is a dynamic process of continuous extracellular matrix (ECM) remodeling in the setting of chronic liver injury, which leads to an excessive accumulation of extracellular proteins, proteoglycans, and carbohydrates. To learn more about the molecular basis of hepatic fibrosis, please see Chap. 30. Hepatic stellate cells (HSCs) play a key role in hepatic fibrogenesis, regardless of the underlying etiology. A more comprehensive account on this cell type is included in Chap. 5. In the normal liver, HSCs comprise approximately 1.4% of total liver volume, or 5–8% of the total liver-cell population. These cells are typically located in the perisinusoidal space of Disse, a recess between endothelial cells of sinusoids and hepatocytes. When the liver is injured, HSCs are activated and transitioned into highly proliferative and contractile myofibroblastic cells that have several new phenotypic characteristics, i.e. enhanced cell migration and adhesion, increased proliferation, production of chemotactic substances capable of recruiting inflammatory cells and other HSCs, and most importantly, acquisition of fibrogenic capacity. After the initial activation of HSCs, other cells present in the normal liver, such as hepatocytes, Kupffer cells, sinusoidal endothelial cells, platelets, and activated HSCs themselves can produce cytokines and other mediators to perpetuate the process of matrix remodeling and accumulation. Leptin and insulin have been shown to stimulate HSC to increase collagenous matrix production [70, 71]. In addition, accompanying these cellular process are the loss of retinyl esters (RE) and increased smooth muscle α-actin (α-SMA) expression [72]. The inhibition of diacylglycerol acyltransferase (DGAT1), an enzyme that plays a critical role in TG biosynthesis, has been shown to reduce HSC activation and liver fibrosis in mice with diet-induced NASH [73]. The proposed mechanism is that RE stores decrease during HSC activation and liver fibrosis, and retinol esterification is mostly catalyzed by lecithin-retinol acyltransferase (LRAT) and DGAT1. While the inhibition of LRAT reduced hepatic RE store without affecting hepatic fibrosis, DGAT1, closely related to LRAT, repressed the transcription of collagen A1 and A2.

Our understanding of the cellular and molecular mechanisms involved in hepatic fibrogenesis has increased exponentially in recent years. Some of the key steps in this process, HSCs and their complex pathophysiologic interactions have been further refined. In addition to the previously mentioned mechanisms, matrix production has been shown to be modulated by HSC apoptosis, which can be affected by the activation of the cannabinoid receptor [74]. Furthermore, studies have demonstrated that HSC activation is characterized by the loss of adipogenic transcriptional regulation that is required for the differentiated phenotype of HSC [75]. Several major transcriptional factors involved in adipocyte differentiation include the CCAAT/enhanced binding protein family (C/EBP), PPAR-gamma, liver X receptor, and SREBP-1 [76]. The expression of these transcription factors and downstream genes are quite abundant in quiescent and differentiated HSC but are lost upon activation. A gain of function approach by treatment with an adipocyte differentiation agent or ectopic

transduction of PPAR-gamma or SREBP-1c restored the quiescent phenotype of HSC [75], thus suggesting the therapeutic potential of adipogenesis antagonism for liver fibrosis. The endocannabinoid system is also related to the development of hepatic fibrosis. In fact, CB2 receptor-dependent effects are also increasingly characterized, particularly it's upregulation during chronic liver diseases and effects on multiple steps of this sequence including hepatocyte injury and inflammation, and fibrogenesis [77]. CB2 receptors signal anti-fibrogenic responses, as demonstrated in both in vitro and in vivo experiments that CB2 receptor activation induces growth inhibition and apoptosis of cultured liver fibrogenic cells, following activation of cyclooxygenase-2 and oxidative stress, respectively [78]. These studies hopefully will shed light in the search of new strategies capable of blocking essential pathways in liver fibrosis.

Genetic Polymorphisms

Based on human studies of hepatic-gene expression in subjects with NASH, genes, particularly those involved in lipid metabolism have been shown to be under-expressed [79]. Specifically, microsomal triglyceride transfer protein (MTTP) that plays a role in the synthesis and secretion of VLDL is of particular interest in the context of dysregulation of lipid metabolism and steatosis. Both in vivo and human studies have shown that a single nucleotide polymorphism in the promoter region reduces the level of MTTP and leads to the accumulation of TAG in the liver, and biochemically it also leads to increased postprandial plasma levels of TAG and FFAs post oral fat-tolerance test [80, 81]. Adiponectin SNPs were found to be associated with the progression of liver fibrosis and insulin resistance, suggesting that adiponectin SNPs might play roles in the occurrence and progression of NAFLD. It has also been reported that a genetic polymorphism in the promoter region of the resistin gene may be an independent predictor of circulating resistin in humans [82]. Other genes involved in glucose metabolism, insulin signaling, inflammation, coagulation, and cell adhesion have also been reported to be significantly associated with steatosis [83]. Genes involved in fatty acid transport (i.e. fatty acid binding protein), amino acid catabolism (i.e. BCAT), and inflammation (CCL2) were found to be unregulated in human subjects with NAFLD [84]. Genetic variation in angiotensin II type 1 receptor (ATGR1) has also recently been recognized to influence the risk of NAFLD and liver fibrosis in NAFLD. Another potential human gene, Patatin-like phospholipase domain containing 3 (PNPLA3) that encodes triacylglycerol lipase, which mediates triacylglycerol hydrolysis in adipocytes has been shown to play a role in the inter-individual differences in hepatic fat content and hepatic inflammation. Several different PNPLA3 alleles have indeed been found to be present in certain ethnic groups, thus rendering varying degree of potential genetic susceptibility to NAFLD in those individuals [85]. Since only a minority of patients who are obese or diabetic will develop severe liver disease, this in part suggests that the progression of NAFLD may also be determined genetically. For this reason, much research efforts to better understand and contain the role of genetics have been recognized, and hence it may be possible to explain the discrepancy between these complex interactions in the mechanisms of disease pathogenesis.

Summary

NAFLD is an emerging metabolic-related disorder and can progress to end stage liver disease. The increased accumulation of intracellular fatty acids within hepatocytes leads to the development of hepatic steatosis, which is followed by the development of necroinflammation and fibrosis. Insulin resistance and abnormal lipid metabolism are key factors in the pathogenesis of NAFLD. There is also mounting evidence that cytokines secreted from adipose tissue, namely, adipokines, are implicated in the pathogenesis and progression of NAFLD. Several cell-signaling and regulatory pathways such as the ER stress, microRNAs, and oxidative stress have been demonstrated to contribute to the final common fate of cell injury via immumodulation and ultimately necroinflammation and cell death. While many studies have revealed the mechanism associated with these signaling cascades, the exact mechanisms related to each of these molecular components, however, have yet to be completely characterized.

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Chapter 35
Alcoholic Liver Disease

Samuel W. French

Introduction

Alcoholic liver disease (ALD) remains a significant health burden in the USA and worldwide. The histological spectrum of this disease spans from simple steatosis to steatohepatitis (characterized by inflammatory cell infiltration, hepatocyte ballooning, apoptosis, and necrosis in addition to fat accumulation) to fibrosis and overt cirrhosis. Steatosis in this setting is mainly macrovesicular and most prominent in the centrilobular region or zone 3. Early stages of alcohol-induced liver injury are reversible and prevention of steatosis in experimental models has been shown to prevent progression to inflammation and fibrosis.

Primer on the Histopathology of Alcoholic Liver Disease

A detailed review of the histopathology of ALD has been published previously [1]. Here, selected photographs of the histopathology will be highlighted. The four main stages of ALD are: (1) Steatosis, (2) Steatohepatitis (formerly designated as alcoholic hepatitis or ASH), (3) cirrhosis, (4) combined steatohepatitis and cirrhosis. Steatohepatitis is somewhat of a misnomer because the steatosis can be minimal or absent at this stage.

Steatohepatitis is illustrated in Fig. 35.1a. The fat globules in the hepatocytes are large and single (macrovesicular fat). When steatosis progresses to steatohepatitis, balloon cell degeneration develops where hepatocytes become round, swell, and have empty looking cytoplasm (Fig. 35.1b). This is a necessary component of steatohepatitis. The balloon cells often form Mallory-Denk bodies (MDBs) which stain for cytokeratins 8 and 18 (Fig. 35.1c). The normal cytokeratin distribution in the cell is lost in these cells. The MDBs stain positive for ubiquitin (Fig. 35.1d). Lymphocyte infiltration is sometimes prominent (Fig. 35.1e). PMN infiltrate is prominent when single cell necrosis develops (Fig. 35.1f). Stellate cell activation (smooth muscle α(alpha) actin positive) develops (Fig. 35.1g) to cause progressive fibrosis seen on the reticulin stain (Fig. 35.1h). Finally, micronodular cirrhosis develops (Fig. 35.1i) with bile duct metaplasia of liver cells.

Molecular Pathology of ALD

Molecular pathology of ALD is understood primarily at the level of gene transcription regulation, which is controlled through epigenetic mechanisms. The dysregulation that occurs during the onset and progression of ALD involves primarily genetic and epigenetic alterations in the liver cells as being at the core of the established diseased state. The epigenetic changes involved in dysregulation of gene expression include a variety of covalent modifications that affect the methylation status of DNA. It also includes the post-translational modifications of histones. The modifications determine the structural features of chromatin that ultimately control the transcriptional outcomes of liver cells in order for them to adapt to requirements. Also small noncoding environmental microRNAs that regulate the expression of complimentary messenger RNAs function as key controllers in a myriad of cellular processes including cellular proliferation, differentiation and apoptosis.

Most experimental studies reported are based on tissue culture models to determine the mechanisms involved in epigenetic changes. In vivo based studies are difficult to interpret and are largely limited to global changes in gene expression as seen by microarray analysis or assays, which involve functional pathways of signal transduction. It is difficult to relate functional or specific pathological changes in the intact liver to changes in DNA methylation, histone modifications or microRNA interference in transcription, translation, and post translational modifications.

Whether the approach is in vivo or in vitro, the important concepts investigated should be focused on mechanism of adaptation to injury by the liver. The concept is that gene expression reprogramming of liver cells in response to injury

S.W. French (✉)
Division of Anatomic Pathology, Department of Pathology,
Harbor UCLA Medical Center, Torrance, CA, USA
e-mail: french7@ucla.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_35, © Springer Science+Business Media, LLC 2011

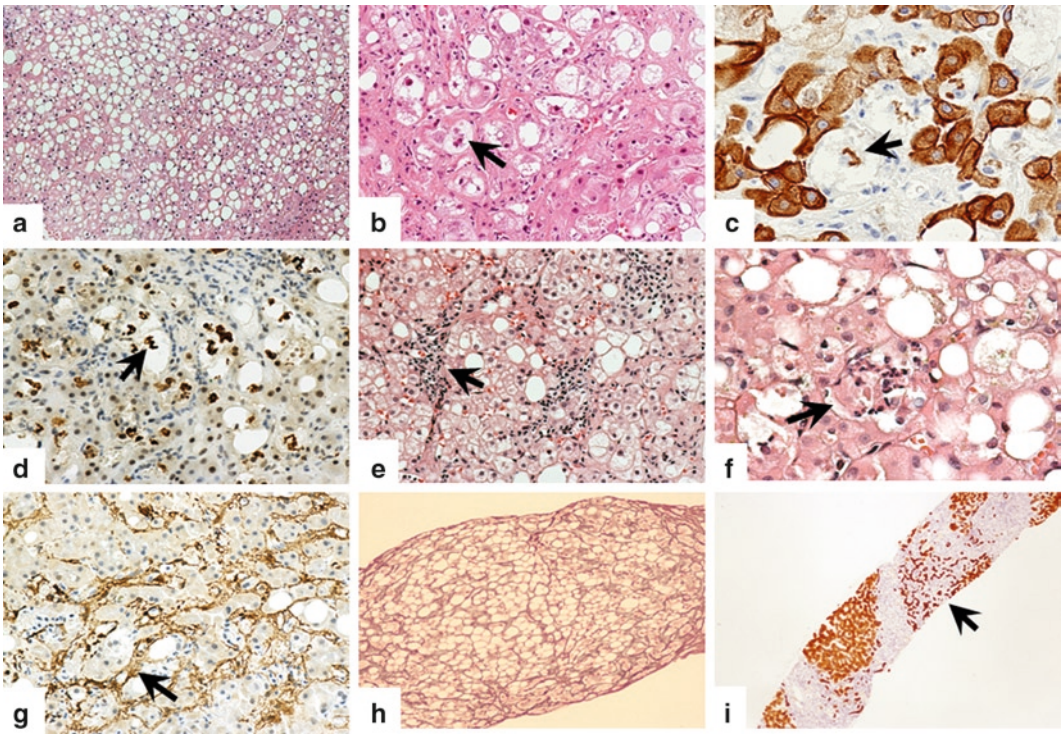

Fig. 35.1 (**a**) Macrovesicular fat (steatosis) × 109; (**b**) Balloon degeneration (arrow) × 218; (**c**) Mallory-Denk body (MDB) formation in balloon cells, empty cells stained for CK8 and 18 × 436; (**d**) MDBs stained for ubiquitin (arrow) × 218; (**e**) lymphocytic infiltration in the lobular parenchyma (arrow) × 218; (**f**) PMN infiltrate, necrotic liver cell (arrow) × 436; (**g**) activated stellate cells stained positive for smooth muscle actin (arrow) × 218; (**h**) pericellular fibrosis, reticulin stain × 109; (**i**) micronodular cirrhosis showing liver-cell nodules, bridging scars and duct metaplasia (arrow), CK8 and 18 stain × 44

creates a newly induced liver-cell phenotype which is heritable. Thus the changed liver-cell pattern of gene expression is permanently altered. In the case of liver cell-injury caused by alcohol abuse, the liver-cell phenotype is altered as an adaptation to alcohol metabolism. The new phenotype created persists in the absence of alcohol (periodic alcohol abstinence) and sensitizes the liver to injury when alcohol abuse is reinstituted. Hence, fatty livers, balloon degeneration, MDB formation, apoptosis and fibrosis develop episodically with progression even in the absence of alcohol abuse. This concept of ALD pathogenesis was recently advocated in a review by Zakhari and Li [2] who recommend that studies on how alcohol causes liver disease should focus on the quantity of alcohol consumed and patterns of drinking.

Microarrays and Global Changes in Gene Expression

Microarray Analysis in Animal Studies

It is logical to start the investigation of the molecular basis of ALD by using a global survey of changed expression of genes using microarray analysis of livers, which have been altered by chronic ethanol feeding. This approach may reveal key changes in gene expression regulation which focus on further investigation. The prior approach has been to focus on specific functional pathways of signal transduction rather than chromatin alterations. This approach starts with environmentally triggered signal transduction events rather than the global integration of changes at the chromatin level. The results of microarray analysis have established global epigenetic changes in gene expression, orchestrating complex, and integrated pathologic processes. It has become obvious that it is naive to suppose that alterations of a single signal transduction pathway could account for a complex disease process, partly because signal transduction responses are short lived and if repeated lead to adaptive mechanisms. This concept applies to the pathogenesis of ALD.

Microarray analyses of the livers from rats fed with ethanol by intragastric tube were first reported in 2001 [3, 4]. The blood alcohol levels (BALs) were high or low at sacrifice in order to determine if the BAL was a determinate of gene expression in a chronic model in which alcohol was delivered at a constant rate (13 g/kg/day) for 2 months. In this model, the livers were very fatty with inflammation, necrosis, and fibrosis. Clontech rat 1.2 array (1,200 genes) and Rat Stress array (472 genes) were used. Some changes in gene expression were confirmed by Northern blot and Western blot. Two

or more fold changes were reported. When the BALs were high (~400 mg%), the expression of some genes were increased: Tyrosine phosphatase, CYP2E1, MAPkinase 3, DNA damage inducible protein 45, D-aminoacid oxidase, catechol-0-methyltransferase, NADH-cytochrome B5 reductase, and ADH-1 and fatty acid binding protein. When the blood ethanol levels were low, expression of some genes were up regulated: IGF-1, retinoid receptor alpha, JNK2, and beta actin. By RT-PCR, MnSOD was decreased at high BAL; NOS was decreased at low BAL; COX2, CTGF, and MCD-1 were decreased at high BAL and increased at low BAL; VEGF and TNFα(alpha) were increased at both high and low BAL; MCP-1 was increased at high BAL; Bax and Bcl-2 were increased at low BAL. Since the expression of genes varies depending on the BAL even when the diet and ethanol ingested were constant, the BAL was the only variable. Therefore, it is necessary to take BAL under consideration when interpreting gene expression changes. It is possible to make a correlation between the changes in gene expression and differences in pathologic changes at high and low levels of BAL. For instance, body temperature and hormone levels are different at high and low BALs [5]. The severity of the liver pathology is worse at high BAL. The serum levels of LPS were higher at high BAL. TNFα(alpha) levels were equally high, both at high and low BAL. ALT levels were higher at low BAL, indicating that necrosis was worse at low BAL. The liver is hypoxic at high BAL. ATP levels were lower at high BAL and NAD levels were lower at high BAL [6, 7]. Nuclear HIF1α(alpha) increased at high BAL [7]. Genes regulated by hypoxia, such as VEGF and erythropoietin are also up regulated at high BAL. The expression of MCP-1 was up regulated at high BAL and correlated with increased inflammation. Increased expression of Bax and Bcl-2 at low BAL correlated with apoptosis (TUNEL) observed at low BAL.

Microarray analyses has been performed on a baboon model of ALD and compared with human ALD in the cirrhotic stage (explants) using human cDNA arrays (1,211) [8, 9]. The baboon model was fed the Lieber–DeCarli diet and liver biopsies were fast frozen for microarrays. The BAL was not stated. In the case of the human ALD livers, they were from alcohol withdrawal patients with end stage cirrhosis. Controls were from the donor livers used for liver transplant. The global gene expression profile (functional pathway changes) showed that in both models, most of the changes in gene expression involved fibrosis/extracellular matrix, metabolism, immune system, transcription, and cell-signaling pathways. Up regulated genes in the baboon model as in the rat model [3] included CYP2E1 and NADH-cytochrome B5 reductase. The results are useful in that both the baboon model and human data provide the understanding of the mechanisms involved in liver fibrosis in ALD. The molecular events set in motion by alcohol abuse continued to be active well after alcohol abuse was stopped.

Rats fed ethanol intragastrically for 1 month had urinary alcohol levels (UAL) averaging 170 mg% [10]. BALs were not stated, but in the intragastric feeding model they are 80% of UAL (136 mg%). The liver pathology included fatty change, inflammation, and necrosis. The livers were subjected to microanalysis (8,740 probe sets). Forty genes were reported to be upregulated and 32 downregulated [10]. Fold change varied from 1.3–2.9 and only four of them increased more than twofold . These minimal changes in gene expression were probably due to low BAL where the changes are minimal especially in the chronic intragastric feeding model of ALD [11]. These results [10] were compared with those of prior studies by Deaciuc et al. [12], Tadi et al. [13], and Ji and Kaplowitz [14]. Alcohol dehydrogenase was upregulated in all three of their studies. Bcl-2 expression was decreased in several studies. CYP-2E1 was increased in all of the studies. Glutathionine S-transferase was increased in six studies but not in the Deaciuic study [12]. LPS binding was increased in four studies. Metalloproteinases were increased in three studies.

Rats fed with ethanol voluntarily using the Lieber–DeCarli diet for 14–15 weeks developed fatty liver with focal inflammation and necrosis, and showed increased serum transaminases (ALT and AST) [15]. Liver tissues from these rats were analyzed using toxicology V34 microarrays (Affymetrix, 8,740 genes on the microchip). Apoptotic gene, CPP32, the caspase-3 precursor was upregulated associated with apoptosis (TUNEL). CD36, a member of the scavenger receptor family, was upregulated. Ligands included long-chain fatty acids, oxidized LDL, anionic phospholipids, and thrombospondin 1. They are linked to TGF-β(beta) activation of stellate-cell fibrogenesis. Squalene epoxidase involved in cholesterol biosynthesis was upregulated. Insulin-like growth factor binding protein-2 precursor, which may be involved in fibrogenesis in the liver, was upregulated by ethanol ingestion. Ethanol downregulated two enzymes, which catalyze desaturation of saturated fatty acids – stearoyl-coenzyme A desaturase-2 and acyl-CoA desaturase. Glutathione S-transferase was upregulated. PKC8-binding protein, which may control growth signaled by the PKC family, was upregulated by ethanol.

Livers from mice fed ethanol intragastrically for 4 weeks were evaluated by microarray analyses [12]. Such mice showed BALs averaging 34 mM and high ALT levels. Their livers showed fatty change and foci of necrosis. Of the 12,423 genes on the chip, 4,867 were expressed by the liver. Gene expression was upregulated for 44 genes and downregulated expression was observed for 42 genes. Upregulated genes of interest included 6 CYPs, glutathionine S-transferase, MMP13, GADD45, and NADPH dehydrogenase. Downregulated genes were ADH, MHC, squalene epoxidase, EGFR, fatty acid synthetase, stearoyl-CoA desaturase, and histone H2a. It should be noted that H2a regulates histone 3 methylation that is important in epigenetic changes.

In a rat study using the intragastric tube feeding model of ALD, the focus was on differential gene expression at low UAL (30 mg%) compared with high BAL (620 mg%) using the Rat Expression Array 230A (Affymetrix) [16]. This showed that the gene expression patterns were different at high and low UAL and indicated that BAL levels need to be the same if comparisons are to be valid. Many published studies where microarray analyses have been done did not state the BALs or whether the rats were withdrawn from alcohol before assay. For instance, the expression of SREBP was decreased at high UAL compared with low UAL. SREBP-1 is an important O_2 sensor, which controls H1Fa activation. It is an important regulator of ADH-1 expression. Gene expression was many folds higher for PPARγ(gamma) coactivator 1α(alpha), betaine-homocysteine methyltransferase, GOT-1, GST Beta 3, and ALDH1. They were all markedly upregulated at high UAL. Markedly downregulated genes at high vs. low BAL were: CXC1 chemokine, cadherin 17, pyruvate dehydrogenase kinase 4, heat shock protein 1α(alpha), leptin receptor, p63, arrestin, LPS binding protein, integrin α(alpha)1, TRAF and Bc12-like1.

In a review on lessons learnt from large-scale gene profiling of the liver from ALD by Deaciuc et al., [15] the expression of two novel genes were singled out that generated new hypotheses on the mechanisms of action of alcohol. One was intestinal trefoil factor expression, which was markedly downregulated by ethanol in mice. It affects large bile duct epithelium. Another novel change identified in gene expression was of galectin-1 and 9, which were up regulated by alcohol. Galectin stimulates activated stellate cell proliferation.

Although CYP2E1 is not upregulated by chronic ethanol ingestion in humans and experimental animals, the level of CYP2E1 protein is increased in the liver because proteolysis of CYP2E1 by the 26S proteasome is decreased by ethanol [17–19]. Increased CYP2E1 by ethanol generates free radicals and causes oxidative stress in the liver when ethanol is ingested. To explore how CYP2E1 contributes to the changes in liver microarrays, transgenic mice which over express CYP2E1 were fed with ethanol intragastrically for 4 weeks [20]. The results were compared with wild type mice fed ethanol. The microarray analysis was done on 14 mice, 3 in each group. Transgenic mice fed with ethanol had higher serum ALTs, liver MDA, CYP2E1 activity, and total pathology score. The highest correlation of the pathology score was related to the CYP2E1 activity. There were 250 gene expression changes when the wild type dextrose fed mice were compared with the wild type ethanol fed mice. The transgenic alcohol treated mice differed from the wild type, ethanol-fed mice, by 65 genes. The largest group of genes with increased expression due to ethanol treatment was glutathione transferase followed by mono-oxidases and hydrolases. When wild type mice and transgenic ethanol-fed mice were compared, the expression of 70 genes was upregulated. These included functional pathways for cell adhesion, extracellular matrix, integral membrane proteins and, cytoskeletal organization. Transgenic, ethanol-fed mice showed an increased expression of TGFβ(beta) induced transcript 4, growth arrest specific 5 (GAS 5) and nuclear receptor for NrOb2. To validate these changes, RT-PCR was done on wild type and transgenic ethanol-fed mice, and the results correlated with pathologic changes. For instance, transgenic mice data on TNFα(alpha) correlated with CYP2E1 activity (–0.5) and Jun D (0.54). GSTα(alpha)4, ERK1, Jun D, and TNFα(alpha) were all significantly increased when transgenic ethanol-fed mice were compared to wild type mice fed with ethanol. The pathology score correlated best with cytokeratin 8 and 18, methionine sulfoxide reductase and succinic dehydrogenase. The changes reflect a reorganization of the cellular machinery towards an increased synthesis of structural proteins at the expense of metabolic function.

Rats fed with ethanol using the intragastric tube feeding model develop inhibition of the ubiquitin proteasome pathway (UPP) in the nucleus, [19] which leads to changes in the turnover of transcriptional factors and histone modifications, affecting epigenetic mechanisms and global changes in gene expression. Histone acetylation and histone acetyltransferase (HAT) are increased. When rats were given the proteasome inhibitor PS341 24 h before sacrifice, the nuclear proteasome activity was markedly reduced as it was with chronic ethanol feeding. Likewise, histone acetylation increased as did expression of HAT. Microarray analysis of the livers treated with the PS341 caused a change in gene expression of a large number of genes (2,082) similar to that observed in chronic ethanol feeding. The MAPK signaling pathway, cytoskeletal pathway, focal adhesion pathway, apoptosis, insulin signaling pathway, tight junctions, cell cycle, TGFβ(beta) signaling pathway, adipocytokine signaling pathway were mostly upregulated. The PPAR signaling pathway and fatty acid metabolism pathways were greatly downregulated. Data mining and gene specific clustering showed that, similar to ethanol feeding, several transcription factors, such as the histone modifying enzymes and the remethylating pathway components such as betaine homocysteine methyl transferase (BHMT), were downregulated. The decrease in BHMT could explain the elevation of serum homocysteine seen in alcohol abuse [19]. This was documented to have also occurred during chronic ethanol feeding. Inhibition of nuclear histone H3K9 me2 was decreased as the result of proteasome inhibition similar to chronic ethanol feeding [21–23]. These results indicate that proteasome inhibition of nuclear proteasome activity makes a major contribution to the global epigenetic changes in gene expression caused by chronic ethanol feeding.

Is the global gene-expression response to an acute ethanol bolus (binge drinking) the same as its response to chronic

ethanol ingestion? This question was answered by microarray analysis of livers from rats fed an acute bolus of ethanol and compared to ethanol-fed rats by intragastric tubing for 1 month. In the chronically fed model, rats were sacrificed at high and low blood ethanol levels. The acute ethanol-bolus fed rats were sacrificed at high (3 h) and low (12 h) BAL. The BAL levels in the acute and chronic models were comparable. Therefore the microarrays reflected effects of acute and chronic ethanol consumption. The number of genes differentially expressed at 3 h with high BAL was 488 compared with high BAL at 1 month, where a number of differentially expressed genes was 1,300. At 12 h low BAL, the number of expression changes was 586 genes. This was compared to the low BAL at 1 month, where expression changes of 230 genes were revealed [9]. The patterns of gene expression for each time period were different. This includes differences in gene expression between low and high BALs in the 1 month chronic ethanol feeding-rat model. There were 400 different gene expression changes when the chronic models of high and low BALs were compared. There was clear evidence in the chronic model that the liver had adapted to the chronic ingestion of ethanol when the BALs were low. However, the liver was supersensitive to high BALs in the chronic model compared to the acute ethanol-bolus model. Key changes in gene expression were validated by RT-PCR. No changes in ALDH1 occurred in the acute model, but ALDH-1 was markedly upregulated (tenfold) in both the high and low BALs of the chronic studies. Sirt1 was upregulated at both high and low BALs in the chronic model. PGC1 and RARβ(beta) were upregulated only at high BALs and PPAR was downregulated only when the BALs were high in the chronic model. The implications were that chronic ethanol treatment upregulated the Sirt/PGC1/PPAR pathway [24, 25]. The ethanol-bolus experiment upregulated gene expressions in all but one functional pathway at 3 h and in all pathways at 12 h, but the gene expressions that were changed differed to a large extent from those gene expressions changed in the chronic model. For instance, the expression of CBP/P300 (HAT), Ahr, Nostrim (NOS), Fabp5, KIf 2 and 3, Jun, CYP1a1, EGR 1, Gadd 45b, and ALDH-1 and 4 were only changed in the chronic model. Proteins involved in gene regulation were only changed in the chronic model. AcH3K9, H3K4me2, H3K27me3, and acH3K18 were up regulated only in the chronic model. The results favor the concept that the liver epigenetic memory only changes after chronic exposure to ethanol. These changes that occur are adaptive changes at low BALs and supersensitivity changes at high BALs (Fig. 35.2).

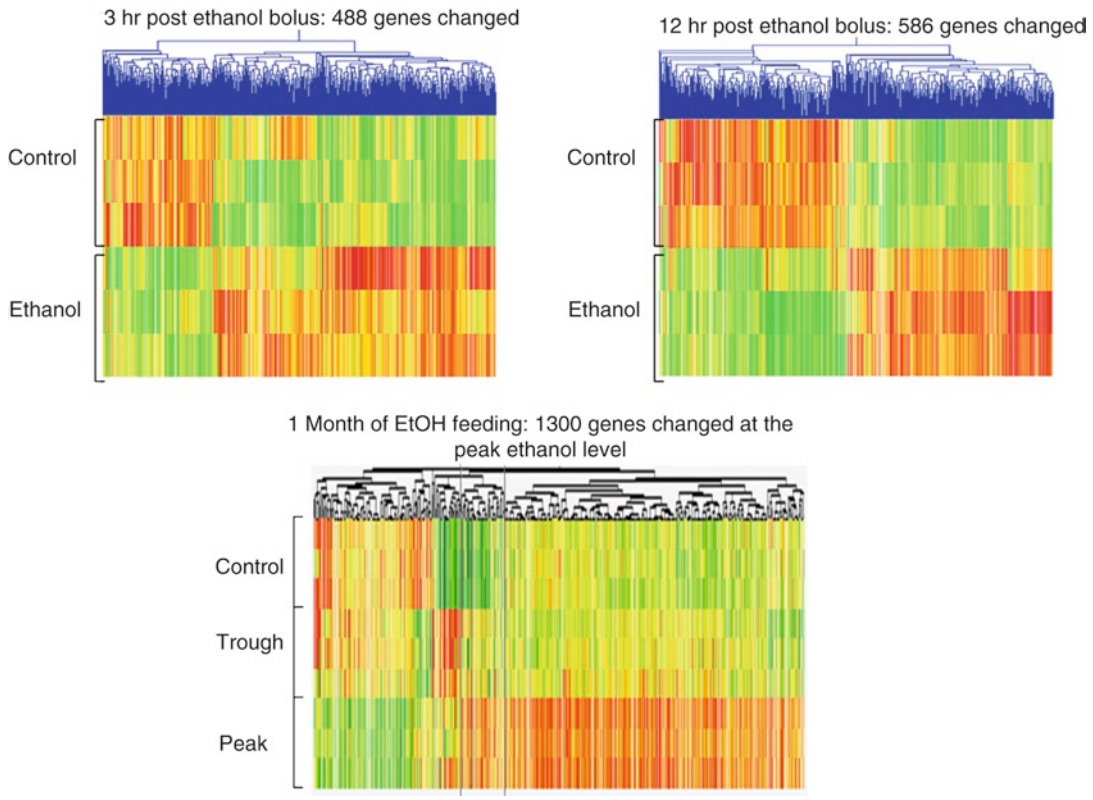

Fig. 35.2 The microarray Heat maps are shown, in which genes upregulated (*red*) or downregulated (*green*) are clustered. Note that the gene expressions change after acute and chronic alcohol feeding when high BAL is achieved, but at low BAL after chronic feeding, the gene expression profile is the same as the controls, indicating that the livers had become adapted to alcohol. From Bardag-Gorce et al. [11] with permission

The microarray data make clear that no single signal transduction pathway or the expression of a small cluster of gene changes can explain all the mechanisms involved in ALD pathogenesis. The disease is multifactorial and the gene expression changes are global. They are dependent on the BALs over an extended period of time of chronic ethanol feeding. The gene expression changes are adaptive and epigenetic in nature, setting the stage for repeated episodes of hepatocellular injury.

The question is how can the global switches in gene expression imposed on the liver by alcohol abuse be prevented. One way would be to methylate histones and DNA to block the transcription of the genes by imposing increased heterochromatin. This would cause gene silencing. To do this, the methyl donor S-adenosylmethionine (SAMe) could be fed with ethanol. If this prevented the global upregulation by ethanol, it would be proof of principle. To test this idea, rats were fed a bolus of ethanol with or without SAMe (1 g/kg/body wt.) or SAMe alone. Microarray analysis was done at 3 and 12 h post bolus. At 3 h, SAMe prevented the upregulation of all pathways induced by the ethanol bolus [21]. SAMe alone also downregulated all the pathways expressed by the glucose pair-fed controls. However, the prevention of ethanol induced upregulation of gene expression by SAMe was no longer evident at 12 h post ethanol bolus. This supports the concept that epigenetic changes in gene expression require prolonged exposure before the changes become permanent.

Microarrays of Tissue Cultures Treated with Ethanol

One of the outcomes of microarray analysis of alcohol treated HepG2 cells that over express CYP2E1, has been the identification of downregulation of six subunits of the 26s proteasome with a consequential loss of 26s proteasome activity [17]. The expression of a large number of genes involved in the ubiquitin-proteasome pathway was also downregulated after 24 h exposure to ethanol (100 mM). Consequently, polyubiquitinated proteins accumulated in these cells, including polyubiquitinated cytokeratins. These formed the Mallory-Denk-like bodies in the cytoplasm of liver cells in culture [17]. These results correlated with in vivo microarray analysis of livers from CYP2E1 over expressing mice, fed with ethanol for 1 month, where it was shown that the cytokeratin 8 and 18 genes were over expressed [20]. Out of 22,000 probe sets studied, CK8 and 18 were number one and two genes that correlated with the total pathology score [20]. Cytokeratin 8 and 18 are the two cytokeratins that form Mallory bodies, an important feature of ALD pathology.

In vitro studies have been done to investigate epigenetic mechanisms of gene expression regulation by ethanol, where primary cultures of hepatocytes were used. For instance, Park et al. [22] reported that ethanol caused an increase in H3K9ac in vitro. Likewise, both acetate and acetaldehyde had the same effect as alcohol. This effect of ethanol was blocked by MAP kinase inhibitors. Ethanol also increased histone acetyltransferase activity (HAT) [23]. The link between the ethanol-induced increase in H3K9ac and the ADH-1 increase was shown in vitro by chromatin immunoprecipitation (CHIP) assay [23]. H3K4 methylation was increased by ethanol in vitro and by chip assays. It was shown to upregulate the expression of ADH and GST. Ethanol stimulated H3K9 methylation, which down regulated L-serine dehydratase and CYP450 2C11 [26]. Ethanol also increased phosphorylation of H3 serine 10 and 28, mediated by p38 MAPKinase in vitro [27]. However, the significance of this phosphorylation of transcriptional events has not yet been determined.

The question is, how well does the microarray analysis of ethanol treated hepatocytes in vitro relate or correlate with those performed in vivo? It is possible that in vitro assessments of gene expression through epigenetic mechanisms may not be mirrored in vivo. Therefore, an effort to correlate in vitro and in vivo ethanol-induced changes in gene expression has been made [28]. Despite a species difference, rats in vivo were compared to human hepatoblastoma cells (HepG2) overexpressing CYP2E1 in vitro. Ethanol concentrations used by both models were comparable (~100 mM), but the duration of exposure to ethanol was very different i.e. 24 h in vitro compared with 1 month in vivo. Despite these disparities the ethanol induced changes in gene expression were remarkably similar. Correlation was less positive when the in vivo BALs were low, i.e., less than 100 mg%. Many functional pathways were upregulated in both models at 100 mM ethanol concentration, but much more so in the in vivo model. The insulin signaling, TGFβ(beta), apoptosis, MAPK signaling, and the wnt signaling pathways were all upregulated. Differences were found in the fatty acid synthesis 1 pathway, which was upregulated in vivo only. Glycosylation enzymes were downregulated in the in vitro model only. Also, in vitro, β(beta)-oxidation by mitochondria and translation factors were downregulated. Catalase and superoxide dismutase in mitochondria were upregulated in vitro. These two catalase enzymes have antioxidant effects. The HAT enzyme CBP/P300-interacting transactivator gene expression was increased in both the in vivo and in vitro models. This would explain an increase in the acetylation of histones. Acetylation of histones generally supports the upregulation of global epigenetic changes in gene expression.

Microarray Studies on Human ALD

Efforts to relate microarray data of alcoholic humans with ALD to baboons fed with alcohol in different stages of progression from alcoholic steatosis, to alcoholic hepatitis,

to cirrhosis, and finally to end stage cirrhosis have been made [29]. The differentially expressed genes in most pathways increased as the ALD progressed from steatosis to alcoholic hepatitis, to cirrhosis, especially genes associated with fibrogenesis and immune/inflammation. Exceptions were the channel transport and protein turnover categories where differential expression was most prominent in steatosis and alcoholic hepatitis stages of ALD respectively. Cluster analysis with a 19,000 gene set distinguished patients between the alcoholic hepatitis and steatosis stages. Patients with steatosis (six patients compared with seven normal controls) showed 98 differentially expressed genes (30 upregulated, 68 downregulated). Changes in gene expression predominantly involved transport, biosynthesis and lipid-metabolism pathways. A total of 211 differentially expressed genes (100 upregulated, 111 downregulated) were found in patients with alcoholic hepatitis. The expression of genes which changed upward included the following pathways: cell adhesions, immune response, oncogenesis, signal transduction, and embryogenesis. Down regulated expression of gene functional categories included: protein biosynthesis, cell growth and maintenance, transcription, signal transduction, and transport. Changes in the expression of seven key genes were confirmed by RT-PCR, in alcoholic hepatitis, cirrhosis, and end stage ALD. Osteopontin (OPN), IL8, annexin A2, TNFRSF14 and claudin were upregulated. CD209 and S-adenosylmethionine synthase (MATIA) were downregulated. OPN is involved in inflammation, leukocyte recruitment, and cell survival. Annexin A2 is involved in enhanced fibrinolysis. Il-8 is involved in PMN recruitment in the liver sinusoid. TNFR5F14 serves as a co-stimulatory molecule that has pro-inflammatory activities in inflammatory diseases where cytokine production by macrophages includes IL-8 and matrix degrading enzymes. Claudin 10 influences liver cellular proliferation and migration, and CD209 bind LPS and are expressed in dendritic cells. LPS response is downregulated after continuation of exposure to ethanol [30]. MATIA is key to S-adenosyl methionine, which is the dominant methyl donor and downregulates global gene expression, an important epigenetic mechanism [31].

Another study on human alcoholic hepatitis used quantitative PCR on transjugular liver biopsies on 23 patients compared to six normal livers [32]. The expression of 46 candidate genes was measured and the results were correlated with liver morphologic changes and the stage of the liver disease. The clinical stage of ALD studied was advanced as judged by extent of liver dysfunction, advanced fibrosis and severe portal hypertension. Not surprisingly, genes encoding extra cellular matrix 1, collagen 1, fibrogenesis mediators (TGFβ(beta)), inflammatory cytokines, and apoptosis regulators (Bc12) were up regulated. Correlation of changes in gene expression with morphologic changes was made for the first time. Fibrogenic regulators such as TIMP-1, several NADPH oxidase components, and GRO-α(alpha) correlated positively with morphologic changes in alcoholic hepatitis. TGFβ(beta) was the main fibrogenic factor. Regulators of collagen degradation included upregulation of TIMP1, MMP2, and ut-PA. TIMP-1 correlated with disease severity. Lymphocytic inflammation correlated with TGFβ(beta) and procollagen. Gro-α(alpha) and Duox-2 correlated with severe granulocytic infiltrate. The increase in the expression of the cytokine Gro-α(alpha) was higher than any other gene (30 fold). TNFα(alpha) was upregulated only slightly (1.7 fold). Angiotensinogen expression was decreased, so also CYP2E1. ACE-1 was over expressed, which was related to local synthesis of angiotensin II. Key components of the phagocytic NADH oxidase (p22 phos and Rac-1) were upregulated. Nonphagocytic NADPH oxidase genes (NOX4, DUOX1 and 2) were also upregulated. Although TNFα(alpha) expression did not correlate with disease severity, it did correlate with infections (systemic inflammatory response rather than a liver disease mediator), as did IL-6. Gro-α(alpha) and phagocytic NADPH oxidases appeared to be the most significant genes involved in the severe stage of alcoholic hepatitis. Mortality within 3 months was predicted by procollagen 4α(alpha) (I), MMP-2, and UPA increased expression.

Single Signaling Pathways

Despite the evidence that ethanol changes the gene expression of a large number of functional pathways in a global manner, studies have focused on a single pathway perturbed by ethanol treatment. Several functional pathways are activated by ethanol by generating oxidative stress and changes in redox status. These pathways include mitogen-activated protein kinases (MAPK), transcription factors such as nuclear factor KB (NFκ(kappa)B), and activating protein 1 (AP-1). It has been stated that MAPK, NFκ(kappa)B, and AP-1 are the major pathways involved in the action of ethanol, its metabolites, and the development of alcohol related disorder [33].

Mezey et al. [34] showed that alcohol abuse increased NFκ(kappa)B binding activity in monocytes, and this did not occur in alcoholics who stopped drinking. Free radicals generated from NADPH oxidase in Kupffer cells activate NFκ(kappa)B, which in turn upregulates the expression of TNF-α(alpha). NFκ(kappa)B also upregulates T-cell mediated hepatitis as the result of the upregulation of chemokines, adhesive molecules like ICAM-1 that recruits leukocytes to the liver. NFκ(kappa)B also downregulates STAT3 and upregulates STAT1 regulated chemokines [35].

Toll-like receptor 4 (TLR4) that recognizes endotoxin, a trigger of inflammation in ALD, activates two signaling pathways utilizing different adaptor molecules, MyD88 or toll/interleukin immune-response-domain containing adaptors

inducing interferon (IFN-β(beta)). In TLR4-KO mice, fed with ethanol, ALD did not develop, but MyD88 KO ethanol-fed mice did develop ALD [36]. TNFα(alpha), IL-6 and, TLR co-receptors CD14 and MD2 were not increased in the TLR4-KO mice. TLR4-KO mice failed to activate oxidative stress induced by CYP450 and the NADPH oxidase complex. Hepatic NFκ(kappa)B was activated in a TLR4 dependent, MyD88 independent manner. Thus, the TLR4 induced and the MyD88-independent pathway plays a role in the pathogenesis of ALD.

One of the hallmarks of alcoholic hepatitis is the infiltration of lymphocytes and PMNs in the liver [37]. How inflammation plays a dominant role in several hepatic pathologies is discussed in Chap. 27. OPN is an important determinant in the pathogenesis of hepatic inflammation in ALD in the early stages [38]. OPN functions to recruit neutrophils and mononuclear cells. OPN is an extra-cellular protein (MCP) which upregulates CD116/CD18 to cause neutrophil infiltrations in the liver. The molecular basis of inflammation through OPN involves CD44 and integrin binding motifs. Integrin binding sites on OPN include 5 integrins and the SVVYGLR sequence. The activation of thrombin exposes the SLAYGRR binding domain, which is responsible for specific signaling functions. In the experimental ALD model in rats, excessive thrombosis is generated and cleaves OPN, which correlates with an increased neutrophilic infiltration [39]. SLYGLR also mediated integrin signaling in rats fed with ethanol plus LPS treatment [39]. Transmigrated neutrophils adhere to hepatocytes where β(beta)2 integrins and 1CAM-1 are expressed on hepatocyte surfaces. They mediate killing of hepatocytes by oxidative stress (NADPH oxidase O_2 burst) and proteases released from the neutrophils [40]. Cofactors involved in hepatocyte killing by neutrophils in ALD include pro-inflammatory mediators (IL-18, TNF-α(alpha), compliment factors, and PAF), vasodilators (eNOS), adhesion molecules (CD-18 and ICAM), and CXC chemokines (IL-8, CIN and MIP2) [40]. The role of LPS activated Kupffer cells in the production of TNFα(alpha) is an established phenomenon, but the exact mechanism of activation of the genes involved is not established [40]. The redox-sensitive NFκ(kappa)B, AP-1, and Egr-1 activation have been implicated in the transcriptional activation of the pro-inflammatory cytokines, adhesion molecules, and chemokines involved in ALD [41]. The potential importance of neutrophilic-liver infiltrates in the pathogenesis of ALD was established when it was shown that leukocytapheresis reversed severe alcoholic hepatitis [42].

Lymphocytic aggregates in the liver are a prominent feature in ALD. The lymphocytes are predominantly T cells, which form "immunologic synapses" binding to the antigen-presenting complex on the hepatocyte membrane [37, 43]. Autoimmunity to CYP2E1 and hydroxyethyl adducts and other liver cell antigens, which are induced in ALD serves as a sensitizing mechanism of lymphocyte mediated liver-cell death [44]. Activated natural killer T cells induce apoptosis by upregulation of FAS pathways in experimental ALD [45]. Patients with ALD have an increased expression of Fas and Fas ligand in the liver, as well as FADD, ICE, and CPP32. There was a positive correlation between the increase of these apoptosis related factors and serum markers of hepatic injury [46].

Apoptosis due to ALD also occurs through the TNFα(alpha)/ceramide/acidic sphingomyelinase/mitochondria pathway, which is activated when the mitochondrial GSH pool is depleted by ethanol feeding [47]. The molecular basis of various forms of liver-cell death is discussed in Chap. 24. Apoptosis that occurs in the intragastric ethanol feeding mouse model is the result of ER stress (unfolded protein response/endoplasmic reticulum, URR/ER stress response genes). ER stress is caused by hyperhomocysteinema [48]. Hyperhomocysteinema is a result of downregulation of methionine synthase by ethanol. The decrease in BHMT which is reduced in the blood of alcohol dependent patients [49] and the liver of rats fed with ethanol [19], may also cause an increase in homocysteine in the blood. The decrease is due to the inhibition of activity of BHMT by ethanol [21]. This effect of ethanol is reversed by feeding betaine with the ethanol. Betaine reduces homocysteine by increasing BHMT. Alcohol-induced ER stress causes apoptosis by way of CHOP. It can also activate SREBP-1c and 2, increasing liver triglycerides and cholesterol [19]. In alcohol-dependant patients, elevated plasma homocysteine is associated with global and gene-specific DNA methylation, which down regulates the ER stress protein HERP [49].

Mechanisms Involved in Ethanol-Induced Fatty Liver

This brings us to the molecular mechanisms of fatty liver due to ethanol. A general account on the molecular basis of fatty liver is found in Chap. 29. Ethanol at high, but not low concentrations increases the NADH/NAD^+ ratio [6]. Sirt1 activity that is dependent on NAD^+ has reduced activity as a consequence. Sirt1 deacetylates SREBP-1 and inactivates it. The activity of fatty acid synthetase is regulated by acetylated SREBP-1. Therefore, when NAD^+ is reduced at high BAL, the activity of Sirt-1 is reduced. Consequently, the activity of SREBP-1 and fatty acid synthetase are upregulated and fatty liver results at high BALs [50]. AMPK activation, which was down regulated by ethanol in vitro also lead to down regulation of SREBP [51]. Ethanol fed to mice decreased hepatic AMPK activity, increased ACC, and elevated malonyl-CoA content in the mouse liver, but the resulting fatty livers exhibited only a threefold increase in

triglyceride [48]. Similar results were found in the micropig fed with ethanol [52]. These results indicate that AMPK may contribute to the development of alcohol fatty liver through regulating SREBP-1 activation and fatty acid oxidation by mitochondria.

Chronic ethanol administration decreases plasma levels of adiponectin associated with fatty liver in mice. Adiponectin, reduced by ethanol, activates AMPK and reduces SREBP-1c expression in mouse livers [51]. Ethanol in rats and mice caused a decrease in plasma adiponectin associated with fatty liver [53, 54]. Adiponectin reduced the fatty liver in these mice presumably by increasing CPT-1 activity, which increases fat oxidation. This reduces fatty acid synthetase and ACC and suppresses hepatic TNFα(alpha) production. TNFα(alpha) induces liver fat by enhancing gene expression of SREBP 1c [55]. Patients who consume moderate amounts of alcohol (10–28 drinks per week) have increased plasma adiponectin and increased insulin sensitivity, which correlated positively [56]. Plasma TNFα(alpha) was unchanged compared to controls. Adiponectin reduces TNFα(alpha) production in vitro by inhibiting NFκ(kappa)B signaling [57].

Adiponectin increases PPAR-α(alpha) ligand activity in hepatocytes [58]. The hepatoprotective effect of adiponectin is due to the coordination of multiple signaling pathways leading to increased fatty acid oxidation and reduced lipid synthesis, which prevents alcoholic fatty liver [59].

PPAR gene expression is downregulated in rats fed with ethanol by intragastric tube to induce fatty liver [60]. PPAR and RXR play a role in fatty-acid metabolism when they dimerize in the nucleus in response to polyunsaturated free fatty acid. Ethanol decreases unsaturated fatty acids in the liver. Using the same animal model, ethanol induced fatty liver and decreased PPAR-α(alpha) activity. This was prevented by clofibrate, a PPAR-α(alpha) activating ligand [61].

1L-6 levels are increased in the serum of patients with ALD [32], but in mice, IL-6 plays a protective role in ethanol-induced fatty liver disease [62]. The effect of IL-6 is likely through the signal transduction pathway of STAT3, which is activated in patients with alcoholic hepatitis and cirrhosis by inhibiting SREBP1 gene expression [63]. IL-6 gene expression was not changed in the liver over 49 days of ethanol feeding in rats with fatty liver [64].

Activation of complement plays a role in experimental ethanol-induced fatty liver [65] by decreasing adiponectin and increasing lipogenic enzymes [66].

Endocannabinoids and their CB1 receptor are upregulated in ethanol-induced fatty liver. Chronic ethanol feeding increases stellate cell production of 2-arachidonylglycerol, which through the CB1 receptor induces upregulation of fatty acid synthesis gene expression inducing SREBP-1 and fatty acid synthetase [67].

Oxidative stress in ALD is generated through CYP2E1 induction and mitochondrial generation of free radicals, i.e., superoxide. Please see Chap. 28 for a more detailed account on this topic. Mitochondria generated nitric oxide results from ethanol-induction of inducible nitric oxide synthase (iNOS). The latter forms peroxynitrate, which increases lipid peroxidation, mitochondrial dysfunction, and apoptosis [68]. As a result, fatty acid oxidation through mitochondrial β(beta)-oxidation could be impeded, if the enzymes involved undergo damage by oxidative stress induced by ethanol. Ethanol modifies mitochondrial proteins leading to their proteolytic digestion. This leads to mitochondrial loss of function [69] and apoptosis.

Role of the Proteasome in ALD

The 26s proteasome plays a regulatory role in terminating or activating signal transductions, cell cycle check points, gene transcription, and protein turn over. In the case of ALD pathogenesis, CYP2E1 is stabilized by ethanol-induced inhibition of proteasome catalytic activity [18]. This is the mechanism of the post translational increase in CYP2E1, which is responsible for free radical attacks on proteins [17]. CYP2E1 catalyzes free radical formation that results from chronic ethanol feeding in rats and generates 4-hydroxynonenal (4HNE) adducts with proteins including the 26S proteasome subunits [70]. Hyperphosphorylation of the 26S proteasome subunits also develops after chronic ethanol feeding of rats [71]. It is likely that these alterations of the 26S subunits account for the loss of proteolysis by the proteasome. This stabilizes CYP2E1 and the formation of Mallory Denk-like aggresomes seen in cultured HepG2 CYP2E1 over expressing cells when incubated with media containing ethanol [17]. Thus ethanol inhibition of proteasome activity may be one mechanism involved in MDB formation. Inhibition of the 26S proteasome is also essential for the activation of the nuclear transcription factor hypoxia inducible factor 1α(alpha) (HIF1α(alpha)), which is key to the activation of expression of genes involved in compensating for the lack of oxygen as well as proinflammatory cytokines like TNFα(alpha). The proteasome digests HIF1α(alpha) preventing it from entering the nucleus. This reaction is initiated by hypoxia that occurs at high blood-ethanol levels [7]. This leads to centrilobular necrosis [72] when liver ATP and NAD^+ levels fall [72]. Another effect of the alcohol-induced inhibition of the 26S proteasome is the prevention of activation of NFκ(kappa)B in the liver. NFκ(kappa)B activation is dependent on the proteasome to digest phosphorylated IKB in order to liberate NFκ(kappa)B for translocation to the nucleus to increase the expression of numerous proinflammatory cytokines such as TNFα(alpha).

Nuclear NFκ(kappa)B levels in the liver are not increased in the chronic, rat intragastric-ethanol feeding model [73]. Several modifiers of the ubiquitin 26S proteasome pathway (UPP) are also altered by chronic ethanol feeding including ubiquitin levels [73] and p62, a scaffolding chaperone, which carries unfolded and misfolded proteins to the 26S proteasome for digestion [74]. Perhaps most important is the role that inhibition of the proteasomal activity by ethanol or PS-341 play in inducing MDB-like formation in vivo and in vitro [17, 19, 20, 75–78].

Mallory-Denk body Pathogenesis

MDBs are an important hallmark for liver disease progression. They form in liver cells that show balloon degeneration. They first form in centrilobular hepatocytes when the fatty liver stage of ALD progresses to the steatohepatitis stage (alcoholic hepatitis) of ALD. In the early stage of steatohepatitis stage only scattered hepatocytes form MDBs. Towards the end of the steatohepatitis stage and in the cirrhotic stage of hepatitis, large numbers of hepatocytes form MDBs. This often predicts the death of the patient even before cirrhosis has developed. In the cirrhotic stage of ALD, MDBs are formed in the liver at the borders of fibrotic septa. The pathogenesis of MDB formation has recently been reviewed [79].

MDBs are cytokeratin aggresomes composed of altered proteins, which resist proteolysis by the 26S proteasome [79]. The proteins that accumulate because of the failure to be digested include cytokeratin 8 and 18, ubiquitin, the proteasomal subunits, tubulin, heat shock protein 70 and 90, transglutaminase, UBB+1 (a frame shift mutant), p62, and VCP/97 [77–84].

MDBs can only form in mouse and human hepatocytes, probably because of the sequence of amino acids and the placement of lysine residues in the ubiquitin molecule, which differ in other species. When MDBs form, the cytokeratin molecular weights increase due to polyubiquitination [85]. The cytokeratins first become hyperphosphorylated, making them targets for ubiquitinization and digestion by the UPP [86–90]. Ethanol induces cytokeratin phosphorylation in vitro [86]. The ubiquitinated cytokeratins accumulate and form MDBs when the activity of the 26S proteasome is inhibited [91] or cytokeratin 8 is in excess of cytokeratin 18 [79]. Newly synthesized nascent cytokeratins go preferentially to MDBs rather than to normal intermediate filaments in vitro [92]. Hepatocytes, which form MDBs lose endocytotic function when assayed in vitro [93]. The MDB proteins show a shift in structure from alpha helix to beta pleated sheet conformation as assessed by infrared spectroscopy [94]. MDBs resemble amyloid ultrastructurally and by their stickiness.

MDBs form in drug-primed mouse liver in response to a large number of different toxins besides ethanol [79, 95]. The level of CK8 and 18 in primary liver-culture cells derived from mice forming MDBs are equal and the rate of synthesis of both CKs are equal in vitro [86]. These two cytokeratins are similarly hyperphosphorylated in vitro [86]. Maximum phosphorylation of the cytokeratins occurs on day 2 and 3 of incubation in primary cultures when MDBs begin to form in vitro [86]. The increase in gene expression for CK8 was maximal on day 3 and 5, at the same time as when MDB formation was maximal in vivo [89]. The gene expression for transglutaminase, which crosslinks the CK proteins covalently was also up regulated maximally on day 3 and 5 when MDBs form maximally [89]. C-myc and c-jun expression also increased on day 3 and 5 when MDB formation was maximal in vivo [89]. PPARα(alpha) and RXRα(alpha) expression decreased on day 3 and 5. Gel retardation assays for AP-1 consensus oligonucleotide was increased on day 5. PPRE was decreased on day 5. The ubiquitin smear (polyubiquitinized proteins) seen in Western blots when MDBs form, begin to appear on day 3 of drug refeeding and this persists on days 4–6 of refeeding in vivo [96, 97].

A number of proteins involved in the UPP system, which carry phosphorylated and ubiquitinated proteins to the 26s proteasome for digestion are called chaperones or scaffolding proteins. These include p62, VCP/p97, and heat shock proteins HSP 70 and 90, and are involved in MDB formation. Using an in vitro model of MDB formation, p62 scaffolding protein proved to facilitate MDB formation in vitro [83]. Using SiRNA knock down and green fluorescent protein (GFP) gene fusion over expression, it was shown that MDB formation increased with P62-GFP transfection and decreased when p62 gene-specific RNA knock down was applied. Using the same technology with VCP/97 the reverse was proven to be true. VCP/97 knockdown increased MDB formation whereas transfecting with GFP/VCP/p97 to over express VCP/p97 did not affect MDB formation [84]. Both GFP/p62 and GFP/VCP/p97 were localized in the MDBs. Heat shock protein 70 and 90 also are involved in the progression of proteins destined to go to the proteasome, targeting the cytokeratins to the proteasome for digestion [82].

In using the in vitro model of MDB formation, it is important to determine the signals which initiate the process. The first is the tissue culture substratum [98]. The integrins and substratum, which signal MDB formation and are optimal, include laminin, the alpha and beta integrins, and ICAM. Fibronectin and collagen were less activating and plastic coverslips were the least activating for MDB formation. Src, MAPKinase (Met), and ERK expression were increased when MDBs formed. When this signaling process was chemically blocked by inhibiting ERK phosphorylation, MDBs failed to form [98]. NFκ(kappa)B activation also was shown to be a key factor in MDB formation using the MDB in vitro model. Chemical inhibition of NFκ(kappa)B 105 digestion to form p50, prevented MDB formation in vitro. NFκ(kappa)

B became progressively more active reaching a peak on the fifth day when MDB formation was maximal [99]. A third signaling pathway involving p38 was also studied using the MDB in vitro model [100]. A chemical inhibitor of p38 phosphorylation/activation prevented MDB formation. P38 expression was upregulated by liver cell isolation and 3 h culture of hepatocytes, indicating that p38 was increased before the MDBs began to form on the first day of culture. The data supported the concept that p38, not ERK, was responsible for the phosphorylation of CK8 and CK18, preparing them for degradation by the 26s proteasome.

MDB/CK-18 fragment M30 is a prognostic indicator in clinical ALD (alcoholic hepatitis). CK-18, a component of the intermediate filaments in hepatocytes and in MDBs is cleaved by caspases during apoptosis leading to M30 formation [101]. However, when ALD livers were immunostained for M30, only MDBs and macrophages stained positive, not apoptotic cells [102] indicating that M30 is not a marker for apoptosis, but rather, it is a marker for MDBs in alcoholic steatohepatitis (ASH). TPS, another split product of degraded CK-18, is also a marker for ASH [103]. TPS levels correlate strongly with the presence of MDBs [104] and serum M-30 levels [103]. M30 immunoreactivity tends to correlate with clinical severity of ASH [103].

Microarray Analysis of Liver Forming MDBs

DDC primed mice were refed with DDC for 7 days with or without S-adenosylmethionine (SAMe) (4 g/kg/day). Large number of genes showed changes in expression after DDC, but refeeding with SAMe prevented these changes and also prevented MDB formation in vivo [105]. Changes in the expression of specific genes upregulated by DDC refeeding and blocked by SAMe feeding included FAT 10, GSTm2, Egf2, KIf6, Afp, p62, caspase 3, HSP 70 and 90, Dnmt3a, and HDAC. SAMe (2 mM) added to the medium of the in vitro model of MDB formation completely blocked MDB formation in 6 day primary hepatocyte cultures [105]. The results indicate that MDB formation is an epigenetic phenomenon, since SAMe is a major methyl donor and thus methylation causes gene silencing by epigenetic mechanism. It methylates histones and DNA. DDC refeeding increases histone acetyltransferase (GCNS) levels and SAMe feeding prevents this increase [106]. DDC decreases H3K9me3 and H3K4me3 levels and SAMe prevents this change. DDC also increased SET 7/9 histone methyltransferase, and histone 2a ubiquitination and SAMe prevented these changes. Microarray analysis also showed that DDC refeeding increased the expression of SAMe metabolizing enzymes such as MATa, AMD, and MTHFR and it reduced the expression of AHEY and GNMT. Inclusion of SAMe prevented these changes. The implication of these results is that SAMe prevents the epigenetic cellular memory involved in MDB formation [106].

Because FAT10 is over expressed during MDB formation in DDC refed drug-primed mice, data mining of the immunoproteasome subunits has also been reported [12]. The genes of these subunits along with FAT10 are located at the MHC-1 locus. They include LMP2, and 7, and MECL-1, the three main catalytic subunits of the immunoproteasome. All of the subunits were upregulated during MDB formation and confirmed by PCR. This upregulation was associated with the upregulation of the receptors of IFNγ(gamma) and TNFα(alpha), and the loss of 26s proteasome activity. It was concluded that the drug caused a shift from the 26s proteasome to the immunoproteasome to augment proinflammation cytokine responses and NFκ(kappa)B activation [107].

Ethanol-Induced Fibrosis of the Liver

Please see Chap. 30 for a detailed pathogenesis of hepatic fibrosis. Fibrosis of the liver is produced by stellate cells, either centrilobular and perihepatocytic, early in the course of ALD, or periportal, secondary to ductular metaplasia in later stages of ALD. Portal-portal, portal-central, and central-central bridging fibrosis are also of stellate cell-myofibroblast transition origin, which leads to micronodular cirrhosis in ALD. Micronodular cirrhosis progresses to macronodular cirrhosis as a result of multilobular necrosis and regenerative nodule formation (for a review see [1, 108]).

While another section in the textbook is focused at molecular mechanisms of hepatic fibrosis, the focus here is on the regulation of scarring by the stellate cell and extracellular matrix in experimental ALD models. It should be noted that significant fibrosis of the liver in preclinical models is only seen in the ethanol-fed baboon model and in the intragastric tube feeding model of ALD [25, 109–115].

In vitro studies including combined cultures of stellate cells with liver cells and stellate cells with Kupffer cells show how ethanol and acetaldehyde induced oxidative stress activate signaling pathways, which lead to stellate cell activation. Activation of TGFβ(beta)1 and TGFβ(beta) receptors subsequently lead to Smad3/4 activation and binding to the promoter of collagen 1 genes. PKC and/or PI-3K kinases are activated by acetaldehyde leading to activation of ERK1/2 and JNK, which activate AP-1. AP-1 activates BTEB, which upregulates TGFβ(beta) type II receptor gene expression in stellate cells. C/EBP and NF-1 transcription factors are also activated, which up regulate collagen I gene transcription in stellate cells [116].

Stellate cells contain enzymes which oxidize ethanol including ADH and CYP2E1. Oxidative stress upregulates

UPA, which activates TGFβ(beta)1 and induces stellate-cell proliferation as well as proinflammatory mediators IL-6, TNF-α(alpha), and malondialdehyde. Malondialdehyde/acetaldehyde (MAA) protein adducts induce cytokines including chemokines MIP-1 and MIP-2 as well as adhesion molecule 1CAM-1 in activated stellate cells [116].

Leptin has profibrogenic activity. It increases collagen type 1 synthesis by hepatic stellate cells, which is mediated by activating JAK-signal transducers and activating STAT and PI3K/AKT signaling pathways. It reduced FAS-ligand associated apoptosis. Leptin-induced HSC collagen production is increased by decreasing LMP-1 caused by the JAK/STAT pathway and JAK-mediated H_2O_2-dependent extra cellular signal kinase 1/2 and p38 pathways [117]. Leptin induces H_2O_2 production and contributes to TIMP-1 expression in stellate cells [117]. Activated stellate cells express leptin [116]. Adiponectin is also expressed by stellate cells and causes conversion of activated cells to quiescent cells. It also induces apoptosis of stellate cells and inhibits their proliferation [118]. Leptin knockout mice develop less fibrosis and adiponectin knockout mice develop more fibrosis than control mice given carbon tetrachloride [118]. The reduction of adiponectin caused by ethanol further allows stellate-cell generated fibrosis [117]. Adenosine is another mediator of stellate-cell function and is released into the blood by ethanol ingestion. It is known to inhibit stellate-cell contraction [119, 120]. Stellate-cell contraction slows blood flow in the liver at the sinusoid level leading to centrilobular hypoxia and ischemic necrosis and contributes to intrahepatic resistance and portal hypertension [121–123]. Catecholamines and thyroid hormone increase centrilobular necrosis by increasing the rate of O_2 consumption by hepatocytes [122, 123].

Innate Immunity and Fibrosis

Innate immunity in the liver is comprised of Kupffer cells, natural killer (NK) cells, NKT cells, and IFN-α(alpha) and γ(gamma) cytokines. Evidence indicates that innate immunity is involved in liver fibrosis due to alcohol abuse. For instance, macrophages limit liver fibrosis by killing stellate cells and increasing degradation of ECM during the recovery stage of fibrosis [124]. IFN-α(alpha) and γ(gamma) inhibit fibrosis by blocking TGF-β(beta)1 signaling and stellate-cell activation [124]. NK cells kill activated stellate cells and reduce liver fibrosis [124]. However, ethanol reduces innate immunity in both animal and human studies by decreasing NK-cell activity and numbers [125]. Chronic ethanol feeding of mice, where liver fibrosis is induced by CCl_4, reduces the antifibrotic effects of NK/IFN-γ(gamma)/STAT1 in the liver [126]. In human ALD, IFN-α(alpha), STAT1 and p48 are upregulated and STAT2 is down regulated [127]. P42/44 MAPKinases were upregulated, which may cause down regulation of IFN-α(alpha) signaling. TGFβ(beta) in humans at all stages of ALD have circulating monocyte upregulation of TGF-β(beta)1 gene expression, which is related to inflammatory activity and fibrosis [128]. Hedgehog signaling is increased along with up regulation of TGF-β(beta)1 expression in mice fed with ethanol as well as in ALD patients [129].

This correlated with more ductal-cell induced fibrosis or ductal metaplasia, more apoptosis and increased collagen 1α(alpha) expression [129]. Smad7 expression in mouse livers attenuates TGF-beta signaling and epithelial mesenchymal transition (EMT), which results in less interstitial collagen and liver fibrosis induced by CCl_4. This indicates that TGFβ(beta) dependent EMT-like phenotypic changes participate in fibrosis [130]. Hepatocytes grown on stiff substratum are activated by FAK via Src, leading to AKT and ERK 1/2 activation of pathways. AKT causes resistance to TGFβ(beta)-induced apoptosis by antagonizing p38, whereas ERK 1/2 initiate EMT [131].

Epigenetic Changes in the Stellate Cells

Proof that myofibroblastic transformation of the stellate cell is a phenotypic change resulting from epigenetic driven cellular transition (activation) has recently been shown by Mann et al. (as reviewed by Tsukamoto [132]). The transition from quiescent to activated phenotype indicates loss of vitamin A stores and increased contractility due to regulation of smooth muscle actin-gene expression. A global gene expression switch is involved in the phenotype change. The DNA methylation inhibitor Aza prevents the switch in phenotype and the loss of Iκ(kappa)Bα(alpha) and PPARγ(gamma). This change in expression is characteristic of the switch that activates stellate cells that have acquired HDACs and HMTs to locate at the Iκ(kappa)Bα(alpha) promoter. This was inferred because of an increase in H3K9me2 and decrease in H3K9ac expression. This would silence the Iκ(kappa)Bα(alpha) gene expression. Aza blocked the increased methylation of H3K9 and caused an increase in H3K9 acetylation. This study showed the role played by epigenetic regulation by MECP2 in stellate-cell activation by using the Iκ(kappa)Bα(alpha) promoter as a model. Also Aza restored PPARα(alpha) expression. Mann's study poses an important question: how do epigenetic phenomena regulate mesenchymal cell plasticity and switching of the stellate cells to the myofibroblastic phenotypic [132]?

Genetic Polymorphism Predisposition to ALD

A-G mutation in exon 1 of the CTLA-4 gene and a C-A polymorphism in the IL-10 promoter at position 627 are both associated with advanced fibrotic/cirrhotic ALD [133] IL-10

released from Kupffer cells inhibits fibrogenesis by stellate cells. Both polymorphisms may result in the loss of tolerance in the development of ALD [133]. The polymorphism in the TNFα(alpha) promoter and gene encoding transcription and an increase in the IL-4 response favor a Th2 response. Th2 responses create the risk of developing fibrosis in ALD [133]. Patients with advanced ALD are more likely to have anti-CYP2E1 autoantibodies compared to patients who are drinking in the presence of fatty liver formation [133]. Patients with CTLA-4 all are also more likely to develop anti CYP2E1 antibodies [133]. Anti CYP2E1 antibodies present in patients and rats fed ethanol intragastrically have significant ALD pathology [134, 135]. These polymorphisms, which are associated with ALD, support a role for the adaptive immune system in the development of ALD [133]. TLR4 D299G and T3991, single nucleotide polymorphisms (SNPs) provide protection from hepatic fibrosis by reducing TLR-4 mediated inflammation and fibrogenic signaling. They lower the apoptotic threshold of activated stellate cells [136]. Patients with alcoholic cirrhosis that have MTHFR C677T SNPs have a significant increased chance of developing hepatocellular carcinoma as well [137].

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Chapter 36
Viral Hepatitis A

Shiv K. Sarin and Manoj Kumar

Introduction

Hepatitis A is generally an acute, self-limiting infection of the liver caused by hepatitis A virus (HAV). Infection may be asymptomatic or result in acute hepatitis. Rarely, fulminant hepatitis may occur. Hepatitis A infections never cause chronic liver disease.

History

The earliest descriptions of contagious jaundice are from ancient China [1]. Although the symptoms that were described are similar to those currently found in people with hepatitis A, a number of other infections produce similar symptoms. The earliest outbreaks of hepatitis that were almost certainly hepatitis A were documented in Europe in the seventeenth and eighteenth centuries, especially during periods of war. Initially, it was known as "catarrhal jaundice" because it was thought to be caused by blockage of the common bile duct by a plug of inspissated mucus. The first suggestion that the disease was caused by an infectious agent was made by McDonald, who was unable to demonstrate the involvement of enteric bacteria and suggested that the infection might be caused by a virus [2]. The first indication of the existence of a second form of hepatitis came in 1833, when an outbreak of hepatitis was observed in shipyard workers who were vaccinated against smallpox with a particular batch of human glycerinated lymph. The disease, which became known as "serum hepatitis," was assumed to be due to a blood-borne infectious agent.

Analysis of epidemics of hepatitis during World War II confirmed the existence of two epidemiologically and etiologically distinct forms of the disease known as *infectious hepatitis* and *serum hepatitis* [3]. Experimental transmission studies in volunteers soon clarified the major features of the two diseases. Hepatitis A had an incubation period of between 15–49 days and was transmitted by the fecal–oral route [4]. Later studies demonstrated that the virus could be detected in feces or blood during the acute infection, the infection could be transmitted experimentally by both the oral and parenteral routes, and that infection was followed by long-term immunity and could be prevented by prior administration of normal human immune globulin (IG); these studies also defined the incubation period, period of infectivity, and period of viremia, and then standardized reagents representing hepatitis A and hepatitis B were developed [5]. In 1973, a 27 nm virus-like particle was detected in the stools of volunteers infected with hepatitis A that were aggregated by convalescent, but not by preinfection, serum, thus indicating that the particles represented the etiologic agent of the disease [6]. The identification of HAV, transmission of the disease to marmosets and chimpanzees, propagation of HAV in cell culture, and molecular cloning of the viral genome led to the development of effective vaccines [7].

Virology

Classification

HAV is a member of the Picornaviridae family. Although originally classified as Enterovirus type 72, HAV now has its own genus, *Heparnavirus,* within the Picornaviridae family [8].

Structure

Physiochemical Characteristics

HAV is a 27–28 nm, spherical, and nonenveloped virus with a surface structure suggesting icosahedral symmetry. Purification of virus from clinical samples or tissue culture

S.K. Sarin (✉)
Department of Gastroenterology, G.B. Pant Hospital, New Delhi, India
and
Institute of Liver and Biliary Sciences, New Delhi, India
e-mail: shivsarin@gmail.com

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_36, © Springer Science+Business Media, LLC 2011

yields three distinct populations of particles: mature HAV virions that band at 1.32–1.34 g/cm^3 in CsCl and sediment at approximately 160 S, a lower-density fraction that bands at about 1.27 g/cm^3 in CsCl and sediments at 70–80 S and may represent empty capsids or particles with incomplete genomes, and a high-density fraction (1.4 g/cm^3) that may represent particles with a more open virion structure that allows increased penetration and binding of CsCl to the viral particle [9]. These high-density particles have been shown to contain RNA but tend to be less stable than mature virions.

HAV is more resistant to heat than other picornaviruses and may be incompletely inactivated (depending on the conditions) by exposure to 60°C for 10–12 h [10]. HAV may survive for days to weeks in shellfish, water, soil, or marine sediment and outbreaks of hepatitis A have been reported after ingestion of steamed shellfish and other foods, suggesting that the internal temperature achieved by steaming sometimes may be insufficient to destroy the virus (see below). However, HAV can be reliably inactivated by autoclaving (121°C for 30 min) [11].

The virus is resistant to most organic solvents and detergents as well as pH as low as 3. HAV can be inactivated by many common disinfecting chemicals, including hypochlorite (bleach), and quaternary ammonium formulations containing 23% HCl found in many toilet bowl cleaners [11].

Currently licensed vaccines are inactivated by 1:4,000 formalin at room temperature for at least 15 days to exceed complete inactivation by at least threefold. As a result of several outbreaks of hepatitis A in hemophiliacs who received factor VIII concentrates that had been treated by a solvent detergent method for inactivation of lipid-enveloped viruses, interest has focused on techniques capable of inactivating nonenveloped viruses without compromising the biologic activity of the proteins of interest. Currently, the used techniques are dry heat (80°C for 24 h), ultraviolet irradiation, γ-irradiation, sequential ultrafiltration through 35-nm and 15-nm membranes and pasteurization at 60°C for 10 h. Pressurization has emerged as a new technique for inactivating pathogenic viruses in blood plasma and plasmaderived products, as pressurization at 400 MPa exerted no effect on the recovery of biologically active plasma proteins, with the exception of factor XIII. Most enveloped viruses are markedly inactivated at pressures below 400 MPa. However, small RNA viruses can vary widely in their sensitivity to high pressure. For example, HAV and poliovirus are both members of the picornavirus family, but they exhibit quite different susceptibilities. HAV is inactivated by 3–5 log10 of infectivity at 420 MPa, whereas poliovirus remains essentially unaffected even at 600 MPa [12].

Cell-adapted HAV strains are generally used to confirm virus inactivation in manufacturing blood products, but the strains may differ in their sensitivity to inactivation treatment. KRM238 may be the best candidate for virus validation to ensure the safety of blood products against viral contamination, as it is harder to inactivate and it replicates better in cell culture than the other strains [13].

Morphology

The structure of the infectious HAV particle remains controversial. Attempts to determine the atomic structure of HAV by X-ray crystallography have not been successful, although such studies have provided high-resolution images of virus particles. However, recently, medium resolution images of the HAV particle have been obtained by cryo-electron microscope [14], which suggests significant differences in its structure compared with other picornaviruses. In particular, no well-defined "canyon" surrounding the particle's fivefold-axes, a prominent feature and the site of cellular receptor binding in other picornaviruses, is present.

Genome Organization

Genome and Proteins

Molecular cloning and sequence analysis have demonstrated that the HAV genome is composed of single-stranded, positive-sense linear RNA of 7,478 nucleotides (strain HM175) and a molecular weight of approximately 2.25×10^6, with an overall structure and gene order typical of picornaviruses [15]. It lacks an envelope. The genome contains a single large open-reading frame.

The 5′ end of the genome does not have a cap structure, but instead has a small, covalently bound protein termed VPg. The genome has a long 5′ untranslated region (UTR) beginning with UU, as found in all picornaviruses. This 5′ UTR folds to form a highly ordered secondary structure known as an internal ribosome entry site (IRES) that directs the initiation of translation at the appropriate internal AUG codon, being the AUG after nucleotide 734, in the case of HAV [14] (Fig. 36.1). This AUG codon begins a single long open reading frame of 6,681 nucleotides that encodes a polyprotein 2,227 amino acid residues in length. The coding region of picornaviruses has been arbitrarily divided into three parts termed P1, P2, and P3, and the peptides that are ultimately cleaved from the translation products of these regions are referred to as 1A, 1B, 1C, 2A, 2B, 2C, and so forth, in order of translation from the 5′ to the 3′ end of the genome [16]. The HAV genome ends with a 3′ noncoding region of 63 nucleotides that is followed by a poly(A) tail.

The single large open-reading frame encodes a polyprotein, in which the major capsid proteins represent the amino terminal third (P1 segment), with the remainder of the polyprotein

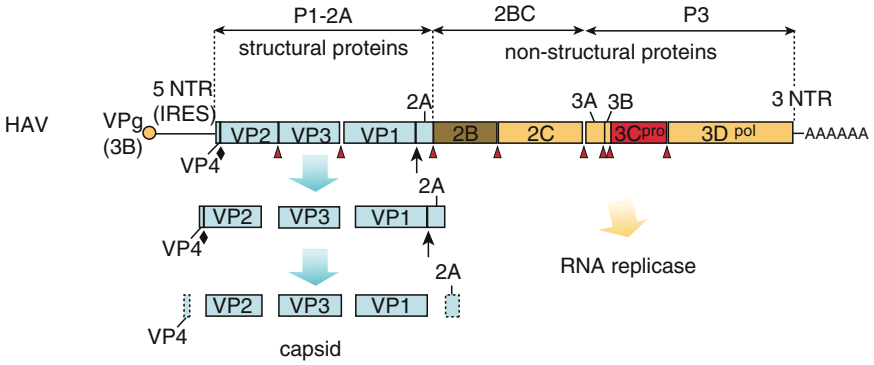

Fig. 36.1 Genome organization and proteins of HAV. The positive-strand RNA genome contains a single open reading frame encoding a polyprotein that is proteolytically processed by the viral protease, 3Cpro (shown in *red*, cleaving at sites identified by *red triangles*), a yet-to-be-identified cellular protease (*arrow*), and an unknown proteolytic activity (*black diamond*) to release the mature structural (*blue*) and nonstructural (*tan* and *red*) proteins. From Martin and Lemon [14]. Used with permission

comprising a series of nonstructural proteins required for HAV RNA replication: 2B, 2C, 3A, 3B (a small protein, also known as VPg that is covalently linked to the 5′ end of the genomic RNA and that probably serves as the protein primer for RNA synthesis), 3Cpro (a cysteine protease responsible for most posttranslational cleavage events within the polyprotein), and 3Dpol (the viral RNA-dependent, RNA polymerase) (Figs. 36.1 and 36.2) [14].

The mature HAV particle is composed of four capsid polypeptides as detected first by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by molecular cloning and nucleic acid sequencing. By analogy with other picornaviruses, these proteins, which are coded within the P1 region, are referred to as virion proteins (VP): VP1 = peptide 1D (molecular weight, 32,800 Da), VP2 = 1B (24,800 Da), VP3 = 1C (27,300 Da), and VP4 = 1A (2,500 Da) [17].

HAV has been adapted to replicate in many types of cultured mammalian cells, including cells of nonhepatic or nonprimate origins (see below). Several features of its replication cycle distinguish it from poliovirus and many other well studied picornaviruses, including its slow and protracted time course, low virus yields, and a propensity to establish persistent infections in cell culture. The viral proteins are translated directly from the messenger-sense genomic RNA, which is delivered to the cytoplasm after uncoating of the viral particle. Proteolytic processing of the polyprotein occurs simultaneously with translation and is largely carried out by the 3Cpro protease (Fig. 36.2). Synthesis of the RNA follows the assembly of a large, macromolecular-replicase complex containing the nonstructural viral proteins spanning the 2B–3Dpol segment of the polyprotein, and occurs on membranes that are usurped for this purpose from the cellular endoplasmic reticulum [18]. RNA transcription is most likely protein primed, with uridylylated VPg (protein 3B) representing the primer for both a negative-sense RNA replication intermediate and subsequent positive-sense progeny RNA molecules.

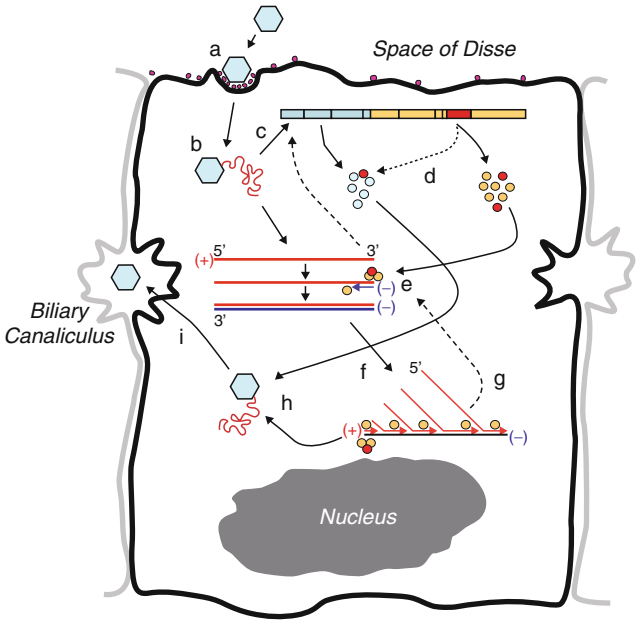

Fig. 36.2 Replication cycle of HAV. (**a**) The virus enters the hepatocyte via a cellular receptor, the identity of which remains uncertain. (**b**) This is followed by uncoating of the viral particle and release of the positive-sense RNA genome into the cell. (**c**) An internal ribosome entry site within the 5′ untranslated segment of the genome mediates cap-independent translation of the viral polyprotein. (**d**) The polyprotein undergoes co- and posttranslational proteolytic processing directed by the viral protease, 3Cpro. (**e**) Nonstructural proteins assemble into a membrane-bound RNA replicase, bind the 3'end of the genomic RNA and commence synthesis of a negative-strand copy of the viral genome. (**f**) The negative-strand copy of the genome is used as template for synthesis of multiple new copies of genomic positive-strand RNA. (**g**) Some of this newly synthesized positive-sense RNA is recycled for further RNA synthesis or translation (*dashed lines*). (**h**) Other positive-strand RNA molecules are packaged into new viral particles formed by assembly of the structural proteins, followed by final cleavage of the VP1–2A precursor by an unknown cellular protease (VP1/2A junction), and the "maturation" cleavage of the VP4/VP2 junction. (**i**) Newly assembled HAV particles are secreted by the cell across the apical membrane of the hepatocyte into the biliary canaliculus, from which they are passed into the bile and small intestine. From Martin and Lemon [14]. Used with permission

Assembly of HAV particles proceeds through several steps (Figs. 36.1 and 36.2). Cleavage of the polyprotein by the 3C protease yields three capsid-related proteins, VP0, VP3, and VP1–2A (also known as PX), that constitute a monomer and subsequently assemble into pentameric subunits. Twelve copies of the pentamer then associate with viral RNA to form provirions, or without viral RNA to form empty capsids (procapsids). The involvement of the VP1–2A precursor in assembly is unique to HAV and the 2A extension is essential for proper processing and assembly of the pentameric subunit [19]. Following assembly, 2A is removed from VP1 by cellular proteases, and in the final maturation step, VP0 is cleaved to yield VP2 and VP4. The VP0 cleavage is dependent on the presence of viral RNA in the particle and procapsids therefore fail to cleave VP0, but in HAV these procapsids are quite stable and indeed have the same antigenic structure as mature virions. Considerable controversy has surrounded two segments of the polyprotein that are likely to be involved in the assembly of virus particles: the extreme amino terminus, which comprises a short polypeptide sequence representing a putative fourth capsid protein, VP4 (also known as 1A), and the segment immediately downstream of the capsid proteins (2A segment), which represents a carboxyterminal extension of VP1, the largest capsid protein (Fig. 36.1). The primary polyprotein cleavage event occurs at the 2A/2B junction, and it is mediated by the 3Cpro protease. The resulting P1–2A structural precursor is further cleaved by the viral protease to generate two capsid protein precursors, VP0 (VP4-VP2) and VP1–2A (also known as pX), as well as the mature VP3 capsid protein. VP1–2A is a critical structural intermediate in virion morphogenesis. Cleavage at the HAV VP1/2A junction occurs late in the process of virion morphogenesis and results from the action of an unknown cellular protease. However, the mature 2A protein has never been identified directly in infected cells. In the case of poliovirus, all of the nonstructural proteins, including the 2A protease, as well as some precursors of the mature nonstructural proteins, are necessary for RNA replication.However, with HAV, this is not the case. Infectious virus can be recovered from recombinant HAV genomes containing exogenous protein-coding sequences inserted in-frame at the 2A/2B junction and flanked by consensus 3Cpro cleavage sites, indicating that the nonstructural 2A polypeptide does not function in *cis* as a 2AB precursor. Viral RNA replication is not impaired when the C-terminal 60% of the 2A sequence is deleted [19]. Furthermore, the complete deletion of 2A has no effect on the replication capacity of subgenomic RNA replicons lacking the P1 capsid protein region [14]. Thus, the nonstructural 2A protein segment is not required for RNA synthesis. Conversely, HAV RNA lacking sequence encoding the N-terminal 40% of 2A is not capable of producing infectious virus particles [19], indicating a role for the 2A segment in capsid assembly. Such a deletion prevents the assembly of the structural precursors into pentamers, important intermediates in capsid morphogenesis that contain five copies of each of the capsid protein precursors VP4-VP2 (VP0), VP3, and VP1–2A. The role of the small VP4 polypeptide in virion morphogenesis is unknown. Most other picornaviruses have four polypeptides within their capsid, including a small VP4 protein located at the amino terminus of the polyprotein. The polyprotein of HAV appears to possess a very short VP4 polypeptide segment at its amino terminus; this putative VP4 moiety has never been demonstrated directly in purified virus preparations. Moreover, whereas N-terminal myristoylation of other picornavirus VP4 proteins is important for virion morphogenesis, the HAV VP4 sequence does not contain a similarly placed myristoylation signal. VP4 may be necessary for assembly of pentamers into empty capsids [20].

In Vitro Culture, Virus–Cell Interactions, and Replication Cycle

HAV was first propagated in marmoset liver explant cultures and a cloned line of fetal rhesus monkey kidney cells (FRhK-6) with a strain of virus (CR326) that had been adapted by multiple passages in *Saguinus mystax* and *Saguinus labiatus* marmosets [21]. Many HAV strains have subsequently been isolated from clinical material, although the procedure may take several weeks. Until recently, only epithelial or fibroblast cells of primate origin have been shown to support growth of the virus. However, in a systematic search for cells that would support HAV replication, growth was detected in cells of guinea pig, dolphin, and porcine origin. The major characteristics of HAV in cell culture are slow growth and low yields relative to other picornaviruses. In addition, the virus remains largely cell associated, does not usually produce a cytopathic effect, and readily leads to persistently infected cell lines. Rapidly replicating variants of HAV have been selected that induce cytopathic effects in some cell lines; these variants have proven extremely useful for virus titrations and studies of inactivation kinetics and virus replication [22].

The growth of wild-type virus is generally poor in cultured cells, and the virus must undergo a process of adaptation before becoming capable of efficient replication. Adaptive mutations that permit HAV to replicate efficiently in cell culture have been extensively characterized.

The kinetics of viral replication and biosynthetic events has been studied in cells infected with cell culture-adapted strains of HAV and reveal a number of differences from most other picornaviruses. Following attachment to cells, the uncoating of virus is delayed for more than 8 h; this exceeds the duration of an entire growth cycle for many picornaviruses. The delayed uncoating appears to be related to the

protracted maturation cleavage of VP0 to VP2 and VP4, as virions are uncoated more rapidly than provirions [23]. As outlined above, translation of the viral polyprotein is directed by the IRES within the 5′ UTR. The initial proteolytic processing of HAV polyprotein is accomplished by the viral 3C protease, and assembly of viral particles proceeds via monomers and pentamers. After infection of cell cultures with rapidly replicating and cytopathic variants of HAV, pentamers are first detected at 9 h post infection and reach peak levels around 18 h even though the amount of viral RNA (and presumably viral translation) increases beyond this time. These cells continue to produce virus for 2–3 days before cell death, whereas most HAV variants progress to a persistent infection with reduced levels of virus production over many weeks and subsequent cell passages [24].

Repeated passage in cell culture has been used to apply mutation pressure to HAV to alter the phenotype. For example, HAV variants have been selected that grow more rapidly or are resistant to neutralization by monoclonal antibodies. Attenuated strains of HAV have been selected by multiple tissue culture passages, and cold adaptation has been achieved by passage at reduced temperature. Some of the mutations responsible for these altered phenotypes have been identified by molecular cloning and sequencing of the mutant. Mutations within the IRES enhance cap-independent viral translation in a cell-type-specific fashion; mutations within the 5′ UTR and mutations within the 2B and 2C coding regions of HAV RNA have been shown to enhance virus replication in vitro [25]. However, mutations within the VP1–2A and 2C proteins appear to be most important for attenuation of virulence. These viruses are cytopathic and appear to cause cell death by inducing apoptosis [26]. However, in most HAV-infected cells, both in cell culture and probably also in vivo, there is no cytopathic effect. Cytopathic variants of HAV have been observed in selected cell culture systems. These cytopathic viruses produce an acute rather than persistent infection. The replication cycle of these variants is shorter (2–3 days) than that observed for noncytopathic HAV, and they produce a much higher viral yield [27].

The virus apparently downregulates its replication in cells commonly used for its propagation (FRhK-4 and MRC-5 cells) [28]. These observations may be of relevance to the mechanisms underlying establishment of persistent infections in vitro. Although a large proportion of the newly replicated virus remain cell-associated, extensive release of progeny virus into cell-culture supernatant fluids occurs by an unknown mechanism. In polarized, human colonic epithelial cell cultures, release of virus occurs almost exclusively into apical supernatant fluids, mimicking the secretion of HAV across the apical canalicular membrane of the hepatocyte into the biliary system [29].

Although it is well established that HAV is transmitted through the fecal–oral route, it is unknown whether there is a primary site of HAV replication in the digestive system or whether the input virus is transported to the blood and then reaches the liver, its target organ. Although evidence of extrahepatic viral replication in humans in tissues other than the liver is sparse, some cells of the gastrointestinal tract are probably susceptible to HAV. Animal studies have demonstrated some evidence of replication in the oropharynx or tonsillar tissue and the upper portion of the small intestine [30].

Most viruses initiate infection by first binding to a specific cell-surface receptor molecule or in many cases may require binding to both receptors and co-receptors to facilitate virus entry and uncoating. Identification of a specific receptor for HAV remained elusive for many years; however, Kaplan and coworkers succeeded in the isolation of one specific receptor molecule, havcr-1, first in cells of simian origin and later in human cells [31]. The HAV cellular receptor 1 (HAVCR1/TIM1) is a type 1 integral membrane glycoprotein consisting of a characteristic six-cysteine immunoglobulin (Ig)-like domain extended above the cell surface by a mucin-like domain that contains variable number of threonine, serine, and proline (TSP) hexameric repeats. Because this molecule is expressed on cells from many tissues that are not susceptible to HAV infection, it is likely that specific coreceptors contribute to the organ tropism of HAV. Soluble receptor forms of HAVCR1/TIM1 bind, alter, and neutralize HAV particles [32], which indicated that additional co-receptors are not required for the initial steps in HAV-cell entry. Since IgA increases the receptor-mediated neutralization of HAV, it appears that additional factors may enhance the cell entry process of HAV. IgA1lambda is a specific ligand of HAVCR1/TIM1 and the association between IgA1lambda and HAVCR1/TIM1, enhances the interaction of HAVCR1/TIM1 with HAV. Thus, the interaction of IgA with HAVCR1/TIM1 may play a role in the pathogenesis of HAV, enhancing viral-cell entry in cells expressing low levels of HAVCR1/TIM1 [33].

A model for HAV replication and gene expression has been proposed (Fig. 36.2) [14]. The virus enters the hepatocyte via an interaction with a cellular receptor, the identity of which remains uncertain (Fig. 36.2a). This is followed by uncoating of the viral particle and release of the positive-sense RNA genome into the cell (Fig. 36.2b). The IRES mediates cap-independent translation of the viral polyprotein (Fig. 36.2c). The polyprotein undergoes co- and posttranslational proteolytic processing directed by the viral protease, 3Cpro (Fig. 36.2d). Nonstructural viral proteins assemble into a membrane-bound RNA replicase, bind the 3′ end of the genomic RNA and commence synthesis of a negative-strand copy of the viral genome (Fig. 36.2e). The negative-strand copy of the genome is used as template for synthesis of multiple new copies of genomic positive-strand RNA (Fig. 36.2f). Some of this newly synthesized positive-sense RNA is recycled for further RNA synthesis or translation (dashed lines) (Fig. 36.2g). Other positive-strand RNA molecules

are packaged into new viral particles formed by assembly of the structural proteins, followed by final cleavage of the VP1–2A precursor by an unknown cellular protease (VP1/2A junction), and the "maturation" cleavage of the VP4/VP2 junction (Fig. 36.2h). Newly assembled HAV particles are secreted by the cell across the apical membrane of the hepatocyte into the biliary canaliculus, from which they are passed into the bile and small intestine (Fig. 36.2i) [14].

Genomic Variability of HAV

A genotype is defined as a group of viruses with >85% nucleotide identity. A subgenotype is defined as a group of viruses with sequence variability of less than 7.5%. Since HAV and poliovirus share many genomic features, the different HAV strains were grouped by comparing the VP1–2A junction, using the method of Rico-Hesse et al., a criterion used at that time for genetic classification of Poliovirus strains [34]. In 1992, using this approach, genetic analysis of 152 HAV strains recovered around the world resulted in the designation of seven genotypes of HAV (I–VII) [35]. However, the majority of strains included in these studies were isolated in the USA and Asia, leaving other regions of the world that have a hyperendemic pattern of HAV, such as South America, North and Central Africa, and India. Moreover, by using the traditional method of genotyping, a few HAV antigenic variants reported recently cannot be detected [36]. Recently, an alternative genotyping method using full-length VP1 sequences (900 nucleotides) has been suggested, which indicates the presence of five distinct genetic groups, all of them supported by high bootstrap values [35]. However, a few sequences were not included in these studies, including one of strain JM-55 (genotype VI) and those representing genotype IIIB, because none of them were available in a public sequence database at that time. Based on these studies, a novel classification of HAV genotypes was proposed to include six different genotypes [37]. A higher degree of genetic relation than previously expected was observed between the previously described genotypes II and VII [14], suggesting that they may be one or two subgenotypes of the same type. Recent work has confirmed the hypothesis that genotypes II and VII are two subgenotypes of genotype II [38]. Therefore, HAV has six different genotypes: three isolated from humans (I–III) and three from a simian origin (IV–VI) [39]. Genotypes I and III are the most prevalent genotypes isolated from humans. The three simian genotypes were each defined by unique nucleotide sequences from the P1 regions of HAV strains recovered from species of Old World monkeys. In addition, all simian HAVs have a distinct signature sequence at the VP3/VP1 junction, which distinguishes these strains from human HAVs. Genotype IV was recovered from a cynomolgus macaque (*Macaca fasicularis*) imported from the Philippines. The prototype strain of genotype V, AGM27, was isolated from an African green monkey (*Cercopithecus aethiops*) imported from Kenya. Genotype VI was also isolated from a cynomolgus macaque (*M. fasicularis*) imported from Indonesia [39].

Different HAV genotypes have a different geographic distribution. Genotype I is most prevalent worldwide, and subgenotype IA is more common than IB. Subgenotypes IA and IB are most often found in North and South America, Europe, South East Asia, and Indian subcontinent [39–41]. Cocirculation of multiple genotypes or subgenotypes has been reported in some regions of the world, as IA and IB in South Africa, Brazil, and France [39] and subgenotypes IA and IIIA and IIIA and IB in India [39, 41].

Recombination in HAV

Genetic exchange by homologous and nonhomologous recombination is a phenomenon that is common among RNA viruses and may lead to hybrid or defective interfering RNA molecules [39]. In HAV, genetic exchange among strains had been observed in cell culture [42], but for many years it was supposed not to occur in nature. This view was challenged by the report of a case of dual infection of a young childcare provider (AUX-23) with HAV strains belonging to different subgenotypes [43]. The first HAV recombinant strain isolated from an infected patient was reported in 2003 [37]. The recombinant isolate, 9F94, comes from a little girl who was hospitalized in France after a 3-month holiday in Morocco. Accordingly, the putative parental strains SLF88 (now classified as genotype II) and MBB (genotype IB) were also originally isolated in North Africa, a region of high endemicity for HAV infection and one in which multiple genotypes cocirculate. The recombination event in strain 9F94 took place in the VP1 capsid protein. This finding indicates that capsid recombination may play a significant role in shaping the genetic diversity of HAV and, as such, can have important implications for its evolution, biology, and control. Nevertheless, the frequency and possible implications of HAV capsid recombination events for the generation of pathogenic HAV strains are not clear at present [39].

Quasispecies Nature and Evolution of HAV

As other RNA viruses, HAV exists in vivo as distributions of closely related variants referred to as quasispecies [44]. Quasispecies dynamics is characterized by continuous generation of variant viral genomes, competition among them, and selection of the fittest mutant distributions in any given environment. Over time, RNA virus evolution is conditioned by perturbations of population equilibrium, that may not be

equal among individual hosts, and therefore, multiple viral sublineages may rapidly be established that differ in the number of rounds of replication (and history of environmental perturbations), and may cocirculate in the same geographical area. To study HAV evolution over time in a specific geographic region, recent studies were carried out on HAV genotype I strains isolated in France from 1983–2001, using a nonhierarchical method developed to study closely related components of mutant spectra of viral quasispecies. These studies have identified different subpopulations of HAV variants that coexist in time and in different environments [44]. Clades isolated from different years, reemerged and were even associated with epidemic strains. These findings suggest that beyond mutations and genetic recombination, HAV exploits this variation strategy in dominance to promote and ensure its survival [44]. The coexistence of different subpopulations are consistent with the presence in each HAV isolate of a mutant spectrum, which provides a repertoire of variants that, while constituting a minority in an infected individual, may become dominant following transmission to a new host individual. These findings fit the general picture of quasispecies dynamics; with the salient antigenic stability of HAV that is probably related to structural constraints of the viral capsid [39]. The HAV mutation rate has been estimated at 1×10^{-3}–1×10^{-4} substitutions per site [45], which is much lower than that found in other members of the family *Picornaviridae.* Nevertheless, further studies are needed in order to establish substitutions/site/year in monophyletic natural populations of HAV [39].

Antigenicity and Serotype

Only a single serotype of HAV exists, despite genetic heterogeneity at the nucleotide level. Individuals infected by HAV in one part of the world are protected from reinfection by HAV from other parts of the world. IG preparations containing anti-HAV, irrespective of their geographic origin, appear to provide protection from disease, and vaccines prepared from virus isolates originating in one region protect from infection worldwide. The antigenic structure of the virus is relatively simple, with a restricted number of overlapping epitopes combining to form a single dominant antigenic site that interacts with virus-neutralizing antibodies. These epitopes are highly conformational and are formed by amino acid residues located on more than one capsid protein. Convalescent-phase sera obtained from hepatitis A patients are reactive primarily to VP1 and to a lesser extent to VP0 and VP3. Empty particles appear to be antigenically indistinguishable from infectious, RNA-containing virions, suggesting that antigenicity may depend on assembly of the major capsid proteins or smaller capsid precursors. An accurately processed and assembled recombinant HAV polyprotein has been produced, which was able to elicit neutralizing antibodies detected by commercial assays [46]. Naturally occurring antigenic variants of HAV have been observed only among strains isolated from Old World monkeys. These viruses are genetically distinct from human HAV isolates and are not recognized by certain monoclonal antibodies produced against human HAV. However, simian HAV binds human polyclonal anti-HAV, and chimpanzees immunized with these viruses had an antibody response that was protective against infection with human HAV challenge [14].

Host Range

Humans are considered to be the only important reservoir of HAV. However, the existence of extrahuman reservoirs of infection remains possible. In 1961, Hillis [47] described an outbreak of hepatitis A among chimpanzee handlers who apparently contracted the infection from the chimpanzees. Epidemiologic data suggested that the animals had become infected during captivity, but before their importation into the United States. Interestingly, although epidemics of hepatitis were recognized in American primate handlers, the disease was rarely seen in Africa, presumably because most handlers were already immune. In 1962, Deinhardt [48] demonstrated liver function abnormalities in chimpanzees that were inoculated with human feces or acute-phase sera known to have transmitted hepatitis A to humans. In 1967, they inoculated tamarins (*Saguinus nigricollis*) with sera from patients who were judged to have hepatitis A and were able to transmit infection [49]. These results were confirmed in other species of tamarins and later in chimpanzees [50].

Widespread screening of nonhuman primates has revealed antibodies to HAV in chimpanzees, gorillas, orangutans, gibbons, macaques, owl monkeys, pig tail monkeys, rhesus monkeys, and several species of South American tamarin monkeys [51–53]. It is unclear whether such primates may serve as reservoirs of infection, or rather as transient hosts after exposure to HAV from human sources.

Epidemiology

Modes of Transmission

HAV replicates in the liver, is excreted in bile, and is found in highest concentrations in stool. Thus, fecal excretion is the primary source of virus. In experimental studies, infectivity of stools has been demonstrated for 14–21 days before to 8 days after onset of jaundice, but the highest concentrations occur during the 2-week period before jaundice develops or

liver enzymes become elevated, followed by a rapid decline after the appearance of jaundice [54]. Data from epidemiologic studies also suggest that peak infectivity occurs during 2 weeks before the onset of symptoms. Shedding of HAV in stool may continue for longer periods in infected infants and children than adults. HAV RNA has been detected in stool of infected newborns for up to 6 months after infection [55]. Excretion in older children and adults has been demonstrated 1–3 months after clinical illness. Although chronic shedding of HAV does not occur, the virus has been detected in stool during relapsing illness [56].

During the period of viremia, which begins during the prodrome and extends through the period of liver enzyme elevation, HAV concentrations in serum are several orders of magnitude lower than in stool. However, in experiments conducted in nonhuman primates, HAV was several orders of magnitude more infectious when administered by the intravenous route in comparison with the oral route, and animals were successfully infected with low concentrations of HAV administered via the intravenous route [57]. Although HAV may occasionally be detected in saliva in experimentally infected animals [58], transmission by saliva has not been demonstrated. Enzyme immunoassays and PCR may detect defective as well as infectious viral particles. Thus, the detection of HAV antigen in the stool by enzyme immunoassays or HAV RNA in the serum or stool by PCR does not mean that an infected person is necessarily infectious, and it is likely that the period of infectivity is shorter than the period during which HAV RNA is detectable. For practical purposes, both children and adults with hepatitis A can be assumed to be noninfectious, 1 week after jaundice appears.

Person to Person

Person-to-person transmission by the fecal–oral route is the primary means of HAV transmission throughout the world [59]. Most transmission occurs among close contacts, particularly in households and extended family settings. Young children have the highest rates of infection and are often the source of infection for others, because infections in this age group are often asymptomatic and standards of hygiene are generally lower among young children compared with adults.

Foodborne and Waterborne

HAV can remain infectious in the environment for long periods of time allowing for common-source outbreaks and sporadic cases to occur from exposure to fecally contaminated food or water. Many uncooked foods have been recognized as the source of outbreaks. Cooked foods can also transmit HAV, if the cooking is inadequate to kill the virus or if the food is contaminated after cooking, which is common in outbreaks associated with infected food handlers [60].

Various types of food products have also been implicated (see below). Waterborne outbreaks of hepatitis A are uncommon in developed countries, but may be common in less developed countries (see below).

Blood-Borne

Transfusion-related hepatitis A is rare because HAV does not result in chronic infection, and, in the developed world, blood donors have been screened for many years for elevated aminotransferase levels. However, transmission by transfusion of blood or blood derivatives collected from donors during the viremic phase of their infection has been reported (see below).

Vertical

Data on the incidence and outcome of hepatitis A during pregnancy are scant. Mother-to-child HAV transmission seems to be very rare. Case reports describe intrauterine transmission of HAV during the first trimester, the risk of transmission from pregnant women who develop hepatitis A in the third trimester of pregnancy to newborns appears to be low (see below).

Specific Groups and Settings

Child Care Centers, Schools, and Institutions

Outbreaks in child care centers have been recognized for many decades. They rarely occur in centers that do not have children in diapers and are more common in larger centers [61]. The outbreaks can be sustained among children with asymptomatic infection and often are not recognized until adult contacts (usually parents) become ill. Despite the occurrence of outbreaks when HAV is introduced into a child care center, studies of child care center employees do not show a significantly increased prevalence of HAV infection compared with control populations [62]. Hepatitis A cases among children in schools usually reflect a disease that has been acquired in the community, although multiple cases among children within a school may indicate a common-source outbreak. Historically, HAV infection was endemic in institutions for the developmentally disabled, but with smaller facilities and improved conditions, the incidence and prevalence of infection have decreased and outbreaks are rarely reported in developed countries [63].

Users of Illicit Drugs

During the two past decades, outbreaks have been reported with increasing frequency among illicit drug users [64]. Cross-sectional serologic surveys have demonstrated that injection drug users have higher prevalence of anti-HAV than the general population in developed countries. Transmission among injection drug users probably occurs through both percutaneous and fecal–oral routes [65].

Homosexuality

Hepatitis A outbreaks among men who have sex with men have been reported frequently, most recently in urban areas in the developed countries and may occur in the context of an outbreak in the larger community [66]. Seroprevalence surveys have not consistently demonstrated an elevated prevalence of anti-HAV compared with a similarly aged general population [67, 68]. Some studies conducted during outbreaks and seroprevalence surveys among homosexual men have identified specific sex practices associated with illness, whereas others have not demonstrated such associations [69, 70].

Transfusions and Other Health Care Settings

Parenteral transmission is extremely rare, but can follow transfusion of blood from a donor who is in the incubation period of the disease [71]. The relatively short duration of viremia in acute hepatitis A, together with the moderate titer of HAV viral load in the blood, diminishes the likelihood of transfusing a unit of blood infectious for HAV. The risk of infection in patients with hemophilia is not known. Multitransfused beta-thalassemic and hemophiliac patients present higher frequency of anti-HAV IgG antibodies than normal population of the same geographic area. This difference is difficult to explain, but it can be attributed to the higher vulnerability of thalassemics to HAV infection and to passive transfer of anti-HAV antibodies by blood transfusions [72]. Outbreaks have been reported in Europe and the United States among patients who received factor VIII and factor IX concentrates [73]. Outbreaks have also been reported in neonatal intensive care units following transmission to hospital staff from a neonate with asymptomatic HAV infection acquired from a blood transfusion [74, 75]. Transmission has also been reported in association with an experimental treatment with lymphocytes incubated in serum from a donor with HAV infection [76]. Nosocomial transmission from adult patients to health care workers is rare, because most patients with hepatitis A are hospitalized after the onset of jaundice, when infectivity is low, but it has been reported in association with fecal incontinence of the patient [77]. Health care workers have not been found to have an increased prevalence of anti-HAV compared with control populations in serologic surveys.

International Travel

Hepatitis A is a common infection among travelers from developed countries who travel to regions with high, transitional, or intermediate endemicity [78, 79]. In prospective studies of American and European travelers, the risk of infection for those who did not receive immunoglobulin was found to be 3–5 per 1,000 per month of stay, of the same order of magnitude as that for malaria, 10–100 times greater than that for typhoid, and 1,000 times greater than that for cholera [80]. The risk may be higher among travelers staying in areas with poor hygienic conditions, varies according to the region and the length of stay, and appears to be increased even among travelers who reported observing protective measures and staying in urban areas or luxury hotels. Travelers who acquire hepatitis A during their trip may also transmit to others on their return [81].

Foodborne and Waterborne

Foodborne hepatitis A outbreaks are recognized relatively infrequently in the developed countries [60]. They are most commonly associated with contamination of food during preparation by a food handler with HAV infection [82]. Implicated foods include those not cooked after handling, such as sandwiches and salads, as well as partially cooked foods. Food contaminated before retail distribution, such as lettuce or fruits contaminated at the growing or processing stage, has been increasingly recognized as the source of hepatitis A outbreaks [83]. Consumption of raw seafood including molluscan shellfish and oysters has also been linked to hepatitis A outbreaks [84, 85].

Waterborne-hepatitis A outbreaks are rare in developed countries, but are common in less developed countries and are related to sewage contamination or inadequate treatment of water [59]. Although results of some serologic surveys conducted among sewage workers in developed countries indicated a possible elevated risk of HAV infection, findings have not been consistent [86].

Incidence and Prevalence and Worldwide Disease Patterns

Hepatitis A occurs worldwide, but major geographical differences exist in endemicity and resulting epidemiologic features (Fig. 36.3). The degree of endemicity is closely related

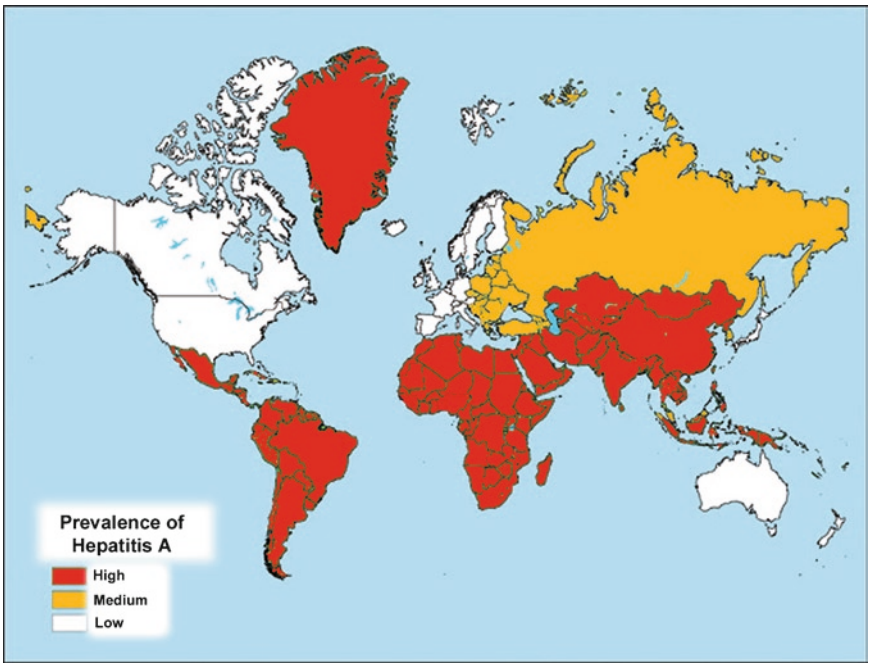

Fig. 36.3 Geographic distribution of hepatitis A. Modified and adapted from Rose and Keystone [208]

to hygienic and sanitary conditions and other indicators of the level of development. In less-developed areas, especially when there is limited access to clean water and inadequate disposal of human feces, HAV infects most people early in life, when infection is rarely clinically apparent. In countries with high standards of hygiene and sanitation, the majority of adults remain susceptible. Distinct patterns of HAV infection can be described, each characterized by particular age-specific anti-HAV prevalence and hepatitis A incidence, and prevailing environmental (hygienic and sanitary) and socioeconomic conditions [87].

In areas of high endemicity, represented by the least-developed countries (i.e., parts of Africa, Asia, Central and South America), poor hygienic and sanitary conditions allow HAV to spread readily. Infection is nearly universal in early childhood, when asymptomatic infection predominates, and essentially the entire population is infected before reaching adolescence, as demonstrated by the age-specific prevalence of anti-HAV [59, 88, 89]. Susceptible adults in these areas are at high risk of hepatitis A, but reported disease rates are generally low and outbreaks are rare because most adults are immune. High endemicity patterns can also be seen in some ethnic or geographically defined groups within highly developed countries, such as aboriginal children in the north of Australia or American Samoa, FSM and Palau [90–92].

In areas of moderate endemicity, HAV is not transmitted as readily because of better sanitary and living conditions, and the predominant age of infection is older than in areas of high endemicity [93, 94]. Paradoxically, the overall incidence and average age of reported cases are often higher than in highly endemic areas because high levels of virus circulate in a population that includes many susceptible older children, adolescents, and young adults, who are likely to develop symptoms with HAV infection [95]. Large common-source food- and water-associated outbreaks can occur, because of the relatively high rate of virus transmission and large number of susceptible persons, especially among those of higher socioeconomic level. Such an outbreak occurred in Shanghai in 1988, with over 300,000 cases associated with consumption of clams harvested from water contaminated with human sewage [96]. Nevertheless, person-to-person transmission in community-wide epidemics continues to account for much of the disease in these countries.

Improved sanitary conditions have generally resulted in a significant decline in the incidence of hepatitis A in various countries. For example, HAV susceptibility in Japan is increasing annually. Particularly, the prevalence of anti-HAV antibody in individuals older than 50 years in 2003 was 50.3%, which is significantly lower than that of corresponding studies in 1994 (74.3%), 1984 (96.9%), and 1973 (96.9%). The growing susceptible population of advanced age results in more frequent HAV infection among them [97]. Similarly, since the mid 1970s, infection with HAV in Thailand has shifted from hyperendemic to mesoendemic. However, as exposure to HAV declines, the risks of symptomatic and potentially severe infection in adulthood (rather than asymptomatic infection during childhood), and epidemics of such infections increases [98]. Also, there has been a marked decline in the prevalence of HAV in Saudi Arabia [99]. Similarly in China, the incidence of hepatitis A has

declined by 90% since 1990, from 56 to 5.9 per 10(5) per year. Declines in age-specific incidence were seen in all age groups, most dramatically among children younger than 10 years. Disease incidence still varies substantially; poorer western provinces have had the highest incidences since 2000. In high-incidence provinces, children younger than 10 years continue to have a high disease incidence. Universal hepatitis A vaccination of young children began to be implemented in 2008 in China [100].

Shifts in age-specific prevalence patterns that reflect a transition from high to intermediate endemicity are occurring in many parts of the world. A feature of this transitional pattern is striking variations in hepatitis A epidemiology between countries, and within countries and cities, with some areas displaying a pattern typical of high endemicity, and others of intermediate endemicity [101, 102]. Considerable hepatitis A related morbidity, mortality, and associated costs occur with this transition, even in developing countries [103, 104].

In the United States, Canada, Western Europe, and other developed countries, the endemicity of HAV infection is low. Relatively fewer children are infected, the incidence of disease is generally low, and disease often occurs in the context of community-wide and child care center outbreaks [85, 105]. Population-based seroprevalence surveys show a gradual increase in the prevalence of anti-HAV with increasing age, primarily reflecting declining incidence, changing endemicity, and resultant lower childhood infection rates over time. The HAV incidence has further decreased by implementation of HAV vaccination. For example, in the US, acute hepatitis A incidence has declined 92%, from 12.0 cases per 100,000 population in 1995 to 1.0 case per 100,000 population in 2007, the lowest rate ever recorded. Declines were greatest among children and in those states where routine vaccination of children was recommended beginning in 1999 [106]. However, in some areas, recent increase in HAV cases have been reported. For example, the continuous improvement of hygiene that occurred in New Caledonia, France during the last two decades, led to a dramatic drop in the frequency of hepatitis A among patients tested, ranging from an average value of 79 cases (14%) for the 1986–1999 period to 0 case from 2002. However, in 2005, a strong increasing number of confirmed cases was notified, mainly among young people (78% were under the age of 20). In 2006, this epidemic reached the island of Futuna where it involved more than 1% of the total population (56 cases). The phylogenetic study has confirmed the clonality of the virus circulating during this epidemic, not related to other regional strains (Fiji, Vanuatu, and New Zealand), nor with a New Caledonian strain from the previous endemic period. This transition situation, with persistence of a high epidemic risk, should encourage the health authorities to implement adapted response strategies, based in particular on systematic case declaration and targeted immunization programs [107].

Pathogenesis

Although HAV shares many virologic characteristics with enteroviruses, it has several differentiating features that influence the pathogenesis and clinical expression of the disease. HAV is resistant to heat, solvents, and acid and grows slowly in living cells, where it has been shown to be relatively noncytolytic and to have little effect on the rate of host protein synthesis.

Incubation Period

Determination of the incubation period of disease is imprecise because the early symptoms of hepatitis are often vague and nonspecific. Jaundice may not be noticed by the patient, so the most useful marker of the onset of the disease is a change in urine color, which is almost always recognized by the patient and is the most common reason for seeking medical attention. The range of incubation is between 2–7 weeks, with a mean of about 4 weeks. Although HAV can be transmitted orally or parenterally, the incubation period is independent of the route of inoculation [108]. Experiments in primates and observations in humans suggest that the incubation period is dependent on the infectious dose [65].

Viral Replication

HAV is generally transmitted by the fecal–oral route. Because the virus is acid resistant, it probably passes through the stomach, replicates lower in the intestine, and is then transported to the liver, which is the major site of replication. Evidence of replication in the oropharynx has been obtained in chimpanzees. HAV, like many other picornaviruses, is highly organ specific with little evidence of significant replication outside the liver. Virus is shed from infected liver cells into the hepatic sinusoids and canaliculi, passes into the intestine, and is excreted in feces. In humans, as well as in nonhuman primates, HAV has been detected in the liver, bile, and feces [69]. The first indirect evidence that virus may replicate in the gut was the detection of coproantibodies in the feces [67], followed by the demonstration of hepatitis A antigen in duodenal-lining cells [109]. Nonetheless, the major pathology is restricted to the liver. The replication cycle has been explained above.

Pathogenesis and Natural History of HAV Infection

Infection with HAV usually occurs by the fecal–oral route of transmission and is associated with extensive shedding of the virus in feces during the 3–6-week incubation period and extending into the early days of the illness (Fig. 36.4). This explains the high prevalence of infection in regions where low standards of sanitation promote transmission of HAV. HAV is exceptionally stable at ambient temperatures and at low pH. These features of the virus explain its ability to survive in the environment and to be transmitted by contaminated foods and drinking water. Resistance to acid pH and detergents also accounts for its ability to transit through the stomach, and to exit the host via the biliary tract. These are important features that contribute significantly to the pathogenesis of hepatitis A.

Chimpanzees, as well as several species of New World monkeys, including marmosets, tamarins, owl monkeys, and Saimiri monkeys, are susceptible to HAV and may be infected by either oral or percutaneous challenge [14]. Much has been learned from these nonhuman primate models of hepatitis A, although they do not recapitulate the disease perfectly. Liver injury is usually mild compared with symptomatic infections in adult humans, although the course of the infection is otherwise very similar. Most of this virus appears to be produced in the liver and to reach the intestinal contents by secretion from infected hepatocytes via the biliary system. Nonetheless, some data suggest that HAV may undergo initial replication within crypt cells of the small intestine before reaching the liver [110]. HAV is primarily hepatotropic; it replicates in the liver, produces a viremia, and is excreted in bile and shed in the stools of infected persons. Feces can contain up to 10(9) infectious virions per gram and is the primary source of HAV infection. Peak fecal excretion, and hence infectivity, occurs before the onset of jaundice, symptoms, or elevation of liver enzymes and declines after jaundice appears. Compared to adults, children and infants can shed HAV for longer periods, that is, up to several months after the onset of clinical illness. Fecal shedding of HAV has been shown to occur as late as 6 months after diagnosis of infection in premature infants [55]. Viremia occurs within 1–2 weeks after HAV exposure and persists through the period of liver enzyme elevation, based on studies in humans and experimentally infected chimpanzees [58, 111]. Virus concentrations in serum are 2–3 log10 units lower than those in stool [58]. An analysis of serum specimens collected prospectively during human and chimpanzee HAV infection showed that HAV RNA was present for 3–4 weeks before the onset of jaundice and that virus concentrations were highest during the period that precedes onset of liver enzyme elevations [112]. Viremia may be present for a much longer period during the convalescent phase of hepatitis A than was previously appreciated, although virus concentration is lowest during this period [112]. The virus is also shed in saliva in most hepatitis A patients. In experimentally infected marmosets, the viral load appears to be 1–3 log10 units lower than that found in serum [113]. However, no epidemiological data suggest that saliva is a significant source of HAV transmission. Liver injury follows, often with marked elevation of serum aminotransferase activities. Viral antigen can be detected within the cytoplasm of hepatocytes, as well as within germinal centers of the spleen and lymph nodes and along the glomerular basement membrane in some primates [114]. Viral antigen typically continues to be shed for 2–3 weeks after the first elevation of enzymes, although sensitive reverse transcription polymerase chain reaction assays can detect continued shedding of viral RNA for many weeks. Prolonged shedding of the virus has only been documented in infected premature infants. Importantly, older epidemiologic studies have demonstrated the disappearance of HAV from closed populations with time, suggesting that long-term, persistent fecal shedding of virus does not occur.

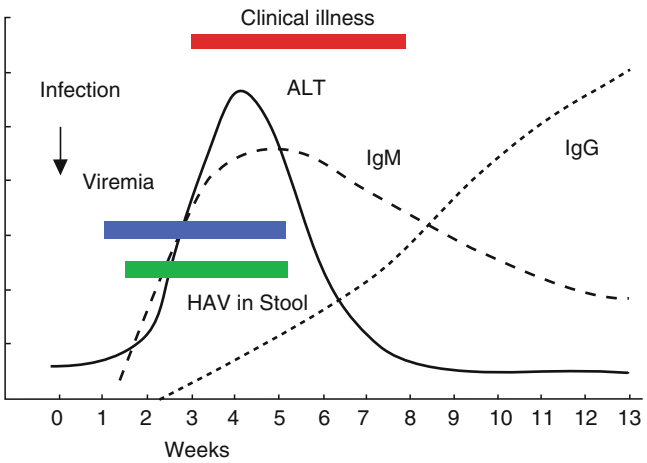

Fig. 36.4 Timeline of hepatitis A manifestations

The mechanisms responsible for hepatocellular injury in hepatitis A are poorly characterized. However, type A hepatitis appears to be attributable to an immunopathologic response to infection of the hepatocyte, rather than to a direct cytopathic effect of the virus. HLA-restricted, virus-specific, cytotoxic, $CD8^+$ T cells have been recovered from the liver in acute hepatitis A (see below).

Although the severity of hepatitis A varies, it is not clear why it is more severe in some patients than that in others. It is thought that disease severity may be dependent on certain characteristics of the individual patients. It has been reported that aging and underlying chronic liver disease could be factors that increase hepatitis A severity [115]. Several studies on analysis of factors possibly contributing to the severity of the disease failed to reveal any significant differences in

patient characteristics including age [116], suggesting that viral factors might determine the severity of the disease. It was reported that mutations in 5′NTR, 2B and 2C of HAV were associated with cytopathic variants in cultured cells, and virulence in tamarins [117, 118]. Fulminant hepatitis patients had fewer nucleotide substitutions in 5′NTR, had a tendency to have more amino acid (aa) substitutions in 2B, and had fewer aa substitutions in 2C than self limited-acute hepatitis patients [119].

Immune Response

HAV is generally not cytopathic in cell culture, and histopathologic findings in experimental animals and humans do not show widespread hepatocyte necrosis, although the vast majority of hepatocytes at the peak of viral replication appear to be infected by immunohistochemical staining. After infection via the gastrointestinal tract, HAV replicates quietly within the liver for several weeks or more during the incubation period of the disease. By the end of this period, high titers of virus are present within liver tissue, bile, stool, and to a lesser extent in blood. Despite this, there is little evidence of liver injury and often no disease. Not until the fourth or fifth week of the infection, do clinical manifestations of the infection appear along with the first evidence of an immune response to the virus.

Recent studies suggest that this prolonged period of clinical quiescence in the face of mounting viral replication may reflect the ability of the virus to disrupt cellular mechanisms by which mammalian cells recognize virus infection and induce synthesis of interferon-γ [28]. In HAV-infected cells, double-stranded RNA (dsRNA)-mediated activation of interferon regulatory factor 3 (IRF-3) is blocked. IRF-3 is a key transcription factor that is constitutively expressed in the cytoplasm. It is phosphorylated after virus infection, leading to its nuclear translocation and subsequent induction of interferon-γ synthesis. Within hepatocytes, this occurs as the result of signaling, transduced through two distinct pathways: one pathway is initiated by dsRNA engagement of Toll-like receptor 3, and the other by the interaction of dsRNA with a novel pathogen-associated molecular pattern receptor, the RNA helicase, retinoic acid-inducible gene I [120]. IRF-3 activation through the retinoic acid-inducible gene I pathway is effectively blocked in cells infected with HAV [121]. Signaling through the Toll-like receptor 3 pathway also may be impaired partially. Limited studies in cell culture suggest that HAV is sensitive to type 1 interferons, but it is unclear whether HAV, expresses proteins that antagonize the specific antiviral effector mechanisms induced by interferons [14]. The adaptive immune response to HAV is robust and extremely effective in eliminating the virus.

It has been postulated that liver-cell damage occurs through a cell-mediated immune response, whereas circulating antibodies are probably more important in limiting the spread of virus to uninfected liver cells and other organs. This hypothesis is consistent with observations in animal models and humans. For example, intravenous inoculation of marmosets with a large dose of HAV resulted in mildly abnormal liver function test results and detectable hepatitis A antigen in hepatocytes within the first week. Enzyme levels stabilized or even declined until the third week after inoculation, when a second, higher peak was observed coincident with the appearance of serum antibodies [74]. One explanation is that the early mild hepatitis was due to a direct viral effect, but the second, more severe, episode was due to an immune response. The presence of large quantities of virus in hepatocytes before the onset of hepatitis also argues against a major direct cytopathic effect of HAV. It has been suggested that virally elicited T cells target infected liver cells and induce immunopathology. Virus-specific, HLA-restricted cytotoxic T-cells have been identified within the liver during acute HAV infection and probably play roles both in viral clearance and in the production of liver injury [122]. In human studies, it has been found that lymphocytes from convalescing patients produced cytotoxic effects against autologous epidermal cell lines infected with HAV and that $CD8^+$ T-cell clones demonstrated cytotoxic activity against autologous fibroblasts infected with hepatitis A. These findings are consistent with the hypothesis that $CD8^+$ T lymphocytes mediate liver-cell damage. Furthermore, natural killer cells have been demonstrated to be capable of lysing HAV-infected tissue culture cells [123]. Recent studies have found impaired function of $CD4^+/CD25^+$ T regulatory lymphocytes in their ability to suppress $CD4^+/CD25^-$ T cells among self limiting cases of acute hepatitis A [124].

Although liver-protein damage occurs at the time the circulating antibodies become detectable, it has not been proven that the pathology is antibody dependent. Circulating immune complexes containing HAV and specific IgM antibodies have been found during infection. However, immunoglobulin and complement deposits were not found at the sites of liver-cell damage, and resolution of disease occurred even when antibody levels were rising and hepatitis A antigen could still be detected in the liver [125]. A humoral immune response to HAV structural proteins occurs prior to onset of symptoms. Immunoglobulin M (IgM) antibodies to HAV (IgM anti-HAV) are detectable at or prior to onset of clinical illness, decline in about 3–6 months, and become undetectable by commercially available diagnostic tests. IgG antibodies to HAV (IgG anti-HAV) appear soon after IgM, persist for years after infection, and confer lifelong immunity [126]. IgA is also produced during infection for a limited time. The role of IgA antibodies in the response against HAV is still unknown. Unlike other *Picornaviridae* family members, HAV does not

seem to elicit an effective intestinal immune response [127]. IgG and IgA anti-HAV are detected in serum, saliva, urine, and feces. Saliva tests have been reported as an alternative to conventional serum testing for anti-HAV due to their simplicity of sample collection [128]. Several studies have demonstrated the benefits of implementing saliva testing as screening tool in outbreak investigations and epidemiological studies [129]. However, the sensitivity of detecting anti-HAV in saliva is 1–3 log10 units lower than that with serum [128]. Antibodies against nonstructural proteins are also produced, although their role in maintenance of immunity is probably less important than that of antibodies to capsid antigens due to their low concentration and lack of neutralization capacity. Antibodies to nonstructural proteins have been detected in humans and experimentally infected chimpanzees, but are absent in vaccinated individuals [130]. However, because of what appears to be a variable host antibody response during HAV replication, a diagnostic test for these antibodies, which could be used to complement current anti-HAV testing and differentiate previously infected from vaccinated persons, has not been developed [131].

Over the past two decades, dramatic increase in childhood asthma prevalence has been reported, and some have hypothesized that it might be related to be due to improved hygienic conditions leading to fewer childhood infections. It has been shown that the prevalence of asthma is lower in children who are seropositive for antibody to HAV [132]. A trait associated with asthma, the T-cell and airway phenotype regulator (TAPR), which controls the development of airway hyperreactivity, is a member of the T-cell membrane proteins (TIMs). The human homolog of TIM-1 is the HAV receptor [133]. This potential association between atopic airway disease and hepatitis A is interesting, but appropriate studies that could evaluate a causal relationship need to be conducted.

Pathology

Liver biopsy is rarely indicated to establish a diagnosis in acute hepatitis because this procedure is associated with a small, but finite, risk and the histopathology is not usually diagnostic. In one study done in Japan, where biopsy for acute hepatitis was routine, 86 patients with serologically established acute hepatitis A were evaluated for quantitative and qualitative light microscopic features, together with biopsy samples from 78 patients with acute hepatitis B and from 76 patients with acute hepatitis non-A, non-B. Hepatitis A was characterized by more pronounced portal inflammation than was hepatitis non-A, non-B, but less conspicuous parenchymal changes such as focal necrosis, Kupffer-cell proliferation, acidophil bodies, and ballooning. Nonspecific reactive hepatitis with slightly raised serum transaminase levels was often seen during recovery from hepatitis A, and needs to be distinguished from the longer-lasting cases of acute hepatitis B and C [134]. Hepatitis A antigen and HAV particles can be detected in the cytoplasm of infected cells by immunostaining techniques or thin-section electron microscopy [114].

Clinical Features

Although the clinical expression of infection varies widely, the disease is self-limited, sometimes subclinical, but typically is symptomatic with jaundice. The most important determinant of the likelihood of clinical expression is the age at which infection occurs. The vast majority of infections in children younger than 5 years are silent, and the proportion of symptomatic infections increases with age. The ratio of anicteric to icteric cases has been reported to vary from 12:1to 1:3.5, depending on the age at which infection occurs. In modeling studies, the estimated average probability of jaundice increased from 7% among children younger than 5 years to 37% among children 5–9 years old and to greater than 70% among adolescents and adults [135].

Symptoms

Patients with hepatitis A often describe a mild illness, the prodrome, which appears 1–7 days before the onset of dark urine, although longer periods have been recorded. In the early stages, flu-like symptoms are common; fever (up to 40°C) may be accompanied by chills, mild headache, malaise, and fatigue. Loss of appetite is a common symptom, with patients reporting that the sight or smell of food, especially fatty foods, is nauseating. Vomiting may occur, but is neither severe nor protracted, and weight loss is common. In addition, patients with hepatitis A often lose their taste for tobacco. Occasionally, children may experience atypical symptoms such as diarrhea, cough, coryza, or arthralgia.

The first specific sign of disease and the one that causes most patients to seek medical attention is the onset of dark urine. Bilirubinuria is usually followed within a few days by pale or clay-colored feces and yellow discoloration of the sclera, skin, and mucous membranes. The return of color to the stool occurs 2 or 3 weeks after the onset of illness and is an indication of resolution of the disease. Itching, a sign of cholestasis, occurs in less than 50% of patients, but may be severe enough to require antipruritics or corticosteroids.

On physical examination, the patient's liver may be enlarged and sometimes tender. The spleen is palpable in 5–15% of patients.

The duration of illness varies, but by the third week most patients feel better, have lost their hepatomegaly, and have normal or nearly normal levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In many patients, the appearance of jaundice is associated with rapid resolution of symptoms.

The clinical course and histologic findings do not differ in pregnancy [136]. Data on the incidence and outcome of hepatitis A during pregnancy are scant. In large, retrospective case series, reported areas of moderate to high endemicity, where the incidence of HAV infection during pregnancy was found to be very low, accounted for <2% of hepatitis cases [137]. However, HAV seroprevalence has substantially declined in the last decades in industrialized countries because of improved sanitation. Hence, in these geographical areas, a growing proportion of adults, including pregnant women, are susceptible to HAV infection. This might be a concern in view of the potential severity of adulthood hepatitis A [138]. It is generally acknowledged that hepatitis A is not associated with a severe outcome or complications during pregnancy [136]. In contrast, Elinav et al. [137] recently reported that 69% of pregnant women with acute hepatitis A infection during the second and third trimesters of pregnancy have gestational complications, including premature contractions, placental separation and premature rupture of membranes, which lead to preterm labor.

Mother-to-child HAV transmission seems to be very rare. Four cases of intrauterine infection have been reported. In two of these cases, HAV infection was associated with fetal ascitis, meconium peritonitis, and perforation of the distal ileum, necessitating postnatal surgery, but the newborns subsequently recovered. Both infections occurred after maternal symptomatic hepatitis A at 20 and 13 weeks of gestation [139, 140]. The two other cases of intrauterine transmission occurred following maternal icteric hepatitis A, 20 days before delivery, resulting in neonatal icteric hepatitis A on the third day of life with subsequent full recovery [141]. HAV mother-to-child transmission following hepatitis onset in the mother later than gestational week 33 have been also reported in four cases, and might be attributed to contact with blood or feces [142–145]. Jaundice was noticed in three mothers [142–144] and mild neonatal acute hepatitis occurred in three cases [142–145]. A potential concern arising from acute hepatitis A in pregnant women and neonates is the risk of transmission of HAV to other infants, mothers, or medical workers. Thus, outbreaks in neonatal intensive care units have been previously described and subsequently ascribed to a case of vertical HAV transmission [55, 142]. To prevent such nosocomial transmission, the infected mother and the neonate should be isolated, and careful hygiene practices emphasized.

Complications

Various complications can occur during the course of hepatitis A.

Prolonged Cholestatic Hepatitis

Cholestatic hepatitis, characterized by fever, pruritus, and prolonged jaundice, has been reported as an occasional complication [146]. Cholestasis develops in around 7% patients [147]. In a detailed description of six patients, peak serum bilirubin levels of 12–29 mg/dL were recorded, and jaundice lasted for 12–18 weeks. In each case, peak ALT levels were below 500 IU/L [148]. Liver biopsies revealed centrilobular cholestasis and portal inflammation. Although the prognosis is universally favorable, a short, rapidly tapered course of corticosteroids may be used to reduce symptoms and hasten resolution.

Relapsing Hepatitis A

Relapsing disease has been reported as an occasional complication in both adults and children. About 3–20% of cases relapse after a typical initial course. Typically, symptoms decrease, but may not completely resolve during the recovery phase, and the relapse is usually milder than the first. In the study of Tong et al. [149], the mean ALT level was 3,500 mIU/mL and the mean bilirubin level was 4.9 mg/dL during the first peak and 1,554 mIU/mL and 2.5 mg/dL, respectively, during the second. Viral excretion during the relapse has been detected. Although the pathogenesis of relapses has not been elucidated, it is important to recognize that these cases resolve without sequelae, although some clinicians have given corticosteroids to hasten recovery.

Coinfection of HAV with Other Hepatotropic Viruses

Coinfection with multiple hepatotropic viruses occurs in various combinations in 7–24% of all patients of sporadic AVH. The most common combination is HAV and HEV coinfection [150, 151]. Simultaneous infection with multiple hepatotropic viruses in the disease states of both AVH and FHF do not adversely affect the outcome. Total serum bilirubin is significantly lower with multiple infections than with single infection for both AVH and FHF. Multiple hepatotropic viral infection thus seems to produce less cholestatic illness and equal outcome if not better as compared to single virus infection. Whether this represents a phenomenon of

mutual viral suppression or viral restitution occuring in multiple virus infection requires further studies [151]. However, another study reported that dual infection with hepatotropic viruses was associated with greater elevation of aspartate and ALT [152].

Acute HEV Superinfection in Patients with Cirrhosis

Data available from a large outbreak of acute hepatitis A in Shanghai in 1988 and from cases of hepatitis A reported to the Centers for Disease Control and Prevention (CDC) between 1983and 1988 demonstrated that HAV infection was more severe in patients with pre-existing CLD [153]. Acute hepatitis A superimposed on chronic hepatitis B virus (HBV) infection was associated with a 5.6-fold and 29-fold increased risk of death, respectively, in the Shanghai outbreak and the CDC analysis of reported cases [154, 155]. In addition, there was a 23-fold increased risk of death in the CDC study in patients with acute hepatitis A superimposed on miscellaneous types of CLD. A matched case–control study using mortality files from the National Center for Health Statistics has confirmed the association between fatal hepatitis A and underlying CLD [156]. In this study, investigators compared the prevalence of CLD in subjects whose immediate cause of death was listed by ICD-9 codes as hepatitis A ($n=1,429$) vs. two controls groups whose immediate cause of death was listed as gastrointestinal hemorrhage ($n=2,376$) or biliary-pancreatic disease ($n=2,477$). The percent of subjects dying secondary to hepatitis A and having a secondary diagnosis of CLD was 63% vs. 8% and 11% with a diagnosis of CLD in the two control groups of gastrointestinal hemorrhage and biliary-pancreatic disease [153]. A prospective 7-year study from Italy demonstrated a remarkably high incidence of acute liver failure (41%) and death (35%) in patients with acute hepatitis A and preexisting chronic hepatitis C virus (HCV) infection [4, 157]. In contrast to the Shanghai and CDC studies cited above, this prospective study did not show higher mortality of acute hepatitis A in patients with chronic hepatitis B, although 1 out of 10 patients had a complicated course with a peak serum bilirubin of 28 mg/dL. Almasio and Amoroso reviewed the clinical course of acute hepatitis A in patients with CLD reported in 18 papers in the literature and noted that the mortality rate ranged from 0–100%, but was generally high [158].

Fulminant Hepatic Failure

The most serious complication of hepatitis A is fulminant hepatic failure, defined by the appearance of severe acute liver disease with hepatic encephalopathy in a previously healthy person [159]. Danger signs include excitability, irritability, insomnia, confusion, and severe vomiting. Laboratory and clinical evidence of deteriorating liver function, especially prolonged prothrombin times, correlates with the histologic picture of almost complete destruction of the hepatic parenchyma, with only a reticulin framework and portal tracts remaining. Occasionally, small groups of surviving hepatocytes can be seen close to the portal tracts, which may represent foci of regeneration. Surprisingly, little indication of a vigorous inflammatory response has been noted. Fulminant hepatitis A is a rare occurrence in the developed world; however, it may be quite common in developing world. Spontaneous survival from fulminant hepatits A occurs more commonly than from fulminant hepatitis of other causes [160].

Other Complications

Other manifestations of hepatitis A rarely include cardiac involvement, although patients with acute hepatitis may have bradycardia and electrocardiograms may show prolongation of the PR interval and some mild T-wave depression. These changes resolve rapidly during convalescence. HAV infection rarely causes pathology of other organs, but occasional cases of pleural effusion, postviral encephalitis, pseudotumor cerebri, Guillain-Barré syndrome, cholecystitis, acute pancreatitis, acute renal failure secondary to interstitial nephritis, acute glomerulonephritis, aplastic or hemolytic anemia, agranulocytosis, thrombocytopenic purpura, hemophagocytic syndrome, or pancytopenia have been reported [161, 162]. Several cases of arthritis, vasculitis, and cryoglobulinemia have also been reported [163]. Some patients may become depressed, and, occasionally, the depression may be severe enough to require treatment, but it is usually mild and self-limited.

Diagnosis and Detection of HAV

Approaches to HAV Detection

Detection of HAV-Specific Antibodies

The humoral immune response plays the pivotal role in the diagnosis of HAV infection and the differentiation of hepatitis A from other types of viral hepatitis. There are a number of commercially available assays for the detection of IgM and total anti-HAV [164]. IgM, IgA, and IgG anti-HAV are usually present at the onset of symptoms. Since hepatitis due to HAV infection is clinically indistinguishable from disease caused by other hepatitis viruses (i.e., HBV, HCV, HDV, and HEV), serologic testing is required to make the diagnosis.

Diagnostically, IgM anti-HAV has been used as the primary marker of acute infection; it is comprised mainly of antibodies against capsid proteins. A number of methods have been used to detect this virus-specific antibody class, including radioimmunoassay, immunochemical staining, enzyme-linked immunosorbent assay, immunoblotting, and dot blot immunogold filtration [165]. IgM anti-HAV enzyme immunoassays are available commercially. The commercially available diagnostic assays are configured in such a manner that although IgM antibodies may be present for long periods of time, the lower concentrations found 4–6 months after the onset of infection do not produce a positive test result [166]. Previous (resolved) HAV infection is diagnosed by detection of IgG anti-HAV. However, commercially available assays detect total anti-HAV (both IgG and IgM antibodies). The presence of total anti-HAV and the absence of IgM anti-HAV can be used to differentiate between past and current infections. Antibodies to structural proteins are produced following immunization with hepatitis A vaccine. A small proportion (8–20%) of vaccinated persons has a transient IgM anti-HAV response [167]. IgG anti-HAV is produced by all successfully immunized persons. However, commercially available tests for total anti-HAV are not sensitive enough to detect antibody concentrations in a significant proportion of immunized persons, especially several years after immunization [168].

Virus or Viral Component Detection

HAV has been grown in several cell types of human and nonhuman origins, including primary and secondary African green monkey kidney cells and fetal rhesus monkey kidney cells. In contrast to most picornaviruses, HAV of human origin requires an extensive adaptation period before it grows in cell culture, and once adapted, HAV produces a persistent infection and becomes attenuated, as shown by not producing disease in experimentally inoculated nonhuman primates. In addition, relatively low concentrations of virus and viral antigen are produced compared to other picornaviruses. Mutations in viral nucleic acid may play a major role in the adaptation of HAV in cell culture and attenuation (see above). HAV replicates in cell culture without cytopathic signs of infection and without apparent host-cell damage. Because of the lack of a cytopathic effect in cell culture, immunological assays are required to detect HAV antigen [169]. Methods commonly used to quantitate infectivity include radioimmunofocus assay, fluorescent focus assay, in situ radioimmunoassay, and in situ hybridization [170].

HAV was first visualized in fecal extracts by electron microscopy using homologous antiserum, and similar virus-like particles were observed in the sera and livers of marmosets experimentally infected with human HAV [171]. HAV antigen has been detected in stool, cell culture, and environmental samples by using radioimmunoassays and enzyme immunoassays. Viremia during HAV infection has been documented both by transmission studies and as a result of outbreaks of posttransfusion hepatitis A. However, detection of antigen in blood has been difficult because fibronectin can bind to HAV and mask antigenic determinants required for immunological detection [172]. HAV capsid polypeptides and viral RNA have been detected in IgM circulating-immune complexes isolated from experimentally infected chimpanzees [173].

Nucleic acid detection techniques are more sensitive than immunoassays for viral antigen to detect HAV in samples of different origins (e.g., clinical specimens, environmental samples, or food). HAV has been detected with techniques such as restriction fragment length polymorphism, single-strand conformational polymorphism, Southern blotting, nucleic acid sequencing-based amplification, nucleic acid hybridization, and reverse transcription-PCR (RT-PCR) and antigen capture RT-PCR [170].

Amplification of viral RNA by RT-PCR is currently the most sensitive and widely used method for detection of HAV RNA. Nested PCR, where products obtained from first-round PCR are used as a template for a second round of PCR, has been used to amplify HAV from clinical and environmental samples where the viral load is expected to be low. Analysis of the PCR product by probe hybridization also has been shown to increase the sensitivity of detection. Multiplex RT-PCR, where genome sequences of more than one organism are amplified simultaneously, provides the most efficient way to detect multiple agents in clinical and environmental samples, compared to conventional RT-PCR amplification for each agent. This method has been developed for simultaneous detection of HAV and HEV [174]. Real-time PCR, which has revolutionized nucleic acid detection by its high speed, sensitivity, and reproducibility and minimization of contamination, has been applied to the detection and quantification of HAV [175].

Nucleic acid sequencing is performed on PCR products to confirm their specificity and provide the ultimate means to identify and characterize the organism. Nucleic acid sequencing of selected genomic regions of HAV has been used to determine the genetic relatedness of isolates (see above).

Laboratory Diagnosis

Hepatitis A is not clinically distinguishable from other forms of viral hepatitis, although the diagnosis may be suspected in a patient with typical symptoms during an outbreak. Liver function tests especially serum levels of ALT and AST are sensitive measures of parenchymal liver damage, but are not specific for hepatitis A. The peak ALT/AST levels may be in thousands and values greater than 20,000 IU/L have been

observed. The ALT levels returned to normal by 6–8 weeks, although it may remain elevated till 28–30 weeks. High ALT levels do not necessarily correlate with an adverse outcome. Alkaline phosphatase levels are usually only mildly elevated, and persisting elevated levels suggest hepatitis-associated cholestasis [148]. The bilirubin levels as high as 38 mg/dL have been reported and are higher in older patients. Elevated levels of total serum IgM, a mild lymphocytosis, and occasional atypical mononuclear cells are commonly found in patients with acute hepatitis A but are not diagnostic of the disease.

The diagnosis of acute hepatitis A is most commonly confirmed by detection of specific IgM in a single acute-phase serum sample. The hepatitis A–specific IgM antibody is usually present at the initial evaluation and may be detectable at the time of the first rise in ALT. IgM anti-HAV can be detected in nearly 100% of patients with acute hepatitis A at their first clinical examination and remains positive in most for 3–6 months and for as long as 12 months in up to 25% of patients. False-positive tests are rare and should be suspected when IgM anti-HAV is found to persist for more than 1 year. Assays for total antibody to the virus are of little diagnostic value because IgG persists for many years and may be related to a past infection. Antibodies to naturally acquired HAV are primarily directed against the virion and do not react well with the individual peptides that make up the virion capsid. Low levels of antibodies to nonstructural proteins are found in the serum of convalescing patients and have been used to distinguish the antibody response to natural infection from the response to a killed-virus preparation [176].

HAV or viral antigen can be detected in the stools of patients 1–2 weeks before symptoms develop, but such detection has little place in routine clinical diagnosis because the tests are not widely available and shedding is often complete before the patient seeks medical attention.

Nucleic acid–based diagnostic techniques, primarily PCR or other nucleic acid amplification assays, have been used in research laboratories when a highly sensitive test for the presence of HAV is required. PCR has been very useful in the study of environmental samples [170].

In response to several outbreaks of hepatitis A associated with pooled-plasma products, screening by nucleic acid testing (NAT) of plasma pools intended for manufacture into various plasma components has been instituted by most plasma fractionators for process testing. While these NAT are now commercially available for testing of plasma, they are not recommended for use as diagnostics for patients with acute hepatitis. The performance of these assays in the diagnostic situation has not been evaluated. Recently oral fluid sample testing has been suggested as a noninvasive method of detecting HAV RNA during HAV outbreaks [177]. Use of dried serum spots (DSS) for the serological and molecular diagnosis of HAV infection has been studied. The DSS method facilitates storage and shipment of samples from routine laboratories to reference centers for further investigations and large epidemiological studies [178].

Detection from Water and Food

HAV is stable in the environment, especially when associated with organic matter, and is resistant to low pH and heating. These characteristics facilitate the likelihood of transmission by contaminated food and water and also improve the likelihood of detection in environmental samples, including water and sewage. Because HAV grows so slowly in cell culture and because of the generally low levels of contamination in environmental samples, virus detection became feasible only with the availability of sensitive nucleic acid detection methods. However, HAV detection in food has not been included as a part of routine analysis of these outbreaks in most parts of the world. The primary reason is that because of the long disease incubation period, implicated foods usually have been consumed or discarded by the time the outbreak is recognized [179]. The same holds true for detection of HAV in waterborne outbreaks, unless there is ongoing contamination. HAV has been successfully isolated from bivalve mollusks, oysters, mussels, and clams [170].

Nucleic acid hybridization assays using labeled probes were initially used to detect HAV in contaminated water [180]. However, this method had low sensitivity and required several logs of virus for detection. Today, PCR is widely used for HAV detection in environmental samples [170].

Therapy and General Management

There is no specific therapy are available for hepatitis A, and management is supportive without hospitalization. In the rare event of fulminant hepatitis, hospitalization and symptomatic supportive treatment become necessary. Identification of patients requiring liver transplantation is difficult because as many as 60% of patients, especially children, with fulminant hepatic failure caused by hepatitis A survive [181]. Transplantation is used for the management of carefully selected patients who have a poor prognosis with medical management alone. The survival rate is reported to be 80%, although reinfection has been reported [182].

In most patients with hepatitis A, admission to the hospital is not indicated, provided that patients have access to good care. If hospitalized, fecally incontinent patients, patients with diarrhea, and small children should be given a separate room and toilet. No objective evidence has been provided that bed rest or restriction of physical activity

affects the outcome of disease. Dietary restrictions, including prohibition of even modest amounts of alcohol, also seem to have little effect on outcome. Nevertheless, recommendation of abstention from alcohol has become conventional because alcohol has been linked with relapse of jaundice [183].

Prevention

General Measures

The most effective method to control hepatitis A and other enteric infections is through improved standards of hygiene and sanitation, especially the provision of clean water. Good hygienic practices with particular emphasis on hand washing and restriction of activities of workers who are ill are of primary importance in the food preparation industry. These general measures are most important to prevent hepatitis A transmission from person to person in families and hospitals. Nosocomial infections have been reported but are not common and transmission usually is from a patient who is not suspected of having hepatitis A [184]. Hence, hospitalized patients need only enteric isolation. Private rooms, gowns, and masks are not necessary unless the patient is incontinent. Gloves should be worn when handling any material potentially contaminated with feces. Frequent hand washing, whether gloves are worn or not, should be emphasized. Hospital personnel in general do not have a higher prevalence of antibody to HAV than matched controls do. However, several outbreaks of hepatitis A in hospital nurseries have been reported with transmission to staff [55].

Travelers to developing countries should be advised to eat only properly cooked food and be careful of uncooked vegetables and shellfish.

Improvements in sanitary systems, although technologically possible, may not be practical in large parts of the world. In more developed areas, sudden deterioration in living conditions because of war or political or economic instability can rapidly degrade sanitary systems. In the developed world, hepatitis A remains a risk associated with travel to exotic areas. Therefore, considerable effort has been expended in the development of hepatitis A vaccines.

Passive Immunoprophylaxis

Before the licensing of hepatitis A vaccines, the mainstay of hepatitis A immunoprophylaxis had been passive immunization with pooled IG. Even with the availability of vaccines, IG still has importance in hepatitis A prophylaxis. IG has proved useful for the prevention of hepatitis A in travelers, Peace Corps volunteers, and military personnel and even in postexposure prophylaxis in common-source or family outbreaks. However, IG has never been successful in altering the epidemiology of hepatitis in a high-risk community because of the transient nature of the protection, coverage rates, and perhaps lack of herd immunity.

IG is manufactured by cold ethanol precipitation from large pools of plasma collected from tens of thousands of donors. Because the prevalence of antibody to HAV in the population has been declining, concern has been voiced that antibody levels against HAV in IG preparations might drop below effective levels. Although no standard for anti-HAV levels exists in IG preparations in the United States even though prophylaxis against hepatitis A is the primary use for this product, anti-HAV levels remain adequate at this time to provide short-term protection. Eventually, the manufacture of IG from selected antibody-positive donors may need to be considered to develop a hyperimmune globulin for hepatitis A prevention analogous to other agent-specific hyperimmune globulins [185]. With the licensure of inactivated hepatitis A vaccines, the use of IG for pre-exposure prophylaxis has been largely eliminated, but IG still has a role in the prevention of hepatitis A after exposure has already occurred, when an exposure is expected before the vaccine would become effective, and in children younger than 2 years, for whom the vaccine has not been approved.

The efficacy of IG was first demonstrated in an outbreak at a summer camp in 1944 and has been confirmed many times since then [186, 187]. A recent metaanalysis of six randomized trials showed that immunoglobulins, when used for pre-exposure prophylaxis, significantly reduced the number of adult patients with hepatitis A at 6–12 months (1,020/286,503 vs. 761/134,529; RR 0.53; 95% CI 0.40–0.70; random-effects model) in comparison with no intervention or inactive control. Four trials showed a similar effect in children aged 3–17 at 6–12 months follow-up (917/210,822 vs. 677/78,960; RR 0.45; 95% CI 0.34–0.59). Comparing different doses of immunoglobulins, higher dosage was generally more effective than lower dosage (1.5 mL better than 0.75 mL and 0.75 mL better than 0.1 mL) in preventing hepatitis A [187]. Active prophylaxis with the recently licensed killed vaccines has largely supplanted the use of IG in this setting. Nevertheless, when administered before exposure or within 2 weeks after exposure, IG is more than 85% effective in preventing hepatitis A. Whether IG completely prevents infection or leads to asymptomatic infection and the development of persistent anti-HAV (passive-active immunity) probably is related to the amount of time that has elapsed between exposure and IG administration [188]. A recent trial compared IG with HAV vaccine for postexposure prophylaxis [189]. Symptomatic infection with HAV was confirmed in 4.4% contacts receiving vaccine and in 3.3% contacts receiving IG (relative risk, 1.35; 95% confidence interval,

0.70–2.67). Low rates of hepatitis A in both groups indicate that hepatitis A vaccine and IG provided good protection after exposure. The slightly higher rates of hepatitis A among vaccine recipients may indicate a true modest difference in efficacy and might be clinically meaningful if vaccine has other advantages, including long-term protection, and it may be a reasonable alternative to IG for postexposure prophylaxis in many situations [189].

Active Immunoprophylaxis

There is still a role for IG in the prevention of hepatitis A, particularly when individuals are recognized to have been exposed to the virus within the preceding 2 weeks [190]. However, its use in pre-exposure prophylaxis has been largely supplanted by inactivated hepatitis A vaccines. These vaccines contain viral particles that are produced in cell culture, purified, inactivated with formalin, and adsorbed to an aluminum hydroxide adjuvant. Inactivated HAV vaccines are highly immunogenic and protect against both infection and disease. This protection is likely primarily antibody based and is broadly directed against all strains of HAV, consistent with the identification of a single serotype of HAV among human strains.

The available vaccines begin to be effective about 2 weeks after a single intramuscular dose. For individuals who expect repeated exposure or require long-term protection, a booster dose is recommended 6–12 months after the initial vaccination.

Clinical trials indicate that inactivated hepatitis A vaccines are safe, highly immunogenic, and provide durable protection against infection; the protection is expected to last at least 10 years for those receiving the primary vaccine plus the booster [191, 192]. While the absolute level of antibody required to protect against infection has not been rigorously established, it is accepted based on comparisons with protective antibody levels associated with passive immunization with IG that antibody concentrations of 10–20 mIU/mL (depending on the assay used) are protective [193]. The licensed inactivated hepatitis A vaccines have all been shown to be highly and rapidly immunogenic. They induce seroconversions to protective levels of antibody in as little as 2 weeks after the initial dose [192]. Therefore, travelers, military personnel, or others who had no previous vaccine could be vaccinated as little as 2 weeks before their expected exposure instead of receiving IG. The level of antibody after vaccination varies with the dose and schedule of the vaccine. However, after a single dose of vaccine, antibody titers are higher than titers produced by known protective levels of IG, but are generally lower than titers measured after natural infection. The quality of the antibody response after vaccination has also been studied by comparing antibodies detected by radioimmunoassay, radioimmunoprecipitation, and in vitro neutralization in sera from persons passively immunized with IG and in persons immunized by vaccine. With the antibody normalized between the two groups by radioimmunoassay, the IG recipients had higher neutralization titers, but negligible radioimmunoprecipitation titers compared with the group who was vaccinated [194]. It must also be understood that there are no direct correlations between in vitro neutralization assays and seroprotection. Regardless of the results of antibody measurements following vaccination, clinical trials have demonstrated that the vaccine is highly effective within a month after the first dose.

Certain factors may reduce the response to the vaccine. Only about 50–75% of HIV-positive vaccinees developed protective levels of antibody, and those that responded had lower antibody titers than vaccinees without HIV [195, 196]. The factors associated with a protective antibody response are an HIV plasma RNA level <1,000 copies/mL at the time of vaccination and male gender [195]. The final antibody concentrations achieved in patients with chronic liver disease were also lower than in normal subjects, but the seroprotection rates were about the same. The common schedule of a single dose followed by a booster dose 6–12 months later produces very high levels of antibody. After the booster dose, it is estimated that protective levels of antibody will persist for at least 20 years [197]. Because the incubation period for hepatitis A is usually 4 weeks, and the anamnestic responses observed after the 12-month booster are rapid and robust, it has been suggested that vaccinees who have seroconverted will be protected even if their antibody levels have fallen below protective levels. Long-term follow-up studies will have to be performed to confirm this hypothesis.

Candidate live, attenuated HAV vaccines have been developed using viruses that have been adapted to growth in cell culture. There was considerable enthusiasm for such vaccines early in the development process, but vaccine candidates were poorly immunogenic [198]. Attenuation seems to have been achieved with these viruses at the cost of their ability to replicate within the liver, not by adaptation to a site of replication within the body that is different from that at which the virus causes disease. Nonetheless, live attenuated hepatitis A vaccine has received relatively wide use in few countries like China and appears capable of inducing protective levels of antibody [199, 200].

Strategies to immunize and protect against HAV infection can be broadly seen in three perspectives. First option is to have a high risk immunization strategy so that individual/group is the focus, and vaccine is administered with the routine immunizations. Second, the intervention is designed to avoid outbreaks of HAV among susceptible population,

wherein mass vaccination in the susceptible population is done. The third option is to vaccinate all children irrespective of their individual risk status of acquiring HAV infection, with the ultimate aim of eliminating the infection from the community. All these options are likely to minimize the risk of HAV disease in susceptible adults [101].

In the past, in industrialized countries, hepatitis A vaccination has been recommended for persons at increased risk of acquiring hepatitis A, including travelers to regions endemic to hepatitis A, users of illicit drugs, homosexual men, patients with chronic liver disease and patients with clotting factor disorders who receive factor concentrates [201]. Immunization also has been recommended for people who are at increased risk of developing fulminant disease should they become infected with HAV, such as those with chronic liver disease. However, in recent years, recommendations for broader immunization of children in regions of the USA having a higher overall incidence of hepatitis A have led to an 88% decline in reported cases of hepatitis A in those states [202]. Such data, coupled with evidence that the vaccine is immunogenic in children over the age of 12 months, recently led the Advisory Committee on Immunization Practices of the U.S. Centers for Disease Control and Prevention to recommend universal childhood immunization against hepatitis A in all regions of the country [203]. The ability of universal immunization to favorably impact the burden of hepatitis A disease is likely to be particularly evident in countries where the average age of infection has increased in recent years as socioeconomic conditions have improved. Such changes have led to increases in the incidence of clinically apparent infections that have an economic cost and also carry a risk of severe disease [204–206].

Cost–benefit analyses conducted in countries of high endemicity, such as Thailand [207] suggest that the benefits of immunization do not justify the expenses incurred, regardless of prior screening for HAV antibodies and of the age group targeted. These conclusions largely reflect the relatively high cost of the vaccine, which relates, in part, to difficulties inherent in propagating HAV in cell culture, but also possibly to the current limited scale of manufacture. However, as mentioned, changes in the epidemiology of hepatitis A may alter the future perspective on immunization in countries where sanitation is undergoing rapid improvement. Among these developing countries, there may be pockets of susceptible population within different regions among older children and adults. Heterogeneous pockets of susceptible and exposed individuals may coexist in rapidly developing societies. Therefore, small localized or large outbreaks of HAV infection remain a threat in such areas. The situation demands that relevant guidelines be evolved for HAV vaccination in these susceptible pockets and communities by characterizing them appropriately [101].

Summary, Conclusions, and Directions for the Future

The availability of safe and extremely effective inactivated HAV vaccines, has contributed to a declining interest in the molecular virology of HAV and the pathogenesis of hepatitis A in recent years. Despite this, hepatitis A remains a public health problem in many countries. At present, hepatitis A vaccination is generally not indicated in developing countries, particularly those with highest endemicity where infection in early childhood is nearly universal and disease is uncommon. Although vaccination strategies could be devised and directed at areas within transitional or intermediate endemicity countries where a sizeable proportion of adults are likely to be susceptible such as urban areas with good water and sanitation facilities, the relative cost effectiveness of hepatitis A vaccination compared with other major public health priorities has not been evaluated. However, the global disease burden associated with hepatitis A is expected to increase in the coming years, particularly in these areas, as a larger proportion of the population remains susceptible to HAV infection into adolescence and adulthood because of continuing improvements in standards of living and sanitary and hygienic conditions, and without proper vaccination or prophylaxis, HAV and its interactions with the hepatocyte represent a fertile field for future investigation. In particular, exploring the nature of this interaction may provide hints as to why this virus does not persist in the infected host.

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Chapter 37
Viral Hepatitis B

Mark A. Feitelson, Alla Arzumanyan, Helena M.G.P.V. Reis, Marcia M. Clayton, Bill S. Sun, and Zhaorui Lian

Introduction

Chronic hepatitis B virus (HBV) infection is the most important etiologic agent of hepatocellular carcinoma (HCC) worldwide. This is remarkable, considering that the virus consists of a DNA genome that is only 3.2 kb in size and encodes proteins from only four open reading frames (ORFs), all of which are located on the same DNA strand of the virus [1]. There are several morphological forms of HBV in the blood of infected patients. Most common is the small, spherical form, which is roughly 22 nm in diameter, and consists mainly of the hepatitis B surface antigen, or HBs envelope polypeptides embedded in the host-derived lipid membrane, derived from the infected cell. Less common are the variably long filamentous forms of HBs, which are also 22 nm in diameter, and have been found in the serum of infected patients. These forms are devoid of virus nucleic acid and are noninfectious, but are often present at very high concentrations. The virion of HBV, or Dane particle, is about 42 nm in diameter, is present in much lower concentrations than the subviral HBs particles mentioned above, and also consists of an envelope containing HBs polypeptides [2]. All HBs particles contain the major HBs protein and glycoprotein in roughly equal amounts, but virions also contain smaller quantities of HBs related polypeptides encoded by surface antigen plus adjacent upstream "preS" sequences encoded by the same ORF [1]. These so called preS/S polypeptides contain the virus-encoded receptor for infection, although the corresponding host-encoded receptor for HBV remains to be identified.

Within the virus envelope is the HBV nucleocapsid, which has icosahedral symmetry, and consists essentially of several hundred hepatitis B core antigen, or HBc polypeptides, encoded by a second ORF in the virus [1]. HBc has a carboxy-terminal region that is rich in arginine, which readily binds to the virus nucleic acid within the nucleocapsid [1]. During the virus replication cycle, some HBc polypeptides are proteolytically cleaved at the amino- and carboxy-terminal regions, resulting in the appearance of a smaller polypeptide, referred to as hepatitis B e antigen, or HBe, which appears in the blood stream of patients, and is a surrogate marker virus replication [3]. It has been shown that the persistence of HBe in the context of chronic liver disease (CLD) is a risk factor for the development of HCC [4]. However, most patients who develop cirrhosis and HCC have long since cleared HBe from serum and have seroconverted to anti-HBe, suggesting that other HBV antigens, and/or alterations in the immune system triggered by one or more HBV antigens, contribute importantly to the pathogenesis of HCC.

The third ORF in the genome of HBV encodes the polymerase, which plays a central role in the life cycle of the virus. Importantly, the polymerase encoded by HBV is a reverse transcriptase because HBV is a DNA virus that replicates by reverse transcription of an RNA replication intermediate [5]. One could think about the life cycle of HBV as a temporally permuted version of the life cycle of retroviruses. Upon HBV infection, the partially double stranded genome of HBV is made fully double stranded by action of the DNA dependent DNA polymerase activity encoded by the virus polymerase. With retroviruses, this step occurs about the time that the proviral DNA appears. Fully double-stranded HBV DNA then becomes supercoiled in the nuclei of infected cells, and encodes a greater than genome length (terminally redundant) pregenomic RNA (analogous to the terminal redundancy in retrovirus RNA). The pregenomic RNA is encapsidated in HBc particles within the cytoplasm and then reverse transcribed by the virus polymerase to yield full length minus strand DNA. The HBV reverse transcriptase also encodes an RNase H activity that degrades pregenomic RNA while the polymerase encoded DNA-dependent DNA polymerase activity partially synthesizes the plus strand DNA prior to virus maturation [5]. Unlike retroviruses, HBV

M.A. Feitelson (✉)
Department of Biology, Temple University, Philadelphia, PA, USA
e-mail: feitelso@temple.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_37, © Springer Science+Business Media, LLC 2011

does not have a provirus step in its life cycle, but as outlined below, fragments of the HBV DNA integrate into many positions within the host genome during chronic infection [6, 7]. While these integration events are not believed to be part of the virus life cycle, they may express one or more virus antigens that contribute importantly to the progression of CLD and development of HCC.

The fourth HBV ORF encodes the hepatitis B x antigen, or HBx, which is the regulatory protein of the virus [1]. As detailed below, after virus infection, HBx upregulates virus gene expression and replication, which are important for the establishment and persistence of the carrier state. HBx also contributes importantly to the pathogenesis of CLD and the development of HCC in a multitude of ways, some of which are described below.

Natural History and Pathogenesis

A hallmark of HBV pathogenesis is its variability [8]. Although a prophylactic vaccine is now available in many parts of the world, HBV was commonly transmitted to infants of infected mothers at birth. More than 90% of infected infants become chronic carriers, and later in life, develop CLD and then cirrhosis. Prior to widespread vaccination, it was not uncommon to see chronically infected patients develop HCC in their 40s and 50s, but with widespread vaccination of newborns in endemic areas such as Taiwan, both the rates of chronic carriers and the incidence of HCC are falling [9]. In adults, the pathogenesis of HBV following infection is quite different. Approximately 65% of adult infections are subclinical, with the appearance of one or more antibodies as the only indication of infection. Another 25% of infected adults develop acute hepatitis, which may or may not be symptomatic, followed by clearance of virus DNA and HBs from the blood, and finally by the appearance of anti-HBs. Some 5–10% of acutely infected adults do not clear virus or HBs within 6 months, and these individuals have a high probability of becoming chronic carriers. Up to 70% of these individuals remain asymptomatic for life, but the remaining carriers then develop CLD, which progresses from hepatitis to fibrosis, cirrhosis, dysplasia, and finally to HCC. At any time during chronic infection, carriers may arrest or resolve CLD. This is often accompanied by the clearance of HBe from the blood and seroconversion anti-HBe [10]. While this seroconversion is often accompanied by the clearance of virus, some infections continue to replicate an HBe negative mutant of HBV, which is also associated with a more intense CLD [11]. The fact that there is a poor understanding of the events that are responsible for this variability underscores the difficulty in treating chronically infected patients and in accurately predicting the long term outcome of such therapeutic approaches to the pathogenesis of CLD and risk of developing HCC.

HBV Vaccine

The HBV vaccine is remarkably safe and efficacious. It consists of the small, spherical HBs particles that were originally identified as “Australia antigen” and later as the whole or part of an infectious agent associated with transfusion transmitted hepatitis [2]. It was originally isolated from the serum of chronic human carriers, inactivated, tested in chimpanzees (that are susceptible to HBV infection), and provided as a series of three injections in alum adjuvant. More recently, the vaccine has been prepared using recombinant DNA technology from yeast, where no infectious virus is present [12]. Successful vaccination is indicated by the appearance of anti-HBs, which is neutralizing, and protection frequently lasts for many years, although it is not clear when and if boosters are needed. The vaccine has virtually eliminated the transmission of HBV by blood transfusion, although there is still a considerable risk of contracting HBV in unvaccinated populations through sexual intercourse, and by intravenous drug abuse involving shared needles. Although there have been reports of vaccine-escape mutants [13], these are still relatively rare, and in some formulations, preS sequences are included with S gene sequences in attempts to broaden the repertoire of neutralizing antibodies [14]. What is quite striking is that the HBV vaccine not only prevents infection, but also prevents the development of liver cancer, making it the first cancer vaccine to be marketed [15]. Another recent example is the introduction of the vaccine for human papilloma virus, which not only prevents infection, but also blocks the development of cervical cancer in women [16]. However, even with the success of the HBV vaccine, the fact that new infections are common in susceptible populations, and that the there are still several hundred million infected people worldwide at risk for CLD and HCC, means that other intervention strategies will be required to reduce the incidence of CLD and HCC among infected patients. In this context, many nucleoside/nucleotide analogs are now in the market or in phase III trials that strongly inhibit HBV replication, although their long term efficacy is still under evaluation [17, 18]. The problem with some of these drugs is that HBV develops resistance with continued use [19, 20], and that they are rather expensive for use in developing countries where the need is greatest. Interferon has also been used to treat chronic hepatitis B, but it is also expensive and may have serious side effects. Finally, there is interest in the development of therapeutic vaccines to treat patients with CLD or HCC, but these are still in preclinical development [21].

Hepatocellular Carcinoma

HCC, or primary cancer of the liver, is the fifth most frequent tumor type worldwide [22]. There are many etiological factors of HCC, but about 80% of this tumor type is associated with chronic HBV infection. Worldwide, there are an estimated more than 350 million carriers of HBV who are at high risk for the development of hepatitis, which may then progress onto cirrhosis (end stage liver disease). About 80% of patients develop HCC on a background of cirrhosis [23]. Due to the very large regenerative capacity of the liver, HCC does not become clinically apparent in most patients until it is too late for curative interventions, such as surgical resection or liver transplantation. Localized radio-ablation or chemotherapy, as well as other approaches, are sometimes effective, but most patients diagnosed with advanced HCC usually have less than a year to live. Part of the problem in managing patients with this cancer is that there are no reliable molecular markers available that could be used for the early detection of HCC (although many have been proposed) [24, 25], and that there are few therapies that specifically target molecules and signaling pathways that are rate limiting in tumor development [26, 27]. Although HBV has been eliminated from most of the world's blood supply, and a highly efficacious vaccine in use for more than a generation has reduced the incidence of HBV infection and HCC in parts of the developing world [28], intravenous drug abuse remains a major source of virus transmission worldwide, and sexual transmission is still important in parts of the world where the infection is endemic and universal vaccination programs do not exist. Therefore, it is expected that HBV infection and HBV associated HCC will continue to decrease among vaccinated populations, but will remain a major public health problem, especially in parts of the world where HBV infection is endemic, for many years to come.

The pathogenesis of HCC, like most other tumors, is multistep, although these steps are only now being tentatively identified and are more comprehensively discussed in a chapter on HCC (Chap. 56). For example, HBV is a noncytopathic virus [29] and there is now considerable evidence that the pathogenesis of HCC is immune mediated [30]. There is increasing evidence that at least some of the immunological abnormalities in chronically infected patients are related to the interactions between cellular components of the immune system and the high concentrations of virus antigens in the liver and/or blood of infected patients.

In addition to immunological abnormalities, there is increasing evidence that epigenetic changes in infected hepatocytes are also very important to the stepwise progression of events that convert a normal cell into a tumor cell [31]. In the case of HBV, one or more virus-encoded proteins may be the driving force behind these epigenetic changes in the liver that, in turn, promote genetic instability and mutations associated with the development and progression of HCC. These epigenetic changes may also alter the ability of the immune system to eliminate virus-infected cells. Among chronically infected patients, the most important risk factors associated with the development of HCC are the establishment and persistence of the chronic carrier state and the progression of CLD [32, 33]. The carrier state is characterized by persistent virus-gene expression (which is sometimes accompanied by virus replication) in the liver and serum, while progressive CLD is characterized by inflammation of the liver (hepatitis), followed by the accumulation of scar tissue (fibrosis and then cirrhosis), and the appearance of dysplastic nodules within areas of cirrhosis [34]. Dysplastic nodules are considered to be a precursor to HCC [34]. In this context, it is the purpose of this review to present some of the major immunological defects that contribute importantly to the pathogenesis of HCC, as well as examples of HBV triggered epigenetic changes among infected hepatocytes that promote both resistance to immune mediated elimination, and altered hepatocellular growth and survival that is characteristic of cells that eventually become tumors.

HBX and the Pathogenesis of HCC

As mentioned above, the genome of HBV encodes a family of related HBs envelope polypeptides from the preS plus S ORF, the nucleocapsid (or HBc) from the C ORF, and the virus polymerase from the P ORF. The X ORF encodes HBx, which is a 17-kDa polypeptide that transcriptionally up- and downregulates the expression of a potentially large number of virus and cellular genes, plays several important roles during HBV infection. First, HBx *trans*-activates HBV gene expression and replication, and in this context, promotes the development and persistence of the chronic carrier state, which is a major risk factor for the development of HCC [35, 36]. Second, HBx contributes to the pathogenesis of CLD by a number of mechanisms (Fig. 37.1). For example, HBx appears to promote the survival of infected hepatocytes by blocking immune mediated apoptosis and by stimulating hepatoprotective pathways that achieve the same end. This also helps to sustain the chronic carrier state, even in the presence of recurring bouts of CLD [36]. As outlined below, HBx also participates in the development of fibrosis and cirrhosis in the chronically infected liver. Importantly, dysplasia, usually seen in cirrhotic nodules, is considered a preneoplastic lesion from which HCC appears. Finally, HBx contributes to the development of HCC, in part, by activating a number of signal transduction pathways in the cell; they promote growth and survival, by inactivating several tumor suppressor genes, and by activating the expression of growth

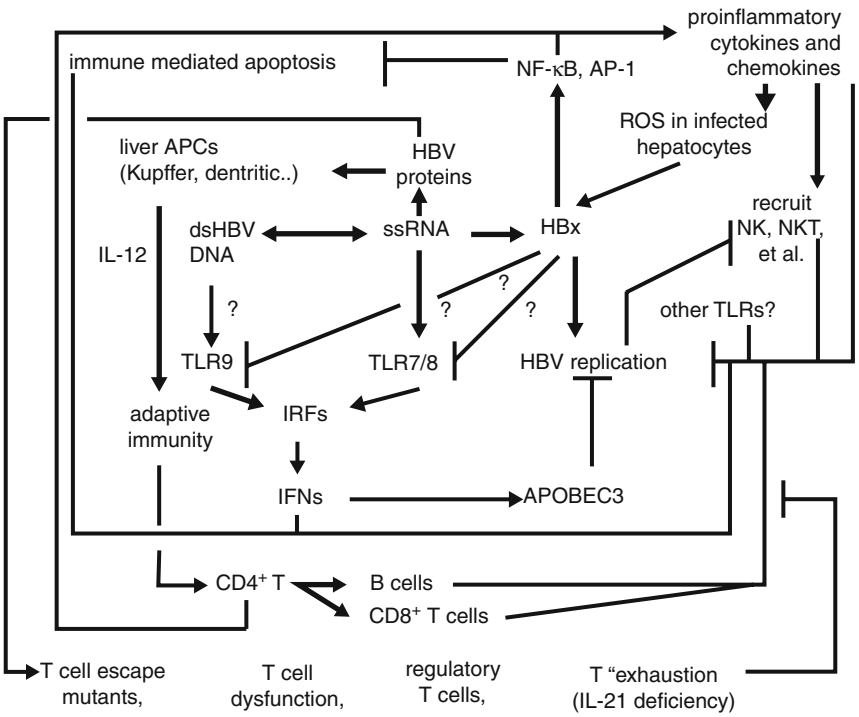

Fig. 37.1 Putative mechanisms of acute, resolving hepatitis and transition to chronic hepatitis. As outlined in this figure, innate immunity appears to be capable of eliminating virus from infected cells by multiple pathways. However, it is not clear that innate immunity is triggered in some acutely infected with HBV, resulting in the development of the carrier state, and in some patients, CLD. Please see the text for additional details

stimulatory pathways [36, 37]. There is also growing evidence that HBx may contribute to early steps in hepatocarcinogenesis by targeting multiple epigenetic changes in the cell [38]. It is proposed that these epigenetic changes provide the foundation upon which infected hepatocytes escape immune elimination, and from which genetic instability appears, resulting in the accumulation and propagation of genetic changes that are characteristic of tumor cells (Fig. 37.1).

Immune Mediated Pathogenesis of HCC

As indicated above, the pathogenesis of HBV associated HCC is immune mediated. While the details are not fully understood, the levels of virus-gene expression and replication, in addition to the timing, amplitude, and quality of immune responses appear to play central roles.

During the incubation phase of acute infection in some patients, innate immune responses may be activated. This may be facilitated by the recognition of HBV replication intermediates, consisting of single stranded (ss)RNA (the virus pregenomic RNA) and double stranded (ds)DNA, by the toll-like receptors (TLR) 7/8 and 9, respectively [39]. These interactions may then result in the activation of interferon responsive factors (IRF) in complex with other factors that trigger the production of interferons alpha and beta (IFNα/β)) (Fig. 37.1). During virus replication, the production of HBx (and possibly preS/S) also triggers activating protein-1 (AP-1) and nuclear factor kappa B (NF-κ(kappa)B), both of which target the production of inflammatory cytokines and chemokines (Fig. 37.1) [40]. These events promote the recruitment and activation of natural killer (NK) and NKT cells [41], which in turn produce IFN-gamma (IFNγ), tumor necrosis factor alpha (TNFα), interkeukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), and macrophage stimulating factor (M-CSF), all of which strongly inhibit virus replication [42, 43]. Elevated IFNα also induces the expression of the APOBEC3 class of RNA-editing enzymes (Fig. 37.1). These host enzymes are part of the innate immune system that inhibit HBV replication by catalyzing the introduction of mutations through cytidine deamination of minus strand DNA, which is the strand that encodes all of the virus proteins [44]. This may contribute to the introduction of lethal mutations into the virus during replication, and introduce mutations in host DNA that contribute importantly to the pathogenesis of HCC. An effective IFNα/β response in acutely infected adults correlates with a decrease in liver and serum levels of virus, the priming of strong, multispecific adaptive immune responses, and resolution of acute hepatitis.

The ability to trigger strong, timely innate immune responses, and subsequent adaptive immunity, are very important for the resolution of acute infection. When these processes are compromised, the chronic carrier state may

develop. Early treatments consisted of IFNα replacement therapy, but this alone was not very effective [45], suggesting that other components of the immune system probably played a major role in clearing virus. What are these other components? A partial answer may lie in the interaction of HBV with the innate immune system. For example, the fact that HBV replication occurs within immature core particles in the cytoplasm of infected cells [5], may sequester these replication intermediates from recognition by TLR7, 8, and 9 in the endosome, thereby blunting the IFN response (Fig. 37.1) [46, 47]. Importantly, there is evidence that TLR signaling inhibits virus replication in HBV transgenic mice [48–50]. In addition, TLR activated-mouse Kupffer cells and liver sinusoidal endothelial cells have been shown to suppress HBV replication [51]. However, HBV may infect and partially suppress the function of NK cells, which make up about one-third of intrahepatic lymphocytes (Fig. 37.1) [43]. This partial suppression may still be enough to promote a nonspecific inflammatory response by a virus that damages liver tissue, but does not clear the virus. This hypothesis is supported by data from HBV transgenic mice, which show depressed numbers and lower activation levels of intrahepatic-NK cells compared to nontransgenic littermates [43]. Alternatively, HBV infected hepatocytes produce IFNα/β and IL-12, which then potently activate intrahepatic-NK cells [43]. Activation of intrahepatic-NK cells has been shown to inhibit HBV replication and resulted in liver injury [52], while inhibiting NK cells did the opposite [53]. IFN-γ produced by NK cells then triggers other lymphocytes to produce IFN α/β and TNFα, which noncytolytically inhibit virus replication [54]. IFN-γ not only reduces the number of infected hepatocytes, but also upregulates major histocompatibility complex (MHC) Class I antigens on hepatocytes, which then present virus antigens to virus-specific cytotoxic T cells (CTLs) that complete the process of killing virus infected cells [55]. Other mechanisms of innate immunity that may limit HBV replication have been proposed [50]. However, there is also evidence that HBV and soluble HBV antigens were capable of suppressing TLR-triggered innate immune responses in hepatocytes and nonparenchymal liver cells [51], resulting in immune responses that damage the liver, but may not be effective in virus clearance. This poses the question of whether virus inhibition of TLR-triggered innate immunity occurs in chronically infected patients (Fig. 37.1). Given that the carrier state and progressive CLD are the two most important risk factors in the pathogenesis of HCC [32, 33], it is perhaps no surprise that the ability of the virus to modulate timely and appropriate innate and adaptive immune responses will have a major impact upon CLD and tumor development.

There are also important links between innate and adaptive immunity in the control of HBV infection. For example, Kupffer and dendritic cells in the liver are thought to present virus antigens to naïve T helper ($CD4^+$) cells (Fig. 37.1) [56]. Activated Kupffer and dendritic cells secrete IL-12, which activate T cells [56]. Combined with the production of IFNα by plasmacytoid dendritic cells (pDC) [57], most adults infected with HBV develop innate and adaptive immune responses that clear the virus from the liver (Fig. 37.1). This may be augmented by activated NK cells, which promote Th1 responses, promote maturation of monocytes and dendritic cells, and induce $CD8^+$ T-cell responses against tumor cells [43]. Subsets of activated $CD4^+$ T helper cells produce a variety of antiviral cytokines, activate B cells to make virus specific antibodies, and trigger virus specific $CD8^+$ T cells, which contribute centrally to the clearance of HBV following acute infection (Fig. 37.1) [58, 59]. If one or more of these events do not occur, the infection may become chronic. In this context, the fact that HBV infects lymphocytes [60] implies that such infections may subvert innate immune responses central for the development and/or persistence of strong $CD8^+$ T cell and antiviral cytokine responses, thereby promoting chronic infection. In addition, chronic infections may result from the emergence of T cell-escape mutations, or T-cell dysfunction, and/or the appearance of T regulatory cells that suppress $CD8^+$ T cell function and other viral immune responses [61, 62]. The role of T regulatory cells, although probably important, is somewhat controversial, since it is not clear whether they act by suppressing T-cell responses against HBV infected cells, and/or by downregulating immune responses that cause liver damage [62]. Alternatively, or in addition, upregulated expression of programmed cell death-1 (PD-1 or CD279) and CTLA-4 (CD152) on $CD8^+$ T cells is associated with the "exhaustion" of $CD8^+$ T cells, which has been observed in both acutely infected patients who do not clear virus and in HBV transgenic mice (Fig. 37.1) [63–66]. In this context, it has been suggested that the blocking of PD-1, CTLA-4, and perhaps other death receptors, in combination with therapeutic vaccination, may block the development and/or persistence of the chronic carrier state [67].

Among patients with chronic infection, the proliferation of hepatic dendritic cells is commonly observed. This often triggers a progressive inflammatory liver disease [68, 69] which is an important risk factor for the development of HCC [32, 33]. The importance of progressive inflammatory liver disease to the pathogenesis of HCC has been recapitulated in HBs transgenic mice, where the forced over-expression of this virus antigen results in massive hepatocellular necrosis, prolonged regeneration, and the eventual appearance of HCC [70]. Although there is no evidence that overexpression of HBs in human liver correlates with intrahepatic inflammation or the development of HCC, these observations underscore the role of persistent and progressive hepatocellular damage to the pathogenesis of HCC. Importantly, the progressive hepatitis seen in chronic human infections is often associated with the recruitment of virus

nonspecific-inflammatory cells that cause liver damage, but do not eliminate virus. In addition, given the centrality of the intrahepatic $CD8^+$ response to virus clearance [63], and that there are often too few $CD8^+$ T cells to effectively clear virus infected hepatocytes [71], it is not surprising that so few chronically infected patients spontaneously resolve the carrier state by seroconversion from HBs to anti-HBs. Recent work in this area has shown that the pro-apoptotic Bcl-2 interacting protein, Bim, is upregulated, and that this contributes to the depletion of HBV specific $CD8^+$ T cells among chronically infected patients [72]. Further, the issue of $CD8^+$ T-cell exhaustion, resulting from a war of attrition between these cells and infected hepatocytes that continuously produce virus antigens, points to another mechanism whereby HBV persists despite ongoing CLD. In this context, it has recently been shown that the ability of $CD8^+$ T cells to resolve a chronic virus infection was dependent upon the sustained production of IL-21 by $CD4^+$ T cells [73–75]. Although IL-21 was shown to promote $CD8^+$ T-cell differentiation and resolve chronic lymphocytic choriomeningitis virus (LCMV) infection in mice, it seems possible that a deficiency in IL-21 production may underlie the T-cell exhaustion associated with the transition from acute to chronic HBV infection and/or the persistence of CLD without resolution (Fig. 37.1).

HBV DNA Integration and HCC

Integration of the HBV genome into multiple sites within the host DNA readily occurs in the chronically infected liver [6, 7], especially during the liver regeneration that accompanies bouts of hepatitis. In this setting, single stranded regions of HBV DNA (which spans the X and S ORFs) integrate within replication forks of host DNA. Sequence analysis of host-virus junctions in chronically infected liver and HCC have shown that most viral DNA integrates within the direct repeat (DR) sequences located at each end of the viral genome [76], although integrations characterized by variable deletions in the 3′ end of the X gene have also been described [77]. Integration with respect to host sequences appears to be random and within many chromosomes [6, 78], although multiple integration events have been found in or around the telomerase gene [79], near or within selected oncogenes or protooncogenes [80], and within or adjacent to fragile sites or cancer susceptibility regions [81]. Although these findings suggest that *cis*-acting mechanisms of hepatocarcinogenesis may occur in nature, these are rare, considering that most integration events are widely dispersed throughout the genome.

Integrated viral sequences sometimes overexpress truncated forms of surface antigen (envelope) polypeptides, which have *trans*-activating properties [82, 83] that may contribute importantly to the pathogenesis of HCC. Preliminary work has shown that truncated preS/S mutant encoding genes from HCC tissue promote hepatocellular growth in vitro [84]. However, these truncated forms of surface antigen polypeptides have not been commonly found in the livers or tumors of patients with HCC [85], which leaves their contribution to HCC unresolved in natural infections [86].

Unlike the preS/S proteins described above, integrated viral sequences isolated from human hepatoma samples commonly overexpress full length or 3′-truncated HBx mRNAs [87, 88] and corresponding HBx polypeptides have *trans*-activation activities [77, 83, 89, 90]. Full-length HBx transformed nontumorigenic liver cells into those that grew in soft agar and formed tumors in nude mice [91, 92]. HBx transgenic mice with sustained high levels of HBx expression also developed progressive lesions in the liver, consisting of altered foci, adenomas, dysplastic loci, and finally HCC [93, 94], suggesting that HBx associated HCC is multistep. HBx is also expressed in roughly 70% of chronically infected patients with HCC, and among these patients, HBx is present in more than 95% of their peritumor liver samples [95, 96], suggesting an early role for HBx in hepatocarcinogenesis. In nontumor liver, staining was observed predominantly in the cytoplasm, although nuclear staining was also seen in hepatocytes within cirrhotic nodules. Among carriers with CLD, the frequency and intensity of HBx staining, as well as the number of patients with detectable HBx in the liver, increases with the severity of intrahepatic lesions [95, 96]. Although not proven, most of this HBx probably comes from integrated HBV DNA templates, which increase in frequency with each bout of CLD (Fig. 37.2). In this setting, HBx may alter the patterns of host gene expression that contribute importantly to the pathogenesis of chronic infection and development of tumors by several different mechanisms. As noted above, HBx is a transcriptional regulatory protein, but instead of *trans*-activating virus gene expression and replication, relatively high levels of HBx expression from integrated templates alters host gene expression by constitutively activating cytoplasmic signal transduction pathways (e.g., NF-κB, src, ras, AP-1, AP-2, PI3K/AKT, JAK/STAT, Smad, and Wnt), and by binding to nuclear transcription factors (e.g., CREB, ATF-2, Oct-1, TBP, and basal transcription factors), which together contribute to increased cell growth and survival (Fig. 37.2) [36, 97]. However, HBx also stimulates the growth of the human hepatoblastoma cell line, HepG2, in serum-free medium and in soft agar, accelerates HepG2 tumor formation in nude mice, and has been shown to promote metastasis [98, 99], suggesting that HBx also contributes importantly to later steps in multistep hepatocarcinogenesis. As presented below, the production of HBx from integrated templates appears to alter the response of infected hepatocytes to cytotoxic cytokines and activated T cells, and also promotes a profibrogenic environment and disease progression.

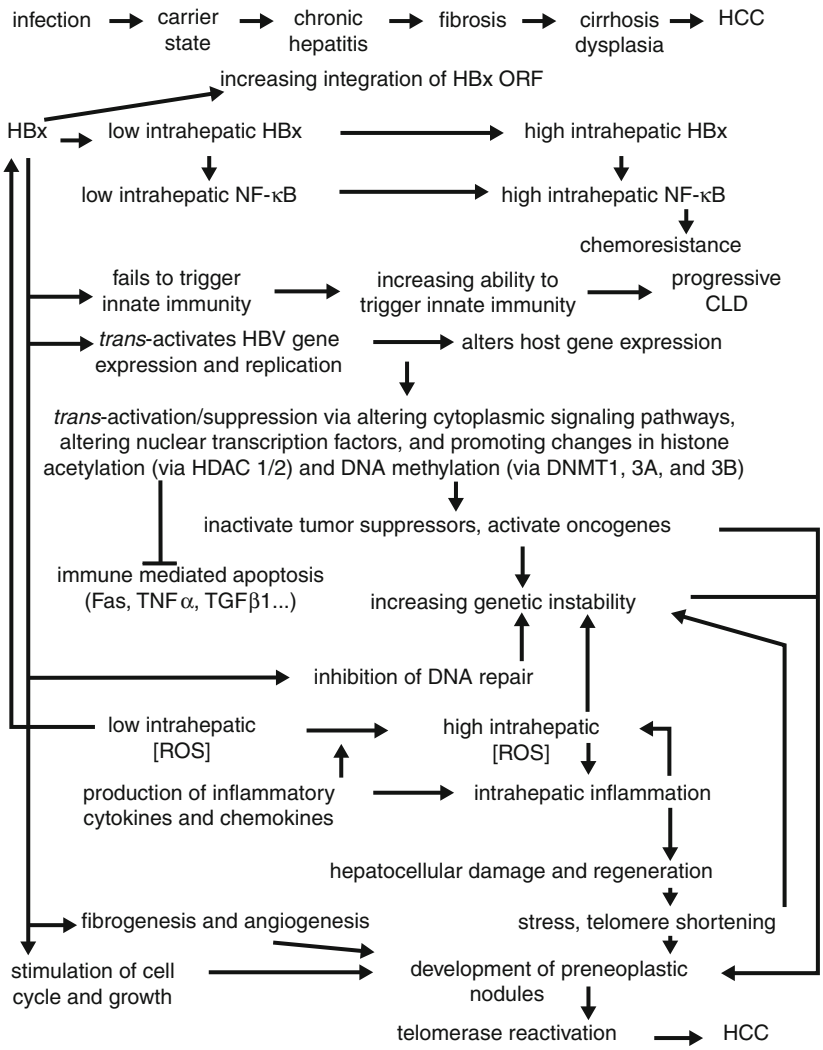

Fig. 37.2 Summary of some HBx related changes occurring in the liver that contribute to the development of HCC. Note that the HBx mediated epigenetic changes that alter hepatocellular gene expression may contribute to the inflammatory liver disease that develops and to the genetic instability that is a hallmark of tumor development. Additional details are included in the text

HBX and Epigenetic Mechanisms of HCC

There is often 20–50 years between HBV infection and the diagnosis of HCC. During this lengthy period, many stepwise changes occur in the liver that promotes the development of preneoplastic lesions. Many of these are epigenetic in nature, and in most cases, appear before the accumulation of point mutations, small deletions, and loss of heterozygosity that are characteristic of tumor cells [100]. For example, DNA hypermethylation has been observed in the livers of patients with chronic hepatitis and cirrhosis [101], suggesting that this is an important early event in hepatocarcinogenesis (Fig. 37.2). Independent work has shown that HBx represses the expression of the gene encoding E-cadherin, an important cell-adhesion molecule, by promoter hypermethylation [102, 103]. Additional observations have shown that HBx mediates these epigenetic changes by activating expression of DNA methyltransferase 1 (DNMT1) [102, 104], which is responsible for the reestablishment of the methylation pattern in chromosomal DNA following replication. HBx has also been shown to interact directly with DNMT3A [104, 105], which catalyzes *de novo* methylation of various promoters (Fig. 37.2). These include the MTIF gene, a member of the metallothionein family that appears to be a tumor suppressor in HepG2 cells and in HCC, and the interleukin-4 receptor (IL-4R), which is multifunctional cytokine receptor that regulates immune responses and apoptosis [106, 107]. Given that the IL-4R triggers human hepatocellular apoptosis through a Fas-independent pathway [108], HBx-mediated downregulation of IL-4R may protect hepatocytes against apoptosis. Importantly, HBx also inhibits apoptosis mediated by Fas, TNFα, and transforming growth factor beta 1 (TGFβ 1) [109–111], at least in vitro, suggesting that protection against immune mediated apoptosis may be an important mechanism whereby HBx infected cells survive immune-mediated attachment during the course of chronic infection and CLD (Fig. 37.2).

The promoter of the cyclin dependent kinase (CDK) inhibitor, $p16^{INK4a}$, is also frequently methylated in HCC and

liver [112]. This methylation correlates with HBx expression, and results in the subsequent activation of G1-CDKs, phosphorylation (inactivation) of pRb, activation of E2F1, and finally transcriptional activation of DNMT1. The observations that HBx upregulates and stabilizes β-catenin [111], that β-catenin transcriptionally targets cyclin D1, that cyclin D1 activates DNMT1 expression, and that the latter methylates the promoter of E-cadherin *de novo* [112], suggest how HBx activation of Wnt signaling may be linked to the methylation of tumor suppressor gene promoters in carcinogenesis. In addition, the fact that DNMT1 expression is activated by ras/AP-1 [113], and that HBx activates both ras and AP-1 [114, 115], suggests another pathway whereby HBx promotes DNMT1 expression. The finding that p53 inhibits DNMT1 gene expression [116], and that HBx functionally inactivates p53 [36], may also contribute centrally to the upregulated expression of DNMT1 in the chronically infected liver. Hence, there is a fine interplay between HBx and DNA methylation patterns that appear to profoundly alter the expression and function of tumor suppressor genes.

HBx has also been shown to induce global hypomethylation of satellite 2 repeat sequences in the pericentric regions of chromosomes 1, 9, and 16 by downregulating the expression of DNMT3B [104]. Hypomethylation of satellite sequences is associated with chromosomal instability [117], providing a link between viral induced epigenetic changes and the genetic changes resulting from chromosomal instability (Fig. 37.2).

In addition to changes in DNA methylation, HBx has also been shown to bind to and transcriptionally activate histone deacetylase 1 and 2 (HDAC1/2) [105, 118], which shuts down expression of selected genes resulting from the tight binding of deacetylated histones to DNA. For example, HBx has been shown to transcriptionally repress insulin-like growth factor binding protein-3 (IGFBP-3) by promoting HBx/histone deacetylase 1 (HDAC1) complex formation and by IGFBP-3 promoter hypermethylation [104]. IGFBP3 is a potential tumor suppressor that is downregulated in HCC [119], and its suppression is associated with poor survival [120]. The fact that the IGFBP-3 promoter has multiple p53 binding sites, some of which are methylated [121] and that HBx inactivates p53 [36], provides yet another mechanism whereby HBx may suppress IGFBP-3 expression. In nature, IGFBP-3 normally binds to insulin-like growth factors (IGFs) in serum and inhibits their activities. When IGFBP-3 expression is suppressed, IGFs strongly promote hepatocellular growth and tumorigenesis [36, 122]. Interestingly, the IGF-1 receptor and IGF-2 are both upregulated by HBx in the livers and tumors of HCC patients [36], suggesting that epigenetic alterations in gene expression may counteract immune-mediated hepatocellular damage and destruction by upregulating the expression of genes that promote growth and survival.

How could this occur? Acetyltransferases, HDACs, and DNMTs often function as part of large protein complexes that may be altered by HBx binding [118]. For example, HBx *trans*-activation activity is supported by its interaction with the CBP/300 complex, which has histone acetylase function [123], while the evidence above states that HBx also binds HDAC [105, 124], suggesting that HBx may interact with the cellular epigenetic machinery to promote or inhibit histone acetylation under different conditions. These observations suggest that HBx mediated changes in DNA methylation and histone acetylation alter host gene expression in the liver during chronic infection (Fig. 37.2) [125]. In addition, acetyltransferases and HDACs alter the stability of nonhistone proteins. For example, HBx transcriptionally induces the expression of metastasis associated protein 1 (MTA1) and HDAC1, which physically bind to hypoxia inducible factor 1 alpha (HIF-1α), resulting in the stabilization of HIF-1α [126]. HIF-1α, which promotes cell survival and growth under conditions of low oxygen tension in cirrhotic nodules and early HCC, was found to be deacetylated (activated) in the presence of HBx [126]. The fact that HBx modules the functions of Rb, E2F, and p53, which are also known to be regulated by acetylation and deacetylation [127], suggest that HBx may alter important pathways in hepatocellular growth and survival by these epigenetic mechanisms.

HBx Overcomes Apoptosis in Chronic Infection

Chronic hepatitis is characterized by immune mediated hepatocellular destruction and ongoing hepatocellular regeneration in order to reestablish homeostasis in the liver. If HBx promotes the development of HCC, then it should contribute to changes in cell behavior that are hallmarks of oncogenesis, which includes self-sufficient growth, insensitivity to negative growth regulatory signals, and evasion of immune mediated apoptosis. In fact, HBx promotes growth factor-independent growth and survival in serum free medium and under anoxic conditions [128], inactivates multiple tumor suppressors [129] and partially blocks immune-mediated apoptosis (mediated by Fas, TNFα, and TGFβ1) (Fig. 37.2). In particular, HBx blocks Fas and TNF α killing by inhibiting caspase 8 activity, which is common to both pathways, and by blocking caspase 3, which is an effector caspase [109, 111]. HBx also increases TGF β1 levels by transcriptional *trans*-activation [130], by suppressing production of the natural TGF β1 inhibitor, alpha 2-microglobulin [110], and by stimulating several signal transduction pathways (e.g., ras, PI3K, and NF-κB) that change the outcome of TGFβ1 activated Smad signaling from growth inhibition to growth promotion (Fig. 37.3) [131]. The latter is also accompanied by elevated extracellular matrix (ECM) production, mild immunosuppression (associated with elevated TGF β1 levels in blood), epithelial to mesenchymal transition (EMT, which is important to the pathogenesis of many tumors),

and the onset of angiogenesis [132–134]. Interestingly, HBx also targets the upregulated expression of the TNFα related apoptosis induced ligand (TRAIL) in HepG2 (liver adenoma) cells, which is effective in killing primary human hepatocytes [135] and may facilitate growth of HCC into the surrounding liver. TRAIL is expressed on the surface of activated, intrahepatic NK cells, T cells, macrophages, and dendritic cells, where it may function to trigger cytotoxicity in infected liver and/or tumor [136]. TRAIL, like TNFα, mediates apoptosis by activation of caspase 8 [118], although both molecules could also be hepatoprotective if NF-κB is activated (Fig. 37.3). In this context, the fact that HBx activates NF-κB [137, 138] may serve to shift immune mediated killing of virus infected cells to one where infected cells become resistant to killing, which not only promotes the carrier state, but is also a characteristic of tumor cells.

Chronic HBV infection is stressful to infected hepatocytes. Among asymptomatic carriers, who do not develop CLD, this stress is often accommodated by anti-oxidant systems in the liver and by the fact that inflammatory cells are not releasing reactive oxygen species (ROS) or cytotoxic cytokines that otherwise greatly amplify oxidative stress. An important concept here is that an oxidative environment, set up in the liver by innate and adaptive immune responses, stimulates HBx activity (Fig. 37.2). Elevated HBx, in turn, stimulates NF-κB, as well as the activity of other oxidation sensitive transcription factors, such as AP-1 and STAT-3. NF-κB not only protects hepatocytes from extrinsic apoptosis (mediated by Fas, TNFα, TRAIL, and TGFβ1), but also blocks intrinsic apoptosis which is otherwise triggered by mitochrondrially associated HBx [139]. Hence, on the one hand, activated NF-κB is hepatoprotective (Fig. 37.3), but also targets the upregulated expression of many proinflammatory cytokines and chemokines [139, 140], which may promote and sustain CLD (Fig. 37.1). Both of these findings may underscore the very close association between intrahepatic HBx expression and hepatic inflammation [36, 95, 96, 141]. In the context of CLD, sustained inflammation inflicts considerable hepatocellular damage and death, followed by hepatocellular regeneration and scarring (fibrogenesis), persistent activation of HBx, and corresponding epigenetic changes that contribute importantly to the appearance of HCC.

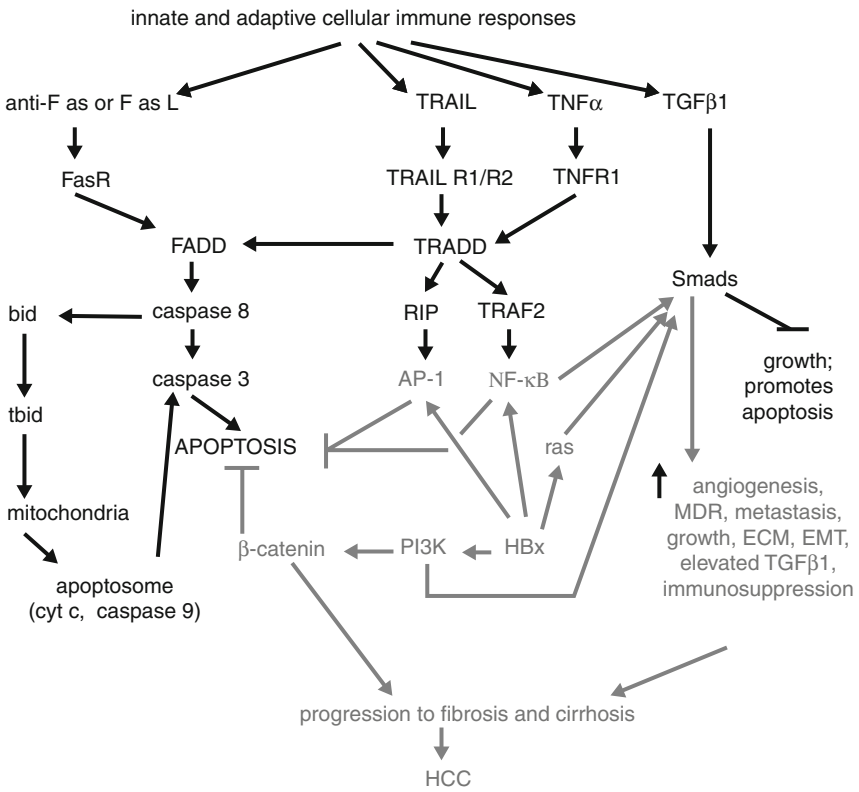

Fig. 37.3 Summary of several signaling pathways triggering apoptosis from innate and adaptive immune responses directed against virus infected cells. HBx alters host signaling pathways (in *gray*) that stimulate growth, inhibit apoptosis, and promote tumorigenesis. It is proposed that epigenetic mechanisms also up- and downregulate the expression of target genes that alter cell behavior. Additional details of these mechanisms are outlined in the text. *Cyt C* cytochrome C; *ECM* extracellular matrix; *EMT* epithelial–mesenchymal transition; *FasR* Fas receptor; *FADD* Fas associated death domain protein (adaptor for Fas signaling); *HCC* hepatocellular carcinoma; *MDR* multidrug resistance; *RIP* receptor interacting protein; *TGFβ1*: transforming growth factor beta 1; *TRADD* TNF receptor associated death domain protein (adaptor for TNF signaling); *TRAF2* TNF receptor associated factor 2; *TRAIL* TNFα related apoptosis induced ligand; *TNFR1* tumor necrosis factor receptor 1

HBX and Innate Immunity

HBx inhibits the action of TNFα [111], and other cytokines, by blocking signaling pathways that would otherwise inhibit virus replication [42, 53, 142], (Fig. 37.3). In addition, when mouse Kupffer cells were treated with various agonists to the TLRs, supernatants from TLR3-and TLR4 stimulated cells were able to potently suppress HBV replication [49]. One of the active agents in the supernatants was IFNβ. Usually, IFNβ production results from signaling through the adaptor molecule MyD88, but in this case, its production was MyD88 independent. In addition, TLR3 recognizes dsRNA, which is not made in the HBV life cycle, while TLR4 recognizes bacterial lipopolysaccharide (LPS), suggesting that HBV-independent TLR ligands may trigger innate immunity against HBV (Fig. 37.1). However, genes reflecting innate immunity are not detectable during HBV entry and expansion [46], suggesting that HBV does not efficiently trigger innate immunity, that HBV may suppress innate immunity in the TLR and other pathways, or that pathogen associated molecular patterns from other microbes trigger innate immune responses that are also effective against HBV. In this context, HBs has been shown to inhibit LPS induced COX-2 expression, rescue IL-18 production by blocking NF-κB, and suppress IFNγ [143]. In addition, HBe positive patients have reduced TLR2 expression in hepatocytes and Kupffer cells [144]. On the other hand, infection of HBV transgenic mice with other microorganisms triggered innate immune responses that were also effective against HBV [145–147]. Independent work conducted cotransfections of human-liver cell cultures (HepG2 and Huh7) with replication competent HBV DNA along with plasmids expressing individual TLR adaptors (e.g., MyD88) that transmit TLR signaling. The results showed that expression of each adaptor strongly inhibited HBV replication. Supernatants from adaptor transfected liver cells did not inhibit HBV replication, suggesting that the antiviral effect was mediated by intracellular factors [50]. The antiviral effect was subsequently shown to be NF-κB dependent. Hence, HBV replication is susceptible to innate immunity, although it may not be readily induced by HBV following acute infections (Fig. 37.2).

The central role of NF-κB in mediating innate immunity the fact that HBx stimulates NF-κB activity [137, 138], that NF-κB turns on many proinflammatory genes [148], that NF-κB is hepatoprotective [149, 150], may help to explain inflammation induced chemoresistance in tumor cells (Fig. 37.2) [151]. At the molecular level, NF-κB promotes chemoresistance by upregulating expression of anti-apoptotic proteins (e.g., c-FLIP and XIAP) and blocking the expression or activity of proapoptotic proteins (e.g., Bax and caspase 9) [151]. While there is an inverse correlation between NF-κB activity and virus replication [50], there is a direct correlation between HBx levels and NF-κB [137, 138]. This is because the longer a person with CLD is infected, the more integration events occur, and the more HBx is observed in the liver, with strong HBx staining observed in greater than 90% of cirrhotic nodules (Fig. 37.2) [95, 96]. While it is not known whether HBx alters the expression or activity of TLRs directly, the ability of HBx to also stimulate MAPK signaling pathways (such as ERK-CREB and JNK-AP-1) [152] may not only induce a proinflammatory environment, but also promote cell growth. During the course of chronic infection, this may establish a positive feedback loop that promotes the progression of CLD from hepatitis to fibrosis to cirrhosis. In this loop, it is proposed that proinflammatory cytokines trigger the accumulation of ROS in the infected cells, which in turn stimulates HBx activity (Fig. 37.1). In this context, there is also considerable evidence that HBx participates in the development of fibrosis and cirrhosis by targeting upregulated expression of fibronectin [153] and the strongly profibrogenic TGFβ1 [110, 130, 133]. As noted above, during HBV entry and expansion, there is no induction of genes reflecting innate immunity. It is proposed that this may be due to the relatively low levels of HBx made during this period, and that the HBx made is dedicated to supporting virus gene expression and replication. This may be sustained for decades among asymptomatic carriers, but among those who develop CLD, which is accompanied by considerable hepatocellular destruction and regeneration, the HBx ORF frequently integrates, resulting in corresponding rises in intracellular HBx. At this later time, HBx may trigger alter the outcome of innate and adaptive immune responses, which promote the progression of CLD to fibrosis, cirrhosis ,and finally HCC (Fig. 37.2). This does not mean that TLR signaling is not important, since necrotic and apoptotic cells release ligands that are known to activate TLRs [154], suggesting that HBx may stimulate a wide variety of TLRs by indirect mechanisms. In this model, innate and adaptive immune responses promote tumorigenesis by activating HBx and downstream epigenetic pathways that contribute to stepwise hepatocarcinogenesis. The challenge for the future is to understand the target genes altered by HBx epigenetic modifications and how they contribute to hepatocellular alterations prior to tumor appearance. It would also be of great interest to determine whether some of the epigenetic alterations in gene expression are triggered by innate immune responses.

HBX and Senescence

There is also evidence that HBx contributes to cells escaping senescence, which results in immortalization, by promoting genomic instability, telomere length stabilization, epigenetic

gene silencing (by altering promoter methylation and histone acetylation), oxidative DNA damage, inactivation of selected cell-cycle regulatory genes (e.g., p53, Rb, $p16^{INK4a}$ and $p21^{WAF1}$) as well as over-expression of cellular oncogenes. Importantly, in the absence of immortalization, cells are unable to undergo malignant transformation [155]. As outlined below, all of these features are altered by persistently high levels of HBx expression, suggesting that overcoming senescence is a central feature of HBx-associated HCC (Fig. 37.2).

Telomeres, which are sequences at the ends of each chromosome, shorten a little with each round of cell replication, thereby limiting the number of regenerative cycles for a given cell type. When telomers reach a critically short length, chromosomal ends become exposed, which activates DNA damage responses that mediate cell cycle arrest or apoptosis until repair could be achieved [156, 157]. In the presence of persistent HBx expression, a number of DNA damage responses are compromised, since HBx inactivates p53 [158], blocks p53 mediated transcription coupled repair [158], and inactivates nucleotide excision repair [159]. The resulting unstable telomeres are highly recombinogenic, resulting in chromosomal fusions, promoting a cascade of many chromosomal abnormalities with each cell division (Fig. 37.2) [155]. Compromised DNA repair, combined with integration of HBV DNA within fragile sites [81], result in genetic instability. This instability often results in cell death, but in some cases, the outcome of this "crisis" in cell survival results in immortalization. Intraheptic HBx, stimulated by the oxidative environment provided by innate and adaptive immune responses, constitutively activates putative oncogenic pathways such as ras, NF-κB, and β-catenin [111, 114, 160, 161], which promotes progressive telomere shortening in cirrhotic livers. Hence, by the time cirrhosis appears, most hepatocytes are approaching the end of their regenerative life span. At this point, cells usually go into senescence, although in addition to telomere shortening [162], oxidative stress [163, 164], DNA damaging signaling [165], and persistent oncogene activation also contribute. In HBV infection, there is also evidence that persistent HBx expression overcomes ras oncogene induced senescence [160]. What makes HBx special here may be the fact that it also blocks expression and/or function of several key tumor suppressor genes, such as those that encode p53, PTEN, Rb, $p21^{WAF!}$, $p16^{INK4a}$, and others (Fig. 37.2). In particular, HBx inactivates p53 by cytoplasmic sequestration [165], promotes phosphorylation (inactivation) of Rb [166], depresses $p21^{WAF1}$ levels by transcriptional downregulation [129], and suppresses $p16^{INK4a}$ by promoter methylation [167]. The findings that HBx mediates sustained over-expression of telomerase in liver cultures [168], and there is co-staining in tumor, peritumor, and cirrhotic tissues [169], and that it blocks immune mediated apoptosis, further favors growth beyond the point of replicative senescence. HBx inactivation of cell-cycle checkpoint proteins [170] is also likely to contribute to getting cells through "crisis," and when this occurs, telomerase is usually partially reactivated in cells that become immortalized (Fig. 37.2).

Conclusions and Prospects

Long term HBV infection, combined with CLD, are major risk factors for the development of HCC. HBx, the virus encoded "oncoprotein," promotes hepatocarcinogenesis by a variety of different mechanisms. First, HBx promotes virus gene expression and replication. Although HBV replication is sensitive to the antiviral activity of innate immune responses, the high levels of virus gene expression and replication that accompany infection may block TLR activity, which is essential for timely IFN production and T-cell activation. This may result in attenuated innate immunity, resulting in a failure to prime appropriate and timely adaptive immune responses. Among the adaptive responses that do appear, the development of $CD4^+$ T regulatory cells and $CD8^+$ T cell exhaustion during chronic infection may prevent virus clearance, but may be sufficient to chronically damage the liver and promote progressive CLD. Hepatocellular regeneration provides many opportunities for the HBx ORF, located at the end of the HBV genome, to integrate into multiple chromosomal sites. Many of these integrated sites produce HBx (and some also produce preS/S) proteins that are activated by the oxidative environment associated with immune mediated CLD. HBx persistently activates oxidation sensitive transcription factors, such as NF-κB and AP-1, which are both hepatoprotective and exacerbate the proinflammatory environment within the liver. High levels of HBx in the liver alters host gene expression that blocks immune mediated apoptosis, promotes cell cycle progression, survival, multidrug resistance, angiogenesis, and fibrogenesis. Moreover, the ability of HBx to alter patterns of host gene expression through targeting upregulated expression of selected DNA-methyltransferases and through histone acetylation/deacetylation provides a framework from which a detailed understanding of stepwise hepatocarcinogenesis can occur, and possibly how HBV continues to evade immune elimination in more than 350 million people worldwide. It will be of great interest to focus upon identification of the host genes that are naturally targeted by HBx in chronic infection. It would not be surprising if some of the target genes found, blocked both innate immunity and promoted tumor development, for these would be the best targets for the development of novel experimental therapeutics that would have the broadest activity against virus and disease.

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Chapter 38
Viral Hepatitis C

Jiaren Sun, Gaurav Chaturvedi, and Steven A. Weinman

Introduction

Chronic hepatitis C is currently the most common cause of cirrhosis, chronic liver failure, and hepatocellular carcinoma in the world [1–3]. While its epidemiology [1], clinical features, and virology [4, 5] have been rather well defined, the mechanisms by which it causes liver injury are among its least understood aspects. The basic outline is that viral infection occurs primarily in hepatocytes and results in both innate and adaptive immune responses. In the short-term, liver injury is caused almost exclusively by T cell-mediated responses and in most individuals is minimal or absent prior to the development of adaptive immunity [6]. However, hepatitis C virus (HCV) infection also directly produces a number of cellular effects in hepatocytes, including alterations in gene expression, signaling pathways, lipid metabolism, cell proliferation, and oxidant production [7].

The infection typically results in a chronic process that slowly progresses to clinically significant disease within 15–40 years. Although liver injury generally does not occur in the absence of the immune response, direct effects of the virus on host cells modulate and modify the chronic course of the disease [8]. This review will describe the pathogenic mechanisms involved in chronic hepatitis C and assess the extent to which these contribute to the development of the disease state.

Natural History of Chronic Hepatitis C

HCV is a single-stranded RNA virus which is classified as a unique species within the family of flaviviruses. HCV undergoes a cytoplasm-based, RNA-dependent replication cycle which is particularly "error" prone; in that it generates a tremendous degree of sequence diversity [9]. There are six major genotypes in which sequences diverge by approximately 30%, and numerous subtypes with lesser sequence diversity. The viral genome includes 5′ and 3′ untranslated regions which are critical for viral function, and a coding sequence of approximately 3,011 bp. This coding region has a major open reading frame that is translated into a single polyprotein that is subsequently processed into ten viral proteins. There are three structural proteins: core, E1, and E2; and 7 nonstructural proteins: p7, NS2, NS3, NS4a, NS4b, NS5a, and NS5b. At least one protein appears to be produced from an alternate reading frame, the F protein [10]. Most, if not all, of these proteins are multifunctional and play a role in both viral replication and modification of the host cell milieu.

Chronic hepatitis C has several pathological phenomena that are responsible for the morbidity and mortality of the disease. The primary process is portal-based lymphocytic infiltration, which is common in many forms of chronic hepatitis, but is particularly evident in hepatitis C in which lymphoid aggregates are frequently observed [11]. Closely associated with these inflammatory infiltrates is the presence of dying (necrotic and apoptotic) hepatocytes, and the overall process is one of a portal-based or interface necroinflammation. In severe cases, lobular necroinflammation is present as well. This ongoing hepatic necroinflammation is associated with elevated aminotransferases, hepatocyte regeneration, and non-specific clinical manifestations such as chronic fatigue. In addition to necroinflammation, chronic hepatitis C is nearly always associated with a process of stellate cell activation, accumulation of an extracellular matrix, and slowly progressive fibrosis [12]. Hepatic steatosis is another common feature of chronic hepatitis C being present in approximately 40–50% of patients. Its occurrence is genotype-specific (see below), and it is predictive of more rapid fibrosis progression. Fibrosis typically progresses via stages from portal only, portal and lobular, bridging fibrosis, and finally established cirrhosis. While fibrosis progression is present in nearly all individuals, the proportion that will develop cirrhosis within their lifetime is estimated to be on the order of 30%. In individuals who develop cirrhosis, the rate of progression is highly variable, and the time interval

S.A. Weinman (✉)
Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kansas, USA
e-mail: sweinman@kumc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_38, © Springer Science+Business Media, LLC 2011

from infection to cirrhosis varies between 15–30 years. Factors that correlate with more rapid fibrosis progression are primarily host characteristics and include older age, male gender, and alcohol consumption [13].

Prior to the development of cirrhosis, patients largely remain healthy and are frequently unaware of their infection. However, once cirrhosis develops, patients are at risk for development of portal hypertension, liver failure, and hepatocellular carcinoma (HCC). It is estimated that the average time from development of cirrhosis to decompensation (portal hypertension and/or liver failure) is 5–10 years, and the risk of development of HCC is about 2% per year [14]. HCV is thus a well-documented human tumor virus, and HCC development in this disease tends to be multifocal, reflecting a diffuse propensity of the hepatocytes for malignant transformation. Progressive fibrosis and HCC are thus the two major disease processes present in chronic hepatitis C.

Immunopathogenesis of Hepatitis C

Immune Contribution to Liver Injury

Although HCV is capable of interfering with a wide range of host physiological processes, it is fundamentally an immune-mediated disease. Since the virus replicates through a stage containing double-stranded RNA (dsRNA) intermediates, it is readily detected by the dsRNA-sensing machinery of the cell [15]. The initial host response to infection is the triggering of innate cellular responses [16, 17], including the production of type-1 interferons (IFN-α/β) and the activation of downstream, antiviral target genes via autocrine and paracrine pathways. The initial phase of infection during the first several weeks is associated with very high viral loads, but minimal liver injury, as judged by normal serum alanine aminotransferase (ALT) levels. However, despite these responses, HCV continues to replicate in the liver regardless of whether the ultimate clinical outcome is resolution or persistent infection.

The adaptive immune response to HCV infection develops over several weeks [18]. Although the reasons for this delay are not understood, it is clear that the magnitude, diversity, and quality of the adaptive immune responses are the determinants of the outcome [18]. Liver injury, as reflected by increases in serum ALT, is clearly caused by the surge of the T-cell responses. It coincides temporally with the expansion of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs), virus-nonspecific NK (natural killer) and NKT (natural killer T) cells, and their acquisition of an activated phenotype [19]. While this acute response has the potential to clear the viral infection, unfortunately, it is unsuccessful at least 50% of the time and the virus has a very strong propensity to develop chronic infections. Recent work clearly indicates that, due to long coevolution with its hosts, HCV has acquired several successful strategies to evade the host response [20, 21]. Development of chronicity is marked by a dramatic decrease in the activity of CTLs and $CD4^+$ Th cells in the liver without achieving viral clearance. Levels of viremia are lower than those in the acute phase and typically remain constant over time in the same individuals, even though the viremia can differ widely between subjects. Since this phase lasts lifelong in most untreated patients, it is the state during which ongoing hepatocyte destruction, progressive fibrosis, and carcinoma occur. During the chronic phase of the disease, immune responses continue to play a prominent pathological role, but direct effects of viral and host factors modify and determine disease phenotype. In the end-stage livers, NK cells become the most abundant lymphocyte subset, followed by T cells, B cells, and NKT cells [22]. Although the significance of this subset distribution is not entirely clear, it may implicate both innate and adaptive effector arms of the immune system in the progressive damage seen in end-stage hepatitis C patients. A summary of the immune processes involved in liver injury due to HCV is illustrated in Fig. 38.1.

Role of Adaptive Immune Responses

$CD8^+$ Cytotoxic T Cells

HCV-specific T cells are capable of migrating to the liver and producing interferon gamma (IFN-γ) locally, leading to either viral clearance and disease resolution, or immunopathogenesis and liver injury, depending on their microenvironment and functional capacity [18, 23]. Interestingly, viral clearance and disease progression are seemingly mediated by phenotypically distinctive $CD8^+$ CTL populations. The $CD8^+$ CTL participating in viral clearance express high IFN-γ levels, but low levels of the activation marker CD38 (IFN-γ^{hi} $CD38^{lo}$) [17, 18]. In contrast, CTL-mediating liver injury are commonly IFN-γ^{lo} $CD38^{hi}$, and the frequency of these cells tends to increase with the inflammation score [24]. However, it is currently unclear whether these phenotypic differences reflect functional status or merely the source of origin of these cells. In addition to these "good" or "bad" HCV-specific CTL, there is a great discrepancy between a high percentage of activated T cells, and the relatively low frequency of antigen-specific T cells, in the liver of hepatitis C patients [25]. It is possible that under a milieu of proinflammatory cytokines (e.g. IL-2, IL-6, and TNF-α), resting and memory T cells could be activated to proliferate and display effector functions. This antigen-nonspecific pathway may play a role in activating effector T cells in the liver and maintaining the clonal size of peripheral memory T cells during chronic hepatitis C [25].

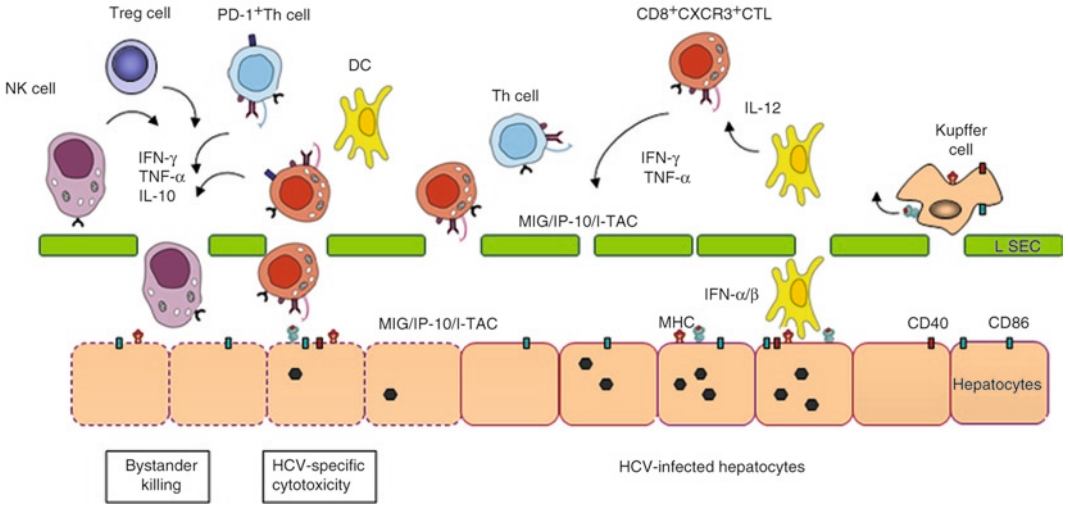

Fig. 38.1 Immunopathogenesis of chronic hepatitis C. HCV is able to evade immune recognition with its genome prone to mutations. The viral proteins may also directly inhibit DC- and T-cell functions by interfering with the hosts' physiological processes. During chronic hepatitis C infection, T cells having a narrow repertoire of TCRs mount a relatively weak response to HCV antigens, and their effort functions (cytokine production as well as cytotoxicity) are often impaired. Many intrahepatic T, B, and NK cells express chemokine receptors CCR5 and CXCR3, and chemokines MIP-1α, MIP-1β, MIG, IP-10, and I-TAC are often upregulated in the liver. Compromised DC functions, development of Treg cells and upregulation of PD-1 on T cells, intrahepatic IL-10, and TGF-β production may all contribute to T cell functional impairment. During the late stage with profound liver function decompensation, large numbers of activated NK cells are recruited to the site and inflict additional damage to the liver

Estimates of the proportion of hepatocytes that are infected vary widely, ranging from less than 10% to almost 50% [26]. However, it is clear that even uninfected hepatocytes can be damaged by the immune response. In an experimental setting, Bertolino and coworkers found that antigen-specific $CD8^+$ CTL secrete IFN-γ and TNF-α, and destroy nearby hepatocytes that do not carry the antigen [27]. This theory of "bystander killing" is supported by studies in which CTL derived from hepatitis C patients recognized a small number of cells presenting HCV core and NS3 antigens (0.8–1.5%), and killed both target and greater numbers of uninfected bystander hepatocytes (10–29%) [23]. These authors argued that the apoptotic pathway involving Fas-FasL interaction is responsible for this bystander killing. This mode of killing has also been implicated in a $CD4^+$ Th1-mediated liver injury in a murine hepatitis B model [28]. While the precise pathways leading to recognition and bystander killing remain debatable, these studies underscore the importance of destruction of cells not directly targeted by effector cells in HCV-induced liver injury [25].

The healthy liver of adult humans maintains tolerance to large varieties of antigens derived from the gastrointestinal tract, including dietary antigens, pathogens, and toxins, by expressing low levels of major histocompatibility complex (MHC) molecules and virtually no immune co-stimulatory molecules. However, under various circumstances, including HCV infection, high levels of MHC class II and co-stimulatory molecules (e.g. B7 and CD40) are expressed in activated Kupffer cells and hepatocytes [29, 30], and the liver can retain large numbers of $CD4^+$ or $CD8^+$ T cells, which can secrete great amounts of proinflammatory cytokines IFN-γ and TNF-α and inflict liver injury [27, 28, 31]. The levels of MHC and co-stimulatory molecules expressed in the liver are closely correlated with those of intrahepatic inflammation and serum aminotransferases. The importance of the co-stimulatory molecule CD86 in HCV-related liver damage has been demonstrated in transgenic mice that conditionally express viral structural proteins [32]. In this model, hepatic CD86 expression resulted in an increased activation of and cytokine production (e.g., IL-2 and IFN-γ) by $CD4^+$ T cells, and the retention of these cells was associated with more pronounced necroinflammatory lesions in the liver. In another murine model infected with adenovirus, CD40 expression in the liver of transgenic animals resulted in severe lymphocytic infiltration, apoptosis and necrosis, accompanied by an exacerbated serum ALT elevation; however, this robust and prolonged expansion of CTL and NK populations with increased IFN-γ and granzyme B production did not facilitate a speedier viral clearance in the liver, but rather exacerbated liver injury (Yan et al., in preparation). These results underscore the key role for a balanced and highly regulated immune response in spontaneous or antiviral-induced disease resolution.

T Helper and Treg Cells

In HCV infection, strong $CD4^+$ Th and $CD8^+$ CTL responses are critical in viral clearance or disease progression [33–35]. Although CTLs can exert limited antiviral activity, even in the

absence of Th cells, they are unable to keep pace with the evolution of HCV. As a result, HCV rapidly accumulates escape mutations in its genome, and persistent, low-level viremia ensues [36]. In studies with large cohorts of chronic subjects and spontaneous resolvers, the presence of virus-specific CTLs that displayed IFN-γ and cytotoxicity did not necessarily correlate with spontaneous viral clearance; however, adequate help from $CD4^+$ T cells was found to be essential to promoting immune protection [37]. Robust Th response with high levels of IL-2 and IFN-γ favors viral resolution. Among resolvers, predominant $CD4^+$ T-cell responses are directed against the nonstructural proteins, and the HCV epitopes are presented by multiple alleles of MHC II molecules [38].

Although vigorous T-cell responses are critical for controlling HCV infection, these responses must be regulated since overly strong responses can lead to excessive liver injury [39]. HCV persistence is associated with a high frequency of $Foxp3^+CD4^+CD25^+$ regulatory T cells (Tregs) that could directly suppress HCV-specific CTL in patients [40]. In acute hepatitis C patients, virus-specific Tregs expand briefly, irrespective of whether they ultimately developed spontaneous resolution or persistence [41]. The resolution of disease is usually associated with the loss of expression of inhibitory receptor-programmed death-1 (PD-1) and decreased functional suppression [42, 43]. In addition to Tregs, high expression of PD-1 on $CD4^+$ and $CD8^+$ cells also contributes to viral persistence as well as to failure of antiviral therapy [44, 45]. A recent study of chronic hepatitis C patients demonstrates that PD-1 not only tempers the functions of effector T cells, but also those of Tregs, which led to a suggestion that both effector T cells and Tregs are tightly regulated by several often overlapping immune mechanisms [46]. In most patients who fail to clear HCV during the acute phase of infection, the course of viral hepatitis lies in part between adequate protective immune response and suppression of immunopathology in the liver.

Dendritic Cells

Dendritic cells (DC) are professional antigen-presenting cells. In viral infections, plasmacytoid DC (pDC) are early and potent producers of IFN-α, followed by activation of myeloid DC (mDC), which produce large amounts of IL-12 and other cytokines and chemokines to attract and activate T cells. A large body of literature demonstrates that DC from HCV-infected individuals do not mature normally and have impaired stimulatory activity. Successful antiviral therapy normalizes many phenotypic and functional abnormities in patients [47]. HCV infection impacts DC populations and their functions through several mechanisms [48, 49]. First, HCV is a relatively weak inducer of IFN-α in pDC, when one compares the levels it induces to those from influenza virus or human herpesvirus type-1, because HCV less efficiently senses viral RNA by TLR7 [50]. Second, HCV appears to inhibit pDC function via a direct and early blockade of TLR9 function during the particle internalization and endocytosis phase, resulting in less efficient sensing of HCV RNA by TLR7. Inhibition of DC function by HCV does not require viral replication or even active viral infection [51]. Several HCV proteins (e.g. core, E1, and NS3) have been shown to impair DC functions through a partial activation and prevention of full maturation [52, 53]. For example, HCV core protein can result in pDC apoptosis and reduced IFN-α production through anomalous monocyte activation and cytokine secretion [54]. In addition, HCV can further inhibit both MHC class I- and II-restricted antigen presentation and induce IL-10 secretion in DCs [55]. Finally, mDC may promote the persistence of the infection by facilitating selective proliferation and expansion of Tregs in patients with HCV [56]. Because of their importance in nearly every stage of the disease, both DC groups are being evaluated as a potential therapeutic tool and viable drug target for chronic hepatitis C patients [57, 58].

Innate Immunity

NK, NT and NKT Cells

NK and NKT cells are the predominant lymphocyte population in the liver, accounting for more than 50% of total hepatic lymphocytes. Both cell types are capable of producing high levels of pro- and anti-inflammatory cytokines upon activation, and are thought to be important in HCV clearance [59]. NKT cells are a diverse group of cells that share phenotypic and functional features of both classical T cells and NK cells. Although both glycolipids and hydrophobic peptides can bind to MHC-like CD1d family molecules on antigen-presenting cells, it is not clear whether their physiological interactions are mediated by specific CD1d-presented antigens or are antigen independent. Like NK cells, upon stimulation, NKT cells can express large amounts of IFN-γ and IL-4 as prototypic type 1 and type 2 cytokines, and display FasL-mediated or perforin-dependent CD1d-specific killing. In patients infected with HCV, NKT cell frequencies in the periphery are lower compared to those of control subjects. These cells in the hepatic compartment switch from a resting phenotype ($CD69^{lo}$) to an activated phenotype ($CD69^{hi}$) with some undergoing apoptosis [60, 61]. In addition to CD1d-restricted NKT cells, a broader group of T cells co-expressing NK cell marker CD56 (natural T cells or NT cells) also plays a critical role in determining the outcome of acute hepatitis C infection [19]. In humans, NT cells comprise approximately 5–15% of the peripheral T-cell pool, and up to 50% of T cells within the liver environment. NT cells possess both

innate and adaptive immune functions capable of MHC-restricted and -unrestricted cytotoxicity and cytokine production. Among resolvers, activated NT cells at baseline are often associated with spontaneous recovery from acute HCV infection. On the other hand, deficient IL-13 production by NT cells and reduced IL-2-activated killing predict the ultimate development of persistence. Classical NKT cells have been shown to play a part in immunopathogenesis of several diseases [62–65]. For example, NKT cells are shown to contribute to the development of hepatitis through the FasL and perforin-granzyme B pathways [66]. Conversely, mice with reduced hepatic NKT cells show an increased sensitivity to lipopolysaccharide (LPS)-induced liver injury [67]. The precise role of NKT cells in HCV immunopathogenesis warrants further investigation.

In patients with chronic hepatitis C, impairment of NK cell functions is often observed [68, 69]. While the effects of NK cells in resistance to HCV infection have been directly demonstrated in patients, their role in the pathogenic process remains unclear [22, 70]. Several lines of circumstantial evidence point to the notion that activated NK cells contribute to HCV-related liver injury. For instance, NK cells expand and become the most abundant cell populations in patients with end-stage liver disease [22]. In the experimental setting, production of IFN-α/β in the liver following HCV infection can activate NK cells, which kill hepatocytes directly via the perforin or Fas-FasL pathways. In other circumstances, proinflammatory cytokines produced by NK cells (e.g. IFN-γ and IL-12) can induce the expression of MIG/CXCL9 and IP-10/CXCL10, resulting in activation and recruitment of T lymphocytes and chronic hepatitis [71]. Depletion of NK cells leads to inhibition of T-cell responses and alleviation of liver injury [72].

Cytokines and Chemokines

HCV clearance and hepatitis have long been thought to reflect direct target destruction. A scenario has emerged in the past few years, which indicates most HCV are purged by locally produced IFN–γ via a novel noncytolytic mechanism [17, 18]. In vitro, IFN-γ can inhibit HCV protein synthesis and viral replication independent of IFN-α/β actions [73]. Although this theory is consistent with the observations made in HCV-infected chimpanzees [16, 26], the exact mechanism(s) responsible for HCV clearance in patients remains a subject of debate. In one recent study designed to dissect the effector functions of virus-specific CTL, the primary CTL clones were reported to degranulate (cytotoxicity) or produce IFN-γ (cytokine production), depending on the antigen concentration [74]. Cytotoxicity can be triggered at antigenic peptide concentrations that are 10–100-fold less than those required for IFN-γ production. These results appear to suggest that the cytokine-vs.-cytolytic nature of the virus-specific CTL effectors is primarily determined by the given moment of virus-host interaction in vivo. Whether this notion is applicable to the acute and chronic stages of HCV infection remains to be tested.

In addition to direct cytotoxic effects of hepatic lymphoid cells, cytokine networks interact with other factors such as alcohol, products of oxidative cell damage, and lipids to play a role in pathogenesis. Kupffer, NK, NKT cells, as well as αβ and γδ T cells in the liver can secrete a common set of cytokines including IL-4, IFN-γ, and TNF-α. In addition to these cells, a large number of hepatic sinusoidal endothelial cells can also be activated in response to viral infection and secrete cytokines such as TGF-β, a powerful immune regulator and promoter of fibrogenesis. It is now known that the increased expression of TNF-α is involved in the pathogenesis and progression of fibrosis in chronic hepatitis C [75]. Other host factors such as alcohol consumption increase hepatic TNF-α and TGF-β expression, as well as lipid peroxidation [76]. These effects may contribute to the activation of fibrogenesis and the development of hepatocellular carcinoma in cases of alcohol abuse and HCV infection. For example, the presence or absence of excessive lipids bathing the liver alters NKT cells, leading to increased apoptosis and inflammation. Furthermore, type-1 immune bias with an increased distribution of NKT cells and TNF-α also leads to other metabolic and signaling effects within hepatocytes, and these play a major role in determining the phenotypic response of the liver to chronic infection.

Chemokines and their receptors are inflammatory cytokines with chemotactic properties. They belong to large families with unique structural features and are involved in regulating the recruitment of lymphocytes to different tissue compartments in both physiological and pathological conditions. In the liver, chemokines are potent activators and chemoattractants for leukocyte subpopulations [22, 24, 77]. Several studies have revealed that the intrahepatic $CD4^+$, $CD8^+$ T cells, NK, NKT cells, and B cells express high levels of the extralymphoid homing receptors CCR5, CXCR3, and CXCR6 in chronic hepatitis C patients [22, 24]. The CXCR3 ligands (MIG/LXCL9, IP-10/CXCL10 and I-TAC/CXCL11) are the most significantly expressed chemokines in chronic hepatitis C. The CXCR3 ligands were upregulated in the sinusoidal endothelium of the liver parenchyma, whereas the CCR5 ligands MIP1α/CCL3 and MIP-1β/CCL4 were largely found within portal tracts [77–79]. The expression of CXCR3 and its three ligands, most markedly IP-10, is associated with higher necroinflammation (particularly in the lobule region) and fibrosis in chronic hepatitis C patients [24, 79]. Human hepatocytes and sinusoidal endothelial cells can produce IP-10, MIG, and I-TAC in response to several inflammatory cytokines (e.g. IFN-α, -γ, IL-1, or TNF-α), a finding

that may imply Th1 cytokines can recruit inflammatory T and NK cells into the hepatic lobule via the CXCR3/ligand pathway. In studies designed to examine chemokines in diagnosis and antiviral therapy, elevations in IP-10, MIG, and I-TAC were observed in sera of patients or chimpanzees with HCV infection [80, 81]. The baseline level of IP-10 was greatest among nonresponders to antiviral therapy. However, following successful antiviral therapy, levels of IP-10 and MIG decreased among resolvers, suggesting that plasma concentrations of IP-10 may be a predictor of responsiveness to combinational antiviral therapy with pegylated IFN and ribavirin [80–82]. Collectively, these results highlight an important role of chemokines and chemokine receptors such as IP-10/CXCR3 in necroinflammation and fibrosis in the liver parenchyma in chronic HCV infection [77–79].

Metabolic Consequences of HCV Infection

Steatosis and Insulin Resistance

Hepatic steatosis is a common feature of chronic hepatitis C [11, 83] and the frequency of steatosis in HCV patients ranges from 40–70% in the US [84, 85], Australia, [86], and Taiwan [87]. These frequencies are higher than those seen in similar patients with autoimmune hepatitis [88] or chronic hepatitis B [89]. Steatosis is a direct cytopathic effect of the virus [90, 91]. This is most clear for genotype 3 infection where the frequency of steatosis is 70–80% [92–94], and occurs independently of the presence of other causes of hepatic lipid accumulation, such as obesity and diabetes. It correlates with viral RNA titers and intrahepatic levels of viral core protein [95]; viral eradication results in disappearance of steatosis and disease-relapse leads to its recurrence [93]. Patients with other viral genotypes, such as genotype 1, also have a higher incidence of steatosis than does the corresponding non-HCV infected population, but the prevalence of steatosis is less than that for genotype 3. In genotype 1, hepatic steatosis development appears to require the presence of other factors, such as obesity and insulin resistance. In this case, HCV infection appears to act like a cofactor thereby allowing steatosis to develop more frequently in the presence of insulin resistance and/or obesity without being sufficient to cause steatosis by itself [96].

Insulin resistance (IR) similarly occurs in about 30–50% of HCV patients. Several recent studies have clearly shown that hepatitis C causes IR. Moucari et al. examined 550 chronic hepatitis C patients and compared them with 100 chronic hepatitis B patients. IR was strongly associated with genotypes 1 and 4, but not with genotype 2 and 3 [97]. When matched for patient characteristics, liver inflammation and fibrosis grade, the presence of IR was strongly associated with HCV but not HBV. It was present in 35% of the HCV patients compared to 5% of those with HBV. These observations have been confirmed in a number of other studies showing the increased risk of IR or diabetes development in HCV patients [97–100].

Whether steatosis causes IR, IR causes steatosis, or if they are unrelated has not been completely resolved. It is well established that circulating free fatty acids are taken up by hepatocytes where they cause both steatosis and IR, but the situation in chronic Hepatitis C appears to be more complicated. Fartoux et al [101] examined the relationship between steatosis and IR and showed that there was a relationship only for type 1 and not type 3. Genotype 1 steatosis almost always occurs in the presence of IR. In the case of genotype 3 infection, there appears to be no relationship between the two, and steatosis is associated with viral load but not IR [101, 102]. Given that similar degrees of steatosis are not associated with IR in genotype 3 infection, the data collectively suggest that in genotype 1, IR is the initiating event that secondarily causes steatosis.

Early retrospective cross-sectional studies reported an association between the presence of steatosis and greater degrees of fibrosis on liver biopsy. This has been seen in several large, multicenter and meta-analyses [84, 103, 104], as well as a majority of smaller single institution studies. More importantly, there have been a number of studies that have examined the fibrosis progression rate directly in patients who have undergone sequential liver biopsies, typically with a several-year interval between biopsies. Most of these studies have also found a correlation between steatosis on initial biopsy and rate of fibrosis progression [105–108]. The association of fibrosis progression with steatosis appears greatest for genotype 1 and less prominent or not observable for genotype 3 [103, 109–111]. Insulin resistance and not steatosis itself is the factor responsible for greater fibrosis progression [109, 110], but this issue has been difficult to resolve since many of the longitudinal studies did not directly examine both steatosis and insulin resistance in multivariate analyses.

Mechanisms of HCV-Induced Steatosis

Hepatic steatosis is an increase in the presence of triglyceride-rich lipid storage droplets in hepatocytes. This can result from an increase in the processes that lead to lipid accumulation, biosynthesis and uptake, and/or a decrease in the processes that remove lipids from hepatocytes, fatty acid oxidation and lipoprotein secretion. Over the past several years, there has been a major increase in our understanding of how hepatitis C produces steatosis. There is an emerging recognition that the virus lifecycle requires it to modify lipid pathways. The virus particle itself is a lipoviroprotein with both a lipid and apolipoprotein components that share characteristics with very-low frequency lipoproteins (VLDL). Viral assembly takes place at the surface

of intracellular lipid droplets, and a virus-induced increase in the number and size of these structures appears necessary for viral particle production. Core binds to the surface of the lipid droplets via hydrophobic sequences in its C-terminal domain and serves as an anchor recruiting other viral proteins and viral RNA as part of the particle assembly process.

One consistent observation in experimental models of HCV is that viral infection or just the presence of core protein induces formation of lipid droplets [112–117]. This effect is present for both genotype 1 and 3 core proteins, but the effect for genotype 3 core is approximately threefold greater than that for genotype 1 [118–121], and core derived from more lipid-inducing viral strains has a greater effect in vitro. Other viral proteins are steatogenic as well. NS2 and NS5a induced steatosis in transgenic mice [122], and NS4b is able to produce lipid accumulation in cell culture.

Defects in Lipoprotein Assembly and Export

The first cellular process shown to contribute to core-induced lipid accumulation is inhibition of lipoprotein formation and secretion. Core protein binds to apolipoprotein AII, and interferes with the export of this apolipoprotein component of high-density lipoproteins (HDL) [123]. More significantly, it also inhibits the activity of microsomal triglyceride transfer protein (MTP), required for triglyceride loading of nascent VLDL. Core protein can thus block export of triglyceride containing lipoproteins [124], leading to intracellular lipid accumulation. The relevance of MTP downregulation to HCV-induced steatosis was further suggested by observations in liver biopsy specimens derived from HCV patients. MTP mRNA levels inversely correlated with the degree of steatosis, and genotype 3 patients had significantly lower MTP enzyme activity compared to that of the other genotypes.

Alterations in Lipid Synthesis

In addition to evidence of impaired lipoprotein secretion resulting from MTP inhibition, there is considerable evidence that hepatitis C also results in increased expression of the enzymes responsible for fatty acid synthesis. Several viral proteins appear responsible for this effect. Expression of core protein in multiple different cell types results in a 50% increase in de novo fatty acid synthesis rates [125], and this results primarily from transcriptional upregulation of the critical enzymes fatty acid synthase and acetyl-coA carboxylase. The clinical relevance of this observation is suggested by the observation that core protein derived from genotype 3 results in a three-fold greater activation of FAS promoter activity [126] in human liver.

The primary mechanism of HCV-induced upregulation of fatty acid synthesis enzymes is via activation of the sterol regulatory element binding protein (SREBP) family of transcription factors. These are ER-resident molecules that are activated by proteolytic processing with subsequent nuclear translocation and activation of many genes required for fatty acid and cholesterol biosynthesis. Infection of hepatoma cells with HCV virus or replication of subgenomic replicons increases the transcriptional activity of SREBP-1c and SREBP-2 by a combination of increased transcription, proteolytic activation, and phosphorylation of the transcription factor. SREBP activation is directly responsible for the increase in FAS and ACC expression and is required for HCV-associated lipid accumulation in these models.

Several studies have investigated the mechanisms of these effects of HCV on SREBP. The viral proteins core, NS2, NS4b, and NS5a have all been implicated. SREBP transcriptional activity is dependent on LXR/RXR [127] and LXR expression and transcriptional activity are both increased by HCV [128, 129]. The mechanism of this effect is unknown, but is dependent on the proteosome activator PA28γ [129].

In addition to LXR-dependent increases in SREBP-1c expression, Waris et al. [127] showed that viral infection resulted in both proteolytic cleavage and phosphorylation of SREBP-1c. Both of these effects serve to activate its transcriptional activity and were induced most strongly by the NS4b protein, but could be produced by core as well, particularly core protein from genotype 3. This activation could be inhibited by PI3kinase inhibitors, Ca^{2+} chelators, or antioxidants leading the authors to conclude that viral proteins initiate a Ca^{2+} and ROS-dependent activation of PI3kinase that subsequently activates Akt leading to SREBP activation. The general outlines of this scheme are supported by a number of other studies, as well with demonstrations that core protein [130], NS2 [128], and NS4b [131] are all able to increase SREBP protein levels and promote proteolytic activation, resulting in the increased expression of fatty acid-synthesis enzymes and fat accumulation in hepatoma cells. These observations in cell culture are supported by the finding that SREBP-1 is upregulated in HCV core transgenic mice that develop hepatic steatosis [113, 129].

One caveat of these experiments in cells and mice is that a single study in patients found that SREBP transcriptional level did not correlate with steatosis in chronic hepatitis C patients [132]. However, this study did show a correlation between SREBP and fibrosis stage. Given the inherent heterogeneity of patients and the tendency of hepatic fat to disappear once cirrhosis develops, the issue remains uncertain.

Effects on Fatty Acid Uptake and Oxidation

There is clear evidence that HCV increases fatty acid synthesis and suppresses lipoprotein export. The other two processes that contribute to lipid accumulation, fatty acid uptake, and fatty acid utilization appear to be regulated as well.

Tanaka et al. [133] demonstrated that lipid accumulation in hepatocytes of transgenic mice expressing core protein was dependent on PPARα. The RXR/PPAR α heterodimer is a key transcription factor responsible for expression of target genes involved in mitochondrial and peroxisomal fatty acid oxidation as well as fatty acid uptake into hepatocytes. Core binds to RXR [134], and decreases expression of PPAR α mRNA and protein [135, 136]. In the case of transgenic mice expressing core protein, core activates the transcriptional activity of the PPARα/RXR heterodimer [134], while at the same time, causing an inhibition of mitochondrial β-oxidation by Ca^{2+} and ROS-dependent mechanisms. The net effect is that PPARα mediated increases in expression of fatty acid translocase and other components of the fatty acid uptake system are able to increase lipid uptake, but the altered mitochondria are not able to support enhanced levels of β-oxidation in spite of stimulation of PPARα-dependent transcription. This results in a PPARα-dependent steatosis induced by core protein [133].

Activation of PPARγ has also been implicated in HCV steatosis. PPARγ activity and recruitment of its co-activator, PGC-1α are increased by NS5a [137], and this was necessary for NS5a-induced steatosis in Chang liver cells, possibly due to increases in lipid uptake. Core also increased PPARγ transcriptional activity [130].

In summary, there is considerable evidence in multiple experimental systems that HCV proteins cause increases in fatty acid synthesis and uptake, while at the same time suppressing fatty-acid oxidation and export. The overall impact of these effects in patients is variable and the multiplicity of mechanisms may explain why HCV-induced steatosis is so heterogeneous between patients and genotypes.

Mechanisms of HCV-Induced Insulin Resistance

As discussed above, hepatic IR is frequently present in HCV infection, particularly in patients infected with genotypes 1 and 4 [97], where it is strongly associated with steatosis and fibrosis progression. On a molecular level, hepatic IR in chronic hepatitis C has been associated with impairment of insulin-induced activating tyrosine phosphorylation of IRS-1 and IRS-2 [138], decreased IRS-1 and IRS-2 expression levels [139, 140], and increased inactivating serine phosphorylation of these substrates as well.

There is compelling evidence that elevated circulating and hepatic TNF-α levels play a central role in HCV-associated IR [141]. In patients, there is an association between TNF-α levels and IR [142, 143]. IR has also been observed in a transgenic mouse model expressing HCV core protein [144]. In this case, elevated circulating TNF-α levels are the cause of insulin resistance and produce this effect by an inhibition of insulin-induced IRS1 tyrosine phosphorylation [145]. This phenomenon has also been seen in human liver biopsy specimens in which chronic hepatitis C also impairs IRS-1 phosphorylation [138]. The TNF-α-induced suppression of insulin signaling requires the presence of the proteosome activator PA28γ, although the precise mechanism of PA28γ involvement in the pathway is unclear [146].

A second mechanism of HCV-induced IR is a SOCS3-dependent ubiquitination and degradation of IRS1 and IRS2 as well [147]. In patients with hepatitis C, there is a core protein-induced increase in the expression of the suppressors of cytokine signaling, SOCS1, and SOCS3. These proteins interact with multiple receptors to regulate cytokine signaling. In the case of the insulin signaling pathway, SOCS3 causes ubiquitination and degradation of both IRS1 and IRS2 and net suppression of the pathway [148]. This has been observed in several studies of HCV patient samples [140, 149] and is associated with insulin resistance. Successful viral eradication results in an increase of IRS-1/2 levels and improvement of insulin sensitivity [139]. Two other mechanisms are likely to contribute to HCV-induced insulin resistance. These are oxidative stress and increased hepatic free fatty acid concentrations as discussed elsewhere [102] and in this review.

Mechanisms of HCV-Induced Oxidative Stress

Hepatic oxidative stress is an intrinsic characteristic of chronic hepatitis C infection and occurs more commonly in HCV than in HBV and other inflammatory liver diseases [150–152]. Based on correlations with greater severity of inflammation, insulin resistance [153, 154], fibrosis progression, and the development of hepatocellular carcinoma [155], oxidative stress appears to play a major role in diverse aspects of pathogenesis.

The mechanism of oxidative stress in chronic hepatitis C is multifactorial involving reactive oxygen species production from macrophages and neutrophils [156], and iron accumulation due to direct HCV-induced suppression of hepatic hepcidin production [157]. However, there is substantial evidence from multiple experimental systems that a major cause of oxidative stress in hepatitis C is due to direct effects of viral proteins, particularly that of core protein on ER and mitochondrial function. Core protein expression in the context of viral replication causes ER stress [158], and this results in the release of Ca^{2+} from the ER lumen [159–161]. In addition, core also directly interacts with mitochondria [162, 163]and upregulates the mitochondrial Ca^{2+} uniporter, thereby causing more rapid Ca^{2+} entry and increased net mitochondrial Ca^{2+} accumulation for a given extramitochondrial Ca^{2+} concentration [164]. Together, both effects result in an increase in mitochondrial Ca^{2+} and a subsequent increase in mitochondrial ROS

production, oxidation of mitochondrial glutathione, and inhibition of mitochondrial electron transport complex I [163].

NS5a [165, 166] and the NS3/NS4a complex also increase ROS formation. In the case of NS5a, this appears to result from ER stress, while NS3/4a forms a protease that binds to the mitochondrial outer membrane where it modulates innate immune signaling [167] and increases mitochondrial ROS production [168, 169]. Pathologically, oxidative stress contributes to many aspects of the disease. Combined with steatosis, it promotes fibrogenesis [153, 170] and sets up positive feedback loops promoting steatosis, oxidative DNA damage, and cell death signaling [171].

Fibrogenesis

The development of progressive fibrosis leading to distortion of liver architecture and ultimately resulting in cirrhosis is the hallmark of significant disease in chronic hepatitis C and precedes the development of liver failure or HCC. Fibrogenesis proceeds primarily from the activation of hepatic stellate cells, which then results in increased production of type 1 collagen and other matrix components. Fibrogenesis occurs in essentially all chronic inflammatory liver diseases, and the general mechanisms of fibrogenesis have been summarized in several excellent reviews [172–174].

In the case of hepatitis C, chronic inflammation activates the fibrogenic process in a manner similar to that of other processes, but several viral-specific effects appear to play a role in greater fibrosis. First, HCV-induced ROS production promotes stellate cell activation and enhances the effects of other activators [175]. Second, HCV infection induces TGF-β production in hepatocytes [176]. TGF-β levels in patients are increased [177, 178], and this is the major cytokine that increases stellate-cell activation and collagen production [172]. Co-culture systems of hepatocytes and stellate cells have demonstrated that HCV-induced TGF-β production is sufficient to activate stellate cells in vitro [179]. A third fibrogenic stimulus associated with HCV is hepatocyte apoptosis. Although several HCV proteins have anti-apoptotic effects under some circumstances, the net effect of viral infection and its associated immune response is to increase hepatocyte turnover and apoptosis. In cell culture, virus infection directly induces increased apoptosis of infected cells via a TRAIL-mediated process [180, 181]. Apoptotic hepatocytes are engulfed by stellate cells and have been recently identified as a potent fibrogenic stimulus, possibly by interaction of hepatocyte DNA with TLR9 [182].

In addition to these direct effects, activated $CD8^+$ T cells have fibrogenic effects as well, and these are an important part of both acute and chronic inflammatory responses in hepatitis C. Finally, there is evidence to support direct interaction of HCV proteins with stellate cells themselves. Core, NS3, and possibly other nonstructural proteins have been shown to directly cause ROS-dependent stellate cell activation and increased stellate cell TGF-β production [183, 184]. While in general, HCV produces a slowly progressive fibrosis, under some circumstances, such as those that occur in immunosuppression after liver transplant or in HIV-co-infected patients, HCV has the capacity to produce a rapid fibrosis response called fibrosing cholestatic hepatitis [185]. While the mechanisms of this phenomenon are not understood, it does demonstrate the intrinsic fibrogenic potential of the virus above and beyond that produced generally by liver inflammation.

Effects of HCV on Cell Cycle Regulation and Growth Control

Development of HCC is one of the most important clinical consequences of chronic hepatitis C. In patients with HCV cirrhosis, the risk of development of HCC is approximately 1.5–2% per year. Given the large population of infected individuals, this makes HCV one of the most important tumor viruses in the world. The association of viruses with specific human cancers has been attributed to viral oncoproteins that manipulate DNA repair and cell-cycle pathways [186]. HCV, like most oncogenic viruses, establishes persistent infections that last the lifetime of the individual. In general, only a small fraction of individuals who harbor these viruses develop cancer, and this usually does not occur until 20–40 years after infection [187].

In the case of HCV, carcinogenesis appears to involve both host and viral factors. Multiple viral proteins including core [188–190], NS3 [191, 192], NS5a [193], and NS5b [194] have the ability to transform cells and promote growth of cells in culture. In addition, cellular mutations are frequent in HCC, and HCV replication has been associated with a pro-mutagenic phenotype possibly by interfering with fidelity of DNA repair [195]. Core protein and NS5a have both been shown to cause tumors or enhance tumor formation induced by other factors. HCV core protein is sufficient to transform immortalized cell lines [196]. Transgenic mouse lines expressing core protein spontaneously develop HCC after 1 year [197], as do transgenic mice expressing the complete HCV open-reading frame [114]. These tumors have phenotypes that resemble HCV-associated HCC in humans [197, 198]. In addition to core protein, HCV NS5a also has tumorigenic properties. Transgenic mice expressing NS5a develop spontaneous tumors, particularly after chronic exposure to LPS [122], or develop synergistic tumor formation induced by endotoxemia [199]. High levels of oxidative stress, abnormalities in cell cycle control, and alterations of growth promoting signaling processes have all been implicated in these effects.

However, it is important to note that HCV-associated hepatocarcinogenesis is a heterogeneous process, and no single step has been identified as critical. At present, our best understanding of the process comes from experimental models, particularly those using cell culture and transgenic mice. Three major classes of effects, alteration of cell cycle checkpoints, activation of pro-proliferation signaling pathways, and oxidative-dependent DNA damage, appear to be central to carcinogenesis. Determining the precise relevance of these effects to human carcinogenesis is the major challenge in the field.

HCV-Induced Alteration of the Cell Cycle Control

HCV Effects on p53

Abnormalities of cell-cycle control are one of the most consistent molecular characteristics of HCC observed in HCV experimental models. The most prevalent abnormality in HCC is inactivation of the tumor suppressor p53 [200], but other oncogenes such as the retinoblastoma protein, Rb, β-catenin, and c-myc have also been implicated [201]. The transcription factor p53 is responsible for induction of genes, particularly the cyclin-dependent kinase inhibitor and p21waf that are required for normal cell-cycle control. In addition, p53 induces expression of several mediators of apoptosis such as PUMA and Noxa [202]. Inhibition of p53, therefore, has major potential to promote carcinogenesis.

NS5a has been most clearly shown to inhibit p53. It directly binds p53 [203, 204], sequesters it in the cytosol [203], represses the p53-dependent induction of p21waf [193, 203–205], and promotes cell growth. NS5a also inhibits the pro-apoptotic effects of p53 [203], possibly via interaction with Bax [206]. Core protein has also been shown to have significant effects on p53, but these are contradictory. Several studies have shown that core, like NS5a inhibits the transcriptional activity of p53, represses p21 induction, and thus, has pro-cycling effects [207, 208]. However, other studies have suggested that core may have the opposite effect and activate p53, resulting in growth arrest [209–212]. One possible explanation suggested by Kao et al. is that the discrepancy may be due to differences in core expression levels, because it is a transcriptional activator at low concentrations and a repressor at higher expression levels [211].

NS3 can also bind p53 and repress its transcriptional activity in a manner that is synergistic with core protein [213], a factor which means that multiple viral proteins can be active by this mechanism. It has been reported that the HCV core binds to both p53 and $p21^{CIP1}$ [210, 214] and impairs cell-cycle regulation in association with the increased expression of $p21^{CIP1}$ [210, 215]. Persistent expression of HCV core does not substantially affect the expression levels of p53 and $p21^{CIP1}$.

HCV Effects on Rb

A second point of interaction between HCV and the cell cycle, linking HCV to HCC, is through the retinoblastoma protein (Rb). The Rb pathway is another checkpoint route that limits uncontrolled cell division in response to DNA insult or oncogene activation. In human HCC, the Rb pathway is defective in more than 80% of all cases [216]. p28/gankyrin, an inhibitor of p53 and Rb, is over expressed in most HCC [217]. pRb plays a major role in controlling the G1- to S-phase transition and mitotic checkpoints through a repressive effect on E2F transcription factors [218]. Rb phosphorylation is dependent on CDK2 activity and is normally downregulated by p53-induced p21waf expression. Increased phosphorylation of Rb activates the E2F family of transcription factors [219]. The Rb/E2F pathway is critical for cell-cycle control and it interacts with p53 in promoting apoptosis. Hyperphosphorylation of Rb and mutations of Rb are associated with a number of tumors, including HCC. Core protein expression has been shown to decrease Rb levels, increase phosphorylation of Rb and consequently activate free E2F1 [219, 220]. Free E2F1 promotes cell cycling via its transcriptional activation, but it can also promote apoptosis via p53, leading to both pro and anti-growth potential. Full genome length HCV replicons produce hyperphosphorylation of Rb and E2F activation [221], but the mechanism of this effect is unknown.

The RNA-dependent RNA polymerase of HCV, NS5b, forms a complex with pRb in cells, leading to its degradation and eventually a reduction in its abundance. This leads to the activation of E2F-responsive promoters in cells containing HCV RNA replicons and promotes progression of the cell cycle from G1- to S-phase in cells expressing NS5b [222]. It has also been documented that pRb is downregulated in human hepatoma cells infected in vitro with different genotypes of HCV [223], and that downregulation of pRb occurs via the ubiquitin-proteasome system, and that pRb is ubiquitinated in an NS5b-dependent manner [222]. These findings lead us to suggest that NS5b-mediated Rb degradation contributes to the loss of cell-cycle control in chronic hepatitis C.

HCV and Cdks

Viruses manipulate cell-cycle check points to create conducive environments for their own replication. This predisposes the infected cells to impaired DNA repair leading to genomic instability, transformation, and tumorigenesis. Since viral activation of DNA-damage repair pathways can lead to checkpoint signaling that would stall the cell cycle at G1/S, viruses inactivate G1/S checkpoints to promote S phase transition. Cell cycle progression is regulated by the controlled expression and degradation of cyclins that regulate the kinase activity

of distinct members of the cyclin-dependent-kinase (Cdk) family. Progression from G0 to G1 is initiated by exogenous mitogens that stimulate expression of cyclin D, which activates Cdk4 and Cdk6 to phosphorylate the Rb protein, resulting in release of the transcription factor E2F from Rb and transcription of target genes, many of which are required for completion of S phase processes [224, 225]. Common mechanisms by which viruses initiate cell cycle progression and activate E2F include promotion of cyclin D activity or dissociation of the Rb/E2F complex in a cyclin-D-independent manner.

HCV modulates these pathways by several mechanisms. As discussed, NS5b binds to Rb for degradation, resulting in cyclin-D-independent activation of E2F [223]. Deregulation of the check point control in the G1 phase has a major influence on genomic instability [226]. Checkpoint control in the G1 phase is precisely regulated by the interactions of cell-cycle regulatory proteins, including cyclins, Cdks, CKIs, p53, and pRb [227].

Considerable evidence has suggested that the overexpression of cyclin E contributes to the development of many types of human cancers [228–230]. HCV core protein promotes cell proliferation by modulating expression levels of the cyclin E, and this property of the HCV core protein plays an important role in hepatocarcinogenesis [231].

Although cell-cycle progression in mammalian cells is mainly governed by cyclins A, D, and E with cyclin-dependent kinase (CDK) 2 and 4, the kinase activities of these CDKs are negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs) p16 and p27. Among these cell-cycle regulators, p16 and p27 are frequently inactivated in HCC cells and considered to be potent tumor suppressors. More significantly, there is a close relationship between p16 and p27 during the progress of HCC [232–234]. It has been demonstrated that extensive DNA methylation is the main cause of p16 inactivation in HCC [235]. Noticeably, HCCs with p16 methylation were only found in individuals with HBV and HCV infections and not in virus-negative individuals [236].

HCV Alterations of Proliferation Signaling Pathways

PI3K/Akt

Aberrant PI3K/Akt signaling plays a major role in liver dysfunction and pathologies associated with HCC. The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a negative regulator of PI3 kinase/Akt signaling and is mutated or deleted in ~30% of all human cancers. It is thus, after p53, the most common tumor suppressor whose expression/activity is altered during carcinogenesis (reviewed in [237]). Mutation/weak expression of PTEN is reported by several studies in human HCC [238–240]. In animal studies, PTEN heterozygous mice were shown to develop tumors in multiple organs, including the liver [241]. Moreover, PTEN liver-specific knock out mouse at age 40–78 weeks developed liver nodular lesions that resembled human-liver adenomas which later evolved into HCC [242, 243]. Additionally, PTEN has been linked to cholangiocellular carcinoma and hepatic angiosarcoma [244]. PTEN also favors epithelial to mesenchymal transition (EMT), which is responsible for migration and invasiveness of cancer cells [245].
HCV infection leads to upregulation of the Akt/PI3K pathway due to suppression of PTEN activity [127]. Downregulation of PTEN in an HCV-positive cirrhotic liver results in concomitant upregulation of iNOS and CoxII leading to tumor progression [246].

MAP Kinases

The MAPK signal transduction pathway is evolutionarily and one of the oldest signal transduction pathways in eukaryotic cells. It contains three different signal tracts: the extracellular regulated protein kinase (ERK and p42/44 MAPK) tract, the stress-activated protein kinase (SAPK, p38 MAPK, and p38-RK or p38) tract, and the c-Jun-NH2-terminal kinase (JNK and p64/54 SAPK) tract, which regulate processes such as cell growth, differentiation, maturation, proliferation, and apoptosis. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of JNK activation, transcription factor-activating protein AP-1 activation is markedly enhanced [134, 247].

In contrast, NS5a inhibits the activity of AP1, and this inhibition is mediated through the ERK pathway [248]. It has also been documented that JNK activation is essential for stimulation of HCV NS3-mediated cell proliferation [249]. MAPK/ERK activation and cell proliferation, driven by HCV E2, is suppressed by blockage of CD81 as well as LDLR [250]. Inhibition of MEK by U0126 also impairs MAPK/ERK activation and cell proliferation induced by HCV E2 [250]. HCV core protein has been shown to promote proliferation of human hepatoma cells by the MAPK/ERK pathway activation via upregulation of TGF-α [251]. TGF-α is upregulated in some human cancers and induces epithelial development and has been shown to stimulate neural cell proliferation. It has been observed that activation of ERK1/2 in HCC indicates aggressive behavior and constitutes an independent prognostic factor [252]. Additionally, HCV infection activates the ERK pathway and thereby might contribute to hepatocarcinogenesis [252]. Koike et al. suggest that induction of oxidative stress in combination with activated MAPK cascade leads to activation of AP-1 and cyclinD1 overexpression and contributes to the development of HCC [253].

Wnt/β-Catenin Pathway

Abnormalities in the Wnt signaling pathway are important in hepatocellular carcinogenesis [254–256]. The Wnt pathway promotes carcinogenesis by signaling through β-catenin and promoting its nuclear localization. Normally, β-catenin's growth-promoting effects are kept in check via phosphorylation and binding to the APC protein. Stabilization of β-catenin either by a reduction in phosphorylation or mutation is a common feature of HCC. NS5a plays a role in this pathway via its activation of Akt/protein kinase B, which phosphorylates and inactivates one of the inactivators of β-catenin, GSK-3β [257]. This, in turn, stabilizes β-catenin by inhibiting its phosphorylation and degradation. Thus, there is evidence that HCV proteins have considerable direct effects on p53, Rb, and β-catenin, and these contribute to cell-cycle dysregulation, apoptosis inhibition, and carcinogenesis.

Various other protein members of the Wnt pathway have been described to function as tumor suppressors (E-Cadherin, APC, Axin1 and 2). Almost 50% of HCC exhibit alterations in the Wnt pathway [258]. E-cadherin is a calcium-dependent, cell–cell adhesion glycoprotein and hypermethylation of its promoter has also been linked to decreased E-cadherin expression, microvascular invasion and recurrence of HCC [259, 260]. The probability of recurrence in the case of HCV-associated HCC has been previously linked to the decreased expression of E-cadherin [261]. More recently, high-tumor *Wnt-1* expression in HCC patients with HCV infection was correlated with increased nuclear β-catenin accumulation, decreased membranous E-cadherin expression, and increased tumor recurrence after curative resection [262]. The disruption of the APC (adenomatosis polyposis coli) gene, another member of the Wnt family, in liver, induces hepatocyte hyperplasia, marked hepatomegaly and rapid mortality. Like other tumor suppressor genes, APC is hypermethylated in HCC relative to non-tumor liver. HCV-associated HCC had increased APC gene methylation compared to non-HCV associated tumors [263].

TGF-β and Epithelial to Mesenchymal Transition

The cytokine TGF-β, a potent inhibitor of epithelial-cell growth and tumor suppressor, is a key regulator of epithelial to mesenchymal transition (EMT) and also has pro-oncogenic functions. Importantly, TGF-β has been shown to induce EMT both in hepatic-stellate cells and hepatocytes [264]. TGF-β signaling is upregulated in fibrosis in HCV-infected patients and stimulates ECM deposition and accumulation. While SMADs are indispensable for EMT, TGF-β signaling via SMAD interacts with other signaling pathways to mediate pro-oncogenic EMT. Pro-inflammatory cytokine interleukin (IL)-1β can activate JNK and shift TGF-β function from tumor suppression to oncogenesis with increased fibrogenesis, cell motility, and transactivation of cell-cycle regulatory genes [264, 265]. Different thresholds of Smad3 activation dictate the TGF-β responses in hepatic cells. HCV core protein may switch TGF-β growth inhibitory effects to tumor-promoting responses by decreasing Smad3 activation [266]. Thus, core protein expression in primary human or mouse hepatocytes prevents the normal growth inhibition response to TGF-β and converts it into a pro-proliferative response. Additionally, this core-induced alteration in TGF-β signaling causes an epithelial to mesenchymal transition (EMT) [266], a process that contributes to the promotion of cell invasion and metastasis.

HCV and DNA Repair

Most DNA viruses target ATM (ataxia-telangiectasia mutated), a DNA-damage checkpoint kinase, leading to inhibition of ATM-signaling pathways [267]. Even though HCV does not have a dsDNA genome, its replication requires ATM pathways [268]. It was previously proposed by Machida et al. that dsDNA breaks are induced by both HCV infection and the expression of HCV NS3 and core proteins [195, 269]. NS3 unwinds dsDNA by virtue of its helicase activity implying its effect on host dsDNA [270, 271]. HCV infection possibly triggers the activation of ATM without a dsDNA genome. Ariumi et al., in 2008, also reported that the ATM-dependent DNA damage response is constantly stimulated in persistent HCV RNA-replicating cells. Interestingly, Lai et al. recently reported that NS3–4A impaired DNA repair and enhanced sensitivity to ionizing radiation through interaction with ATM [272]. Ariumi et al. suggest that HCV NS5b could bind to both ATM and Chk2, thereby enabling HCV to utilize ATM and Chk2 for its replication, resulting in the impairment of DNA repair, enhancement of mutation frequency, and development of HCC [268]. Persistent expression of the HCV core has been reported to lead to malignant transformation of the host cell in vitro [273, 274] and in vivo [197]. HCV core has also been shown to modify the cellular apoptotic cascade under various stimuli [273, 275–277].

Conclusions and Perspectives

Liver disease from hepatitis C virus is characterized by its relatively long duration, its absolute requirement for an adaptive immune response to trigger injury, and the numerous

ways that each of the viral proteins interacts with host-cell processes. Liver injury is determined by a long-term interplay between viral and host factors. In the immediate term following infection, the virus has very limited direct cytopathic effects, and these rarely become clinically significant except in cases of extremely high viral loads in immunosuppressed patients. After some weeks of infection, an adaptive immune response occurs that is the major initiator of liver injury. While this starts out as a specific T-cell phenomenon, cytokine-mediated changes rapidly lead to an innate immune response, bystander hepatocyte killing, and a more profound injury reaction. As the disease enters its chronic phase, viral levels are decreased, and a number of direct cytopathic effects of viral proteins play an increasingly important role in the overall picture as the disease evolves over decades. The overall course is one of chronic inflammation, oxidative stress, steatosis, IR, fibrogenesis, and disordered cell proliferation in the setting of oxidative DNA damage leading to HCC.

While most of the focus in HCV research is appropriately directed toward improvements in antiviral therapy, it seems likely that for the foreseeable future, many patients will either fail to respond or not tolerate therapy and will have to live with chronic infection. Further understanding of the mechanisms of fibrogenesis and carcinogenesis, in particular, hold out the promise that it will be possible to ameliorate disease severity, even in those who remain chronically infected.

Acknowledgments Work from the author's laboratories was supported by NIH grants AA012863 (SW) and AI069142 (JS). We gratefully acknowledge Mardelle Susman and Elizabeth Zeller-Last for assistance with manuscript preparation.

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Chapter 39
Viral Hepatitis D

John M. Taylor

Introduction

Viral hepatitis D, also known as hepatitis delta virus (HDV), was first discovered by Mario Rizzetto in 1977, in a study of Italian patients infected with hepatitis B virus (HBV), who seemed to have a more damaging liver disease. For more information on HBV, please see Chap. 37. In liver biopsies from such patients, a serum antibody detected a novel nuclear antigen that was named the delta antigen (δAg) [1]. An early interpretation was that δAg was expressed from a more pathogenic variant of HBV. In contrast, it was shown that δAg is a protein encoded by HDV, a virus separate from HBV and yet dependent upon HBV for the provision of envelope proteins essential for the assembly of new virus particles and for the process in which HDV is able to attach to and infect new susceptible cells [2]. Therefore, in nature, productive HDV infections can only be associated with HBV. This dependence provides a complication in understanding how HDV can make HBV infections more damaging. However, as will be explained, it is experimentally possible to assemble HDV in the presence of HBV envelope proteins, but absence of infectious HBV. Also, it is possible to assess the effects of HDV genome replication in cells in the absence of the processes of virus entry and infection and/or virus particle assembly.

This chapter will consider forms of molecular pathology associated with HDV replication, whether in patients, experimental animals, or in cultured cells. However, we must begin with a brief introduction to the molecular biology of HDV replication. For more detailed information, there are recent reviews [3–5] and two monographs [6, 7].

As represented in Fig. 39.1, the genome of HDV, as found inside virus particles, is a small single-stranded RNA. It is about 1,700 nucleotides in length, has a circular conformation, and can fold into an unbranched rod-like structure with about 74% of all nucleotides involved in pairing. Also, inside the particles are 70–200 molecules of δAg. This protein is 195 amino acids in length and its properties include localization to the cell nucleus and an ability to bind to the rod-like folding of the genomic RNA. This form of the delta antigen is referred to as small δAg (δAg-S), since during HDV genome replication, as a consequence of posttranscriptional RNA editing, there arises an RNA that is translated to make a longer protein, the 214 amino acid large δAg (δAg-L). Both forms of the δAg undergo several forms of posttranslational modification (phosphorylation, methylation, acetylation), but it is only the δAg-L that undergoes posttranslation farnesylation, and it is this modification that enables an interaction with the envelope proteins of HBV, so as to achieve assembly of HDV genomes into infectious virus particles.

The HDV life cycle depends on both forms of δAg. δAg-S supports the replication and accumulation of new HDV RNAs, while the δAg-L does not support replication but does provide the ability for the assembly, in the presence of HBV envelope proteins, of new virus particles.

The replication of the HDV RNA is different from that of the HBV helper virus, which uses reverse transcription. HDV uses RNA-directed RNA synthesis in a process that involves redirection of host RNA polymerase activity. The host RNA polymerase II is involved and it remains controversial as to whether an additional host polymerase is also required [3].

The process of HDV genome replication is represented in Fig. 39.2. In cells undergoing HDV replication, the circular genomic RNA can accumulate to beyond 300,000 copies per cell [8]. Also present but in somewhat less amounts is an exactly complementary RNA, the antigenome. The production of these two circular RNAs is considered to arise from the processing of greater-than-unit-length RNA transcripts. The processing is mediated at a unique location by a ribozyme, a small sequence that in the absence of protein is able to produce a site-specific RNA cleavage. The linear unit-length RNA products are then ligated, presumably by a host enzyme, to produce mature RNA circles [9]. Such RNA circles not

J.M. Taylor (✉)
Fox Chase Cancer Center, Philadelphia, PA, USA
e-mail: john.taylor@fccc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_39, © Springer Science+Business Media, LLC 2011

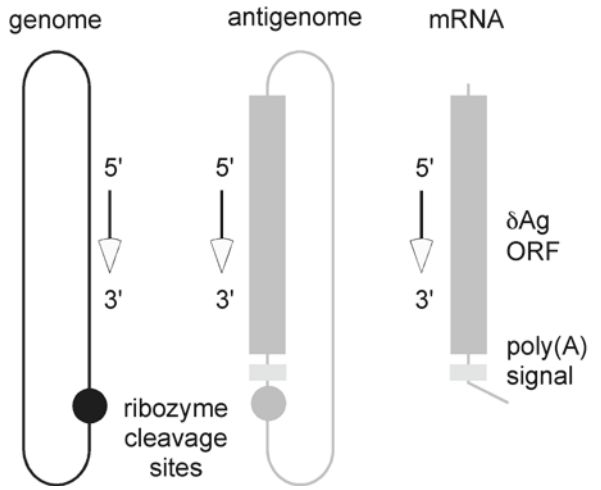

Fig. 39.1 The RNAs of HDV. The genome of HDV is a small single-stranded RNA, with a circular conformation and the ability by about 74% intramolecular base pairing, to form an unbranched rod-like structure. Inside an infected cell there is also present relatively less amounts of an exact complement, called the antigenome. The antigenome contains the open reading frame (δAg ORF) for the one protein of HDV, the δAg. However, the template for δAg translation is a third RNA, the mRNA, which is much less abundant and less than full length. It has a 5'-cap structure, and as directed by a poly(A) signal, undergoes polyadenylation. Both the genome and antigenome contain their own unique ribozyme and ribozyme cleavage sites. Adapted from Taylor [60], copyright 2006, with permission from Elsevier

only bind δAg, but also are considered to be more stable within a host cell than the corresponding linear RNAs [10].

Since HBV and HDV share the same envelope proteins, it is considered that both viruses enter susceptible cells using the same host receptor(s) and the same viral ligands. Studies so far have not yet defined the host receptor or receptors for HBV [11]. These two viruses are almost exclusive in their tropism for hepatocytes, although there is evidence that HBV can replicate in some lymphocytes [12]. The liver tropism for HBV is often explained in part by liver-specific requirements for promoters and enhancers on the viral DNA [13]. However, HDV infections are strictly liver tropic and yet, if appropriately introduced, can replicate in most cell types. Thus, it seems more reasonable to interpret that both HBV and HDV show a preference for specific attachment and entry into hepatocytes because of the presence of the required receptor(s) and/or some hepatocyte-specific pathway of endocytic transport.

HDV infections have now been reported in many parts of the world. Nucleic acid sequencing of such isolates has detected major differences, leading to the definition of what may be as many as eight major clades [14]. Some geographic associations have been made for these clades. Also, some associations have been made between these HDV clades and the eight genotypes that have been defined for the helper virus HBV [14].

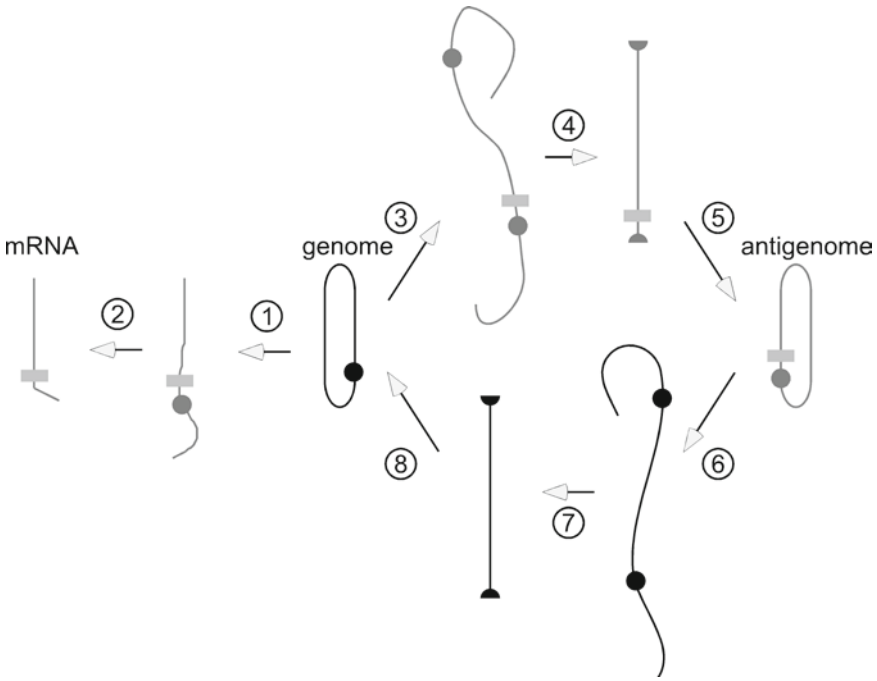

Fig. 39.2 Transcription and processing of HDV RNAs. The genomic RNA by definition is the species present within virus particles. Infection delivers this RNA to the nucleus where it is used as a template for the transcription of antigenomic RNAs that become by processing either new mRNA (Steps 1–2) or – for the processing to greater-than-unit-length transcripts by the unique ribozyme – release unit-length RNAs, which in turn are ligated, to produce new circular antigenomes (Steps 3–6). These in turn act as templates for transcription of new greater-than-unit-length RNAs, which can be processed by a unique ribozyme, and then ligated to form new genomic RNAs (Steps 6–8). Adapted from Taylor [60], copyright 2006, with permission from Elsevier

Pathology in Patients

Initial infections with HDV can be considered as either coinfections with HBV or superinfections of patients already infected with HBV. Both types can produce an acute HDV infection within several weeks with a significant chance of proceeding to fulminating hepatitis.

If the infection is not life threatening and continues for at least 6 months, it is defined as being chronic. In the case of a coinfection, such chronicity for HDV can only be achieved if the HBV infection also becomes chronic; the chance for this is less than 10% in an adult. In contrast, for a superinfection the HBV is already chronic, and so the likelihood of the HDV also becoming chronic is very high, maybe >85%. It has been reported that relative to HBV alone, such superinfections increase the risk of fulminant hepatitis and cirrhosis by tenfold [15, 16].

As a test for infection, detection of the viral RNA in serum is maybe the most sensitive, since one can use PCR procedures, and with an appropriate standard, determine the number of genome equivalents per ml. However, in the absence of this assay, δAg can be measured by an immune assay. The presence in serum of antibody specific for δAg is less reliable, since even in a chronic infection the presence of antibody is low and variable. A liver biopsy for diagnosis of HDV infection is usually unnecessary.

As reviewed elsewhere [4], infection by HDV is limited to the liver, and the histological changes, which can include necrosis and inflammation, are not different from what is detected for other forms of viral hepatitis, except that for HDV, they may be more severe.

While HDV was first discovered in 1977, subsequent retrospective studies of hepatitis cases in the Amazon basin of South America revealed HDV infections that were much more damaging and associated with fulminant hepatitis [17, 18]. In such cases it has not yet been settled as to what contributes to such damage: the HDV clade, the associated HBV genotype, or the immune reactivity of the patients in which the infection was initiated [14].

No mRNA expression data or proteomic analysis has yet been reported for patients infected with HDV. Such studies could have difficulty in resolving changes due to HBV alone relative to those corresponding to HDV plus HBV or to HDV only. Furthermore, changes in cellular composition within the liver, such as due to cirrhosis or presence of inflammatory cells, could be the cause of the mRNA or proteome changes.

Pathology in Experimental Animals

Much more is known about the initiation of infections by hepadnaviruses than for HDV. The initiation of hepadnavirus infection of the liver can be very efficient. Just one genome equivalent of duck HBV is sufficient to establish infection in a duckling [19]. And, it is known that the virus spreads rapidly with a doubling time of 16 h and has reached >95% of hepatocytes by 14 days. This is indicative of no significant antiviral response. Recent detailed studies show something similar for HBV injected into a chimpanzee [20]. These studies show that the amount of inoculum can determine whether the infection will go chronic or be resolved by an adaptive immune response. That is, there is a relationship between the kinetics of viral spread and CD4 T cell priming, and this can determine the outcome of the infection.

Part of the initial studies to help define HDV involved experimental infection of chimpanzees. In 1984 it was first shown that HDV could be transmitted to the eastern woodchuck using not HBV as the helper virus, but woodchuck hepatitis B virus (WHV) [21]. Chronic WHV infections lead to hepatocellular carcinoma (HCC) in woodchucks within only 1–4 years, as compared to 10–30 years for HBV in humans. However, it is possible to superinfect WHV carriers and observe after several weeks a major acute HDV infection, which typically progresses to chronic infection.

In mice made transgenic for the expression of either form of the δAg, no particular cytopathic effects have been observed [22]. Even animals made transgenic for the expression of HDV genome replication show no effects [23]. However, it must be noted that in such situations the helper virus is not present, so that there is neither assembly of HDV nor spread by infection.

If mice are injected with HDV-containing serum, either i.p. or i.v., it is possible to detect a low level of HDV replication in liver hepatocytes [24]. Again, there is no spread of infection, and within 15 days most of the infected cells are no longer present. The rate of disappearance is comparable in SCID mice, indicating that clearance is not mediated by an adaptive immune response.

Several approaches have been developed for the engraftment of human hepatocytes into mice. With such systems, it has been possible to achieve HDV infection and HBV-mediated spread; however, no studies of associated pathogenesis have been reported [25, 26].

The titers of HBV and HDV in the blood of an infected patient or experimental animal can vary greatly and also in relation to each other. In a woodchuck the HDV titer can reach 10E11 [11] particles/ml of serum. Not surprisingly, these absolute and relative values can impact on the consequences of an infection, whether natural or experimental. If HDV reached a hepatocyte in the absence of HBV helper, there can be replication but no assembly and release. Whether such an infection can be considered as "latent" and subsequently become productive due to HBV superinfection is still controversial [27, 28].

Again, mRNA array data and proteomic studies have not yet been reported for HDV infections. Extensive studies of

experimental HBV infections of chimpanzees have been reported [29–31]. A conclusion from such studies is that HBV, unlike HCV, in the first weeks of an infection does not induce an innate response. HBV is thus referred to as a "stealth" virus, able to get under the radar [32]. At later times, damage arises in the liver in parallel with the appearance of an adaptive immune response. For experimental infections of chimpanzees with both HBV and HDV array, data have been interpreted as evidence for an early activation of the innate response (personal communication from Robert Purcell et al.).

Pathogenesis in Cultured Cells

There has been little success in studying pathogenesis of cultured cells infected with HDV and HBV. Several labs have used cell culture to assemble HDV in the absence of infectious HBV and study infection. However, as mentioned earlier, HDV will only infect hepatocytes. Primary cultures of human and other primate hepatocytes, and woodchuck hepatocytes, can be set up and are susceptible, although infection typically involves <20% of cells [33–35]. The only other known susceptible cells are hepRG cells, which are derived from the liver of an HCV-infected patient and can, after several weeks in culture under defined conditions, demonstrate susceptibility that is comparable to primary human hepatocytes [36].

To date, no one has reported detecting pathogenic effects of HDV infection of cultured human hepatocytes. The HDV studies have been limited to the use of cell lines and the expression and/or replication as induced by procedures other than infection with virus.

As mentioned in the previous section, mice transgenic for expression in the liver of either δAg-S or δAg-L show no obvious effects. In cell culture, expression of δAg can have an effect. In an avian cell line it can cause apoptosis [37] and in mammalian cells it can interfere with outgrowth of the cells to form colonies [38].

In early experiments HDV cDNAs were used to transiently transfect the liver cell line, Huh7, and initiated HDV genome replication. By immunostaining for δAg, it was possible to demonstrate small colonies of cells in which the replication was maintained; that is, several cell divisions were possible following HDV replication [39]. More recently, two studies by Mota et al. have made use of Huh7 cells that had been transfected with HDV cDNA and then selected for stable expression [40, 41]. The accumulation of as many as 24 host proteins was changed at least twofold in these cells relative to untransfected Huh7 cells. While some of these changes were considered as consistent with overall deregulation of RNA transcription in the transfected cells, the fact is that the selected cells continued to replicate.

In another system, monolayer cultures of human 293 cells, derived from embryonic kidney, were transfected to express δAg-S under tetracycline inducible conditions [42]. Within 20 h, these cells accumulated more than a million copies of δAg-S and yet there were no obvious cytopathic effects (although there was some effect in that after several weeks of such induction, the cell population no longer expressed δAg). If these same cells were allowed to replicate an RNA copy of the HDV genome, the 20 h induction increased not only the δAg-S, but also the accumulation of maybe 10,000 copies per cell of the HDV genome and somewhat less of the antigenome. In this situation the cells went into G1/G0 cell cycle arrest within 48 h and by 72 h they detached from the monolayer [42].

When whole genome mRNA analysis was applied to the two inducible replication systems, it was found that δAg-S alone caused at least twofold changes in the accumulation of only 94 host mRNAs. In contrast, when HDV RNA accumulation was allowed, there was within 20 h changes in 956 host mRNA. Most of these changes were increases in expression rather than decreases (Fig. 39.3 and unpublished data of Taylor et al.). These studies demonstrate that induced changes can be mainly associated with HDV RNA accumulation, rather than just the accumulation of δAg.

It thus seems that for HDV, and maybe many other RNA virus infections, pathogenic effects are more associated with RNA accumulation than with viral proteins. And, as major precedents for this assertion, the plant viroids, as next discussed, are pathogenic RNAs with no protein coding capacity.

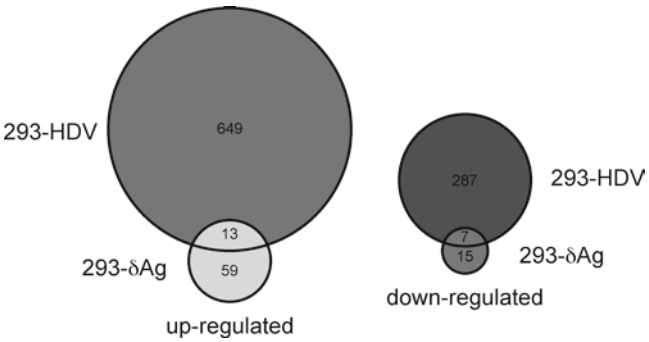

Fig. 39.3 Host mRNAs whose mRNA levels were changed due to HDV expression. As described elsewhere, 293-δAg is 293T cell line that expresses δAg under TET control. 293-HDV was created from this by transfection of a genomic HDV RNA [42]. Agilent whole genome array analysis was performed on both cell lines at 20 h for induced relative to uninduced cells. Quantitation was made of mRNA levels changed at least twofold and with a >95% confidence, based on assays of four pairs of individual experimental samples. Note that the induced 293-HDV cells had 10-times more changes than 293-δAg, which expressed only the δAg. In both situations, there were more mRNAs upregulated than downregulated (unpublished observations of Taylor et al.)

Analogy to Plant Viroids

As reviewed elsewhere, HDV genome structure and replication have many similarities to the plant viroids [43, 44]. These are small noncoding single-stranded RNAs of 250–400 nt in length. Like HDV, they depend on redirecting one or more host RNA polymerases to achieve RNA-directed RNA transcription [44–46]. In further similarity to HDV, some of these viroid RNA transcripts have much rod-like folding, are processed by ribozymes, and can be ligated to achieve a circular conformation.

In addition, and of particular relevance here, is that there are many families of such plant viroids and they all have been recognized by their obvious cytopathic and/or developmental effects on the host. As recently reviewed by Tsagris, there are many and varied ways in which viroids achieve such effects, but they are all linked to the replication and accumulation of the viroid RNAs [44]. For example, several studies show that small interfering RNAs (siRNA) can be cleaved from viroid RNA. One study reports that such siRNA can target specific host mRNAs and thus create a pathogenic response [47]. There are also studies indicating specific host proteins, such as VirP, that need to bind a viroid RNA to support the accumulation and spread of that RNA [48].

At its simplest level, it is the accumulation of viroid RNAs that leads to disruption of the homeostasis of the host cell, producing pathogenic and/or developmental problems. This viroid precedent and the HDV data presented in Fig. 39.1 support the interpretation that the major impact of HDV pathogenesis is mediated in one or probably many complex ways, but always as a consequence of the accumulation of the viral RNA.

Prevention

Without question, the application of the HBV vaccine also prevents HDV infection [2, 49, 50]. The impact of HBV vaccination has already been noted in Italy, where the incidence of HDV is decreasing faster than that of HBV. HDV has thus been referred to as a vanishing disease [50]. However, in certain other areas of the world among nonvaccinated individuals, and especially among intravenous drug users, HDV infections are actually increasing [51].

Remember that approximately 400 million HBV carriers worldwide are vulnerable to HDV superinfection. With this in mind, several attempts have been made to make anti-δAg vaccines. However, tests of this strategy in woodchucks have failed to demonstrate protection against HDV [52].

Treatment

Since HDV is entirely dependent upon host enzymes, especially RNA polymerase activity, this might leave little, if any, scope for antivirals. However, the following three experimental treatments have been considered.

The large δAg, δAg-L, is known to require posttranslational modification by farnesylation before it can support the assembly of the HDV genome into particles with the envelope proteins of the helper virus HBV. Glenn, who discovered this modification and its importance [53], has further shown that farnesyl transferase inhibitors will block HDV assembly both in cultured cells and in mice into which these have been transplanted with human hepatocytes [26]. There is thus the possibility that such inhibitors might help suppress HDV in humans. The inhibitors were actually developed to block the farnesylation of host proteins such as the oncogene *ras,* a kinase activated in many human tumors.

The other approach has been to use relatively high doses of alpha-interferons. In a small number of cases, such treatments over a period of months have led to clearance of HDV [54]. It is not clear to what extent such treatments act on the HDV replication vs. that of the helper HBV. Certainly, studies with cultured hepatocytes or cell lines have failed to detect any effect of interferons on HDV RNA accumulation [55, 56].

A third and more drastic treatment approach, driven by life-threatening compromise in liver function, is liver transplantation. This needs to be in association with anti-HBV treatment to reduce the chance of disease recurrence [57, 58].

Summary and Outlook

Contrary to an earlier observation of HDV infections becoming less frequent in Italy, there are other sites where infections are on the rise. The infection is certainly one that can be prevented by prior HBV vaccination, although treatment of chronic infections is problematic. Continued study of HDV has additional value that is nonclinical. HDV replication is an intriguing phenomenon in itself, most especially because of the ability of its small RNA to divert host machinery and achieve efficient RNA-directed synthesis of new RNA species. Studies of the molecular pathogenesis of HDV will be aided by the precedents of pathogenesis induced by plant viroids, which have many similarities to HDV. All infections, not just those by HDV and viroids, are by definition events that at least transiently perturb the homeostasis of the host cell. For many reasons, this perturbation can increase with time after infection, with the increased expression of novel proteins and nucleic acids. However, for HDV there is only one protein and the data seem to indicate that expression of

this protein, even up to millions of copies per cell, is only modestly deleterious. In contrast, the process of transcription and/or the increased accumulation of HDV RNA can be deleterious within 48 h. In the case of the plant viroids, it is this RNA transcription and/or accumulation that create the deleterious effects. While the transcription process for HDV and viroids unquestionably involves the redirection of one or more host RNA polymerase activities, it would seem at this point that much of the deleterious perturbing effects can be ascribed to the processing and accumulation of RNA species. This could be by multiple mechanisms and it is maybe naïve to presume that a single predominant mechanism can be ascribed. For example, many of the numerous host RNA-binding proteins might be diverted by the presence of more than 300,000 copies per cell of the HDV genomic RNA. The concept of such diversion has already been demonstrated by elegant experiments in which cells are subjected to the expression of multiple copies of small structured RNAs, such as the human Alu RNA or the mouse B2 RNAs [28, 59]. Such RNAs divert specific host proteins and create a transcriptional response almost identical to that observed during a heat shock response, which after all is another form of perturbation of the homeostasis of the host cell.

Acknowledgments The author was supported by grants AI-26522 and CA-06927 from the NIH and by an appropriation from the Commonwealth of Pennsylvania. Certain unpublished studies cited were performed in collaboration with Ziying Han, Severin Gudima, Suresh Peri, Michael Slifker, and Yue-Sheng Lee. William Mason, W. Thomas London, and Richard Katz gave constructive comments on the chapter.

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Chapter 40
Viral Hepatitis E

Shiv K. Sarin and Manoj Kumar

Introduction

Hepatitis E virus (HEV) is an enterically transmitted (other routes of transmission may exist) RNA virus that causes an acute, self-limiting hepatitis in immunecompetent subjects, but may also cause chronic infection in immunesuppressed subjects. Infection with HEV may be asymptomatic or may cause hepatitis varying in degree of severity from mild to fulminant disease. Fulminant hepatitis E has been reported with increased frequency in pregnant women.

History

Serologic studies of water-borne epidemics of acute hepatitis in India in the late 1970s provided evidence for an enterically transmitted virus different from the hepatitis A virus (HAV), where it was demonstrated that patients involved in such epidemics of hepatitis in the Kashmir region and in Delhi, India, lacked serologic evidence of recent HAV infection and only showed evidence of past infection [1]. Balayan et al. in 1983 provided the first proof of the existence of a newly identified form of acute viral hepatitis by transmitting hepatitis to a volunteer from a patient involved in an outbreak of enterically transmitted non-A, non-B hepatitis in central Asia [2]. The volunteer, who had preexisting antibody to HAV, developed a severe hepatitis, shed 27–30-nm virus-like particles in his feces detected by immune electron microscopy (IEM), and developed antibodies to the virus-like particles during convalescence. The researchers also inoculated cynomolgus monkeys with the new virus; again, the monkeys developed hepatitis, shed virus-like particles, and developed an immune response to the particles. In 1990, Reyes et al. cloned and sequenced a part of the genome of the virus [3]. The new form of non-A, non-B hepatitis came to be known as epidemic non-A, non-B hepatitis or enterically transmitted non-A, non-B hepatitis (ET-NANBH), and later, the name of the disease was changed to hepatitis E.

S.K. Sarin (✉)
Department of Gastroenterology, G.B. Pant Hospital, New Delhi, India
and
Institute of Liver and Biliary Sciences, New Delhi, India
e-mail: shivsarin@gmail.com

Virology

Classification

HEV was originally classified in the *Caliciviridae* family because of its structural similarity to other caliciviruses; however, it is now the sole member of the *Hepeviridae* family [4].

Structure

Physiochemical Characteristics

HEV is a spherical, nonenveloped particle that is approximately 27–34 nm in diameter and has a icosahedral symmetry. The buoyant density of HEV is 1.35–1.40 g/cm^3 in CsCl with sedimentation coefficient of 183 S [5]. The virus is relatively stable to environmental and chemical agents. In a recent study comparing thermal stability of virulent HEV and HAV, HEV was found to be less stable than was HAV, although some HEV would most likely survive the internal temperatures of rare-cooked meat [6].

Morphology

HEV has an indefinite surface structure that is intermediate between that of the Norwalk agent (a member of the Caliciviridae family) and that of HAV (a member of the Picornaviridae family). HEV contains an RNA genome enclosed within a capsid. The viral capsid protein is encoded by ORF2 near the 3′ end. The ORF2 capsid protein contains a total of 660 amino acid residues. The viral capsid protein induces

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_40, © Springer Science+Business Media, LLC 2011

neutralizing antibodies by its immunization or during the course of infection. A typical signal sequence at the N terminus and three potential *N*-glycosylation sites (Asn-X-Ser/Thr) are well conserved in the capsid protein derived from all mammalian genotypes. The receptor-binding site has been mapped to the second half of the polypeptide chain [7]. As an alternative to in vitro propagation of HEV, the baculovirus expression system opens the prospect of studying HEV capsid assembly, since HEV-like particles (HEV-LP) with protruding spikes on the surface can be formed in insect cells infected with a recombinant baculovirus expressing the capsid protein of a genotype 1 strain [8]. Cryo-electron microscopic (cryoEM) analysis has revealed that HEV-LP is a $T=1$ icosahedral particle composed of 60 copies of truncated products of ORF2 [8]. HEV-LP appeared to be empty due to a lack of significant density containing RNA inside [8]. The HEV-LP which displays $T=1$ symmetry with a diameter of 270 Å is smaller than the native HEV particle, which displays $T=3$ symmetry with an estimated diameter of 350–400 Å [9]. The surface of the HEV-LP is dominated by 30 dimeric protrusions, and each capsid subunit appears to have two domains [9]. Recently, the crystal structure of HEV-LP determined to 3.5-Å resolution has been reported. Each HEV capsid protein contains three linear domains, S (118–313), P1 (314–453), and P2 (454–end), the final two of which are linked by a long, flexible hinge linker. The S domain forms a continuous capsid shell that is reinforced by threefold protrusions formed by P1 and twofold spikes formed by P2. It adopts the jelly-roll β-barrel fold that is most closely related to plant $T=3$ viruses. P1 and P2 contain compact, 6-stranded β-barrels that resemble the β-barrel domain of phage sialidase and the receptor-binding domain of calicivirus, respectively, both of which are capable of polysaccharide binding. The highly exposed P2 domain likely plays an important role in antigenicity determination and virus neutralization. Structural modeling shows that the assembly of the native $T=3$ capsid requires flat capsid protein dimers with less curvatures than those found in the $T=1$ VLP, suggesting that additional N terminal sequences may be involved in particle size regulation [10]. However, the HEV-LP retained the antigenicity and capsid formation of the native HEV particles and is therefore a promising candidate for use in vaccine development.

Genome Organization

Genome and Proteins

Its genome consists of a single-stranded, positive-sense RNA of approximately 7.3 kb in length. It contains a short 5′ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and a short 3′ UTR that is terminated by a poly(A) tract [11]. The genome is organized as 5′-ORF1–ORF3–ORF2–3′, with ORF3 and ORF2 largely overlapping (Fig. 40.1). Although a single serotype has been proposed, extensive genomic diversity has been observed among HEV isolates.

The 5′ and 3′ UTRs are highly conserved and are likely to play roles in RNA replication and encapsidation. The 5′ end of the genome has a 7-methylguanosine cap.

ORF1, the largest ORF, begins at the 5′ end of the viral genome after a 27-bp noncoding sequence and extends 5,079 bp to the 3′ end (in the Burmese prototype strain) and encodes about 1,693 amino acids encompassing nonstructural, enzymatically active proteins probably involved in viral replication and protein processing. Based on the identification of characteristic amino acid motifs, the following genetic elements have been identified, in order, from the 5′ to the 3′ end of the ORF: (1) a methyl transferase, presumably involved in capping the 5′ end of the viral genome; (2) the "Y" domain, a sequence of unknown function that is found in certain other viruses, including rubella virus; (3) a papain-like

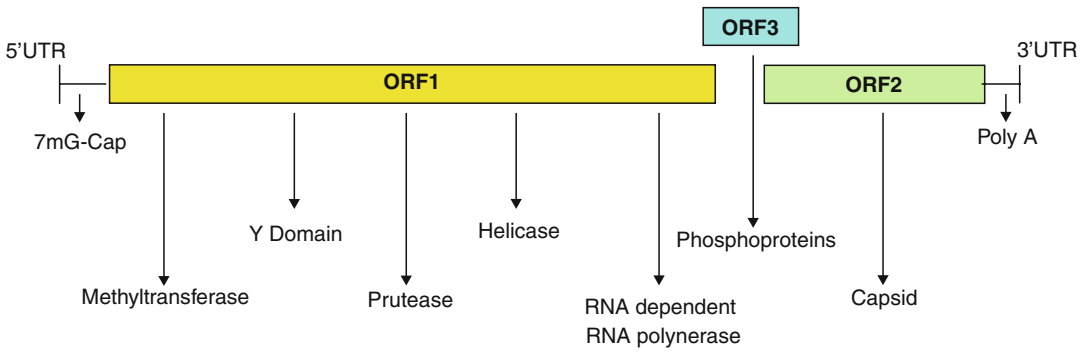

Fig. 40.1 Genome organization and proteins of HEV. The positive-strand RNA genome of HEV is capped at the 5′ end and polyadenylated at the 3′ end. It contains short stretches of untranslated regions (UTR) at both ends. There are three open reading frames (ORFs). ORF1 encodes the nonstructural polyprotein (nsp) that contains various functional units – methyltransferase, papain-like cysteine protease, RNA helicase, and RNA-dependent RNA polymerase. ORF2 encodes the viral capsid protein. ORF3 encodes a small regulatory phosphoprotein

cysteine protease, a type of protease found predominantly in alphaviruses and rubella virus ; (4) a proline-rich "hinge" that may provide flexibility and that contains a region of hypervariable sequence; (5) an "X" domain of unknown function that has been found adjacent to papain-like protease domains in the polyproteins of other positive-strand RNA viruses; (6) a domain containing helicase-like motifs similar to those found in viruses containing type I (superfamily 3) helicases; and (7) an RNA-dependent polymerase, with motifs most closely related to those found in viruses containing an RNA polymerase of superfamily 3 [12]. In vitro expression of the HEV ORF1 produced a polyprotein that was processed into two products following extended incubation [13]. When expressed in insect cells, ORF1 was processed and this was partially blocked by a cell permeable cysteine protease inhibitor [14]. The presence of methyltransferase motifs in ORF1 suggested HEV to have a capped RNA genome. A 5′-methylguanosine residue in the HEV genome is essential for infectivity and replication. The GDD motif in RdRp is also important for HEV replication. Two predicted stem-loop (SL) structures at the 3′ NCR and the polyA tract were necessary for RdRp binding during HEV genome replication [15]. Except for the methyltransferase [16], none of the other putative components of ORF1 have been expressed, purified, and biochemically characterized.

ORF2, approximately 2,000 nucleotides in length, begins approximately 40 nucleotides after the termination of ORF1 and consists of a 5′ signal sequence, a 300-nucleotide region rich in codons for arginine, probably representing an RNA-binding site, and three potential glycosylation sites. ORF2 encodes a 660-amino-acid protein, most likely representing one or more structural or capsid protein(s) of HEV. The pORF2 is an 80-kDa glycoprotein with a potential endoplasmic reticulum (ER) directing signal at its N-terminus (a region containing high concentrations of arginine and lysine). The ORF2 protein enters the ER, but a fraction retrotranslocates to the cytoplasm to trigger a stress pathway [17]. When pORF2 is expressed in mammalian cells, a large proportion of the nascent protein is modified by N-glycosylation. Mutations in the pORF2 glycosylation sites prevented the formation of infectious virus particles and had low infectivity in macaques [18]. When pORF2 is expressed in insect cells, it is cleaved at a site between amino acids 111 and 112 and at various other sites within the C-terminus of the protein. Some of these truncated forms of the pORF2 have the ability to self-assemble into HEV-LPs or subviral particles. The structure of a self-assembled HEV-LP was solved by cryo-electron microscopy and showed the capsid to be dominated by dimmers. This dimerization property may not be amino-acid sequence-dependent, but instead is a complex formation of a specific tertiary structure that imparts to pORF2 its property to self-associate (see above). The ORF2 protein also contains RNA-binding activity and specifically binds to the 5′ end of the HEV genome. A 76-nucleotode region at the 5′ end of the HEV genome was responsible for binding the ORF2 protein. This interaction may be responsible for bringing the genomic RNA into the capsid during assembly, thus playing a role in viral encapsidation [19].

ORF3, <400 nucleotides in length, overlaps ORF1 by one nucleotide at its 5′ end and overlaps ORF2 by over 300 nucleotides at its 3′ end. It codes for a 123-amino-acid, 13.5-kDa non-glycosylated protein (pORF3), which is a very basic protein (pI 12.5), and is the most variable protein among the HEV strains. Recently, it was proposed to be translated from a bicistronic subgenomic RNA and to be nine amino acids shorter at its *N*-terminus [20]. While ORF3 was dispensable for replication in vitro [21], it is required for infection in monkeys inoculated with HEV genomic RNA [22]. Expression of pORF3 in mammalian cells showed it to interact with various cellular proteins. It colocalizes with the cytoskeleton and binds a MAP kinase phosphatase [23]. It also interacts with hemopexin, an acute-phase plasma glycoprotein [24]. The P1 region contains the phosphorylated serine residue that is conserved in all HEV strains, except the Mexican isolate, and the P2 region contains a motif that binds several proteins containing src-homology 3 (SH3) domains [25]. ORF3 protein of HEV also interacts with the Bβ chain of fibrinogen resulting in decreased fibrinogen secretion [26]. The ORF3 protein is likely to regulate the host cell environment through its interaction with various intracellular pathways (Fig. 40.2) [4]. It activates the extracellularly regulated kinase (ERK) by binding and inhibiting its cognate phosphatase [23]. Prolonged activation of ERK would generate a survival and proliferative signal (Fig. 40.2a). Higher levels of hexokinase and oligomeric voltage-dependent anion channel (VDAC) were found in ORF3-expressing cells, which displayed attenuated mitochondrial death signaling [27]. Recently, ORF3 protein has been shown to interact with microtubules and interferes with their dynamics which might be needed for establishment of an HEV infection [28]. The ORF3 protein might act as an adaptor to link intracellular transduction pathways and this might promote HEV replication and assembly. pORF3 localized to early and recycling endosomes and delayed postinternalization trafficking of epidermal growth factor receptor (EGFR). This is likely to prolong endomembrane signaling and promote cell survival [4] (Fig. 40.2). Another effect of this is reduced nuclear translocation of pSTAT3 and attenuation of the acute-phase response. Thus, pORF3 might reduce the host inflammatory response, further creating an environment favorable for viral replication (Fig. 40.2). The α-1-microglobulin and bikunin precursor protein (AMBP) and its constituents α1-microglobulin and bikunin were also identified as pORF3 binding partners [29]. There was increased secretion of α1-microglobulin from ORF3-expressing cells [30]. Since α1-microglobulin is immunosuppressive, this is proposed to

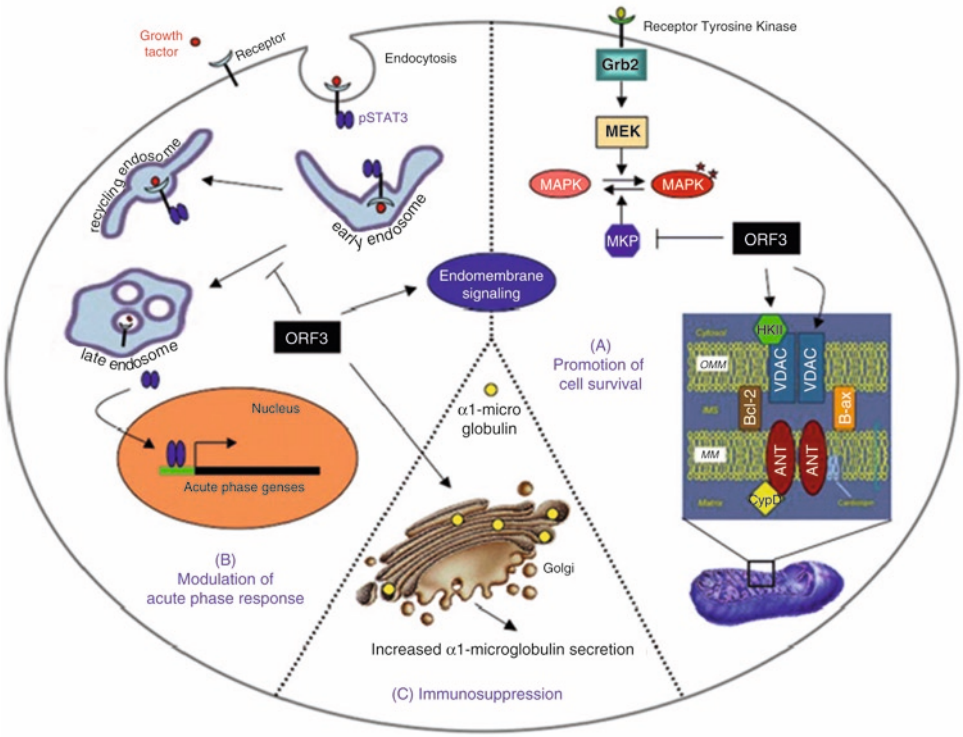

Fig. 40.2 Role of the ORF3 protein in HEV pathogenesis. Three broad functions for the ORF3 protein have been proposed. (**a**) Promotion of cell survival. The ORF3 protein activates MAP kinase by binding and inactivating its cognate phosphatase (MKP). Additionally, it upregulates and promotes homooligomerization of the outer mitochondrial membrane porin, VDAC, and increases hexokinase levels, thus reducing mitochondrial depolarization and inhibiting intrinsic cell death. (**b**) Modulation of the acute-phase response. The ORF3 protein localizes to early and recycling endosomes and inhibits the movement of activated growth factor receptors to late endosomes. This prolongs endomembrane growth factor signaling and contributes to cell survival. Through this mechanism, pORF3 also reduces the nuclear transport of pSTAT3, a critical transcription factor for the expression of acute phase response genes. (**c**) Immunosuppression. The ORF3 protein promotes the secretion of α1-microglobulin, an immunosuppressive protein that could act in the immediate vicinity of the infected cell. From Chandra et al. [4]. Used with permission

protect virus-infected cells (Fig. 40.2c). Recently, it has been shown that HEV ORF3 protein stabilizes HIF-1α and enhances HIF-1-mediated transcriptional activity through p300/CBP, which leads to increased activity of glycolytic pathway, thus promoting survival of the host cell [31].

Two broad roles are thus predicted for pORF3 in HEV pathogenesis (Fig. 40.2) [4]. The first is promotion of cell survival through ERK activation, prolonged endomembrane signaling, and attenuation of the intrinsic death pathway (Fig. 40.2). The second is to downregulate innate host responses through reduced expression of acute-phase proteins and increased secretion of α1-microglobulin (Fig. 40.2) [4]. Recent study has shown that ORF3 protein is responsible for virion egress from infected cells and is present on the surface of released HEV particles, which may be associated with lipids [32].

Replication Cycle

Little is known about the cellular receptors for HEV or its entry process. A recent study showed that a truncated peptide p239 spanning aa 368–606 of pORF2 formed 23 nm particles that bind and penetrate HepG2, Huh-7, PLC/PRF5, and A549 cells [33] and prevent further infection of these cells. The cell surface molecules that bind HEV or its capsid protein are not known. A model for HEV replication and gene expression was proposed based on similarities and sequence homology to better characterized positive-strand RNA viruses [4]. This is shown in Fig. 40.3. Following entry into a permissive cell (Fig. 40.3a), the viral genomic RNA is uncoated (Fig. 40.3b) and translated in the cytosol of infected cells to produce the ORF1-encoded nonstructural polyprotein (nsP) (Fig. 40.3c). Cleavage of the ORF1 nsP is achieved by cellular proteases, possibly with help from the viral PCP. The viral replicase (RdRP) replicates the genomic positive strand into the negative strand replicative intermediates (Fig. 40.3d1). ER has been identified as the site of replicase localization and possible site of replication [34]. These serve as template for the synthesis of additional copies of the genomic positive strands as well as subgenomic positive strands (Fig. 40.3d2). This is akin to alphaviruses and a region homologous to alphavirus junction sequences is

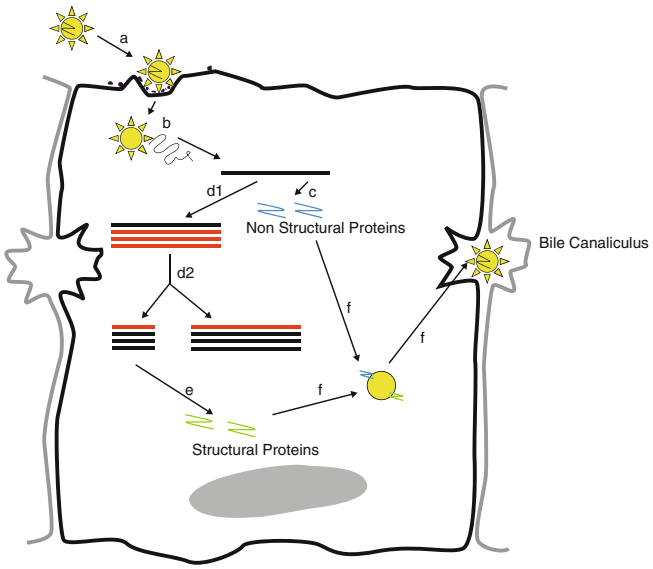

Fig. 40.3 Replication cycle of HEV. (**a**) The virus enters the hepatocyte via a cellular receptor, the identity of which remains uncertain. (**b**) This is followed by uncoating of the viral particle and release of the positive-sense RNA genome into the cell. (**c**) The genomic RNA is translated in the cytoplasm into nonstructural proteins. (**d**) The replicase thus synthesized replicates the positive strand genomic RNA into negative strand RNA intermediates (**d1**) and reverse (**d2**). (**e**) The positive strand subgenomic RNA is also synthesized that is translated into structural proteins. (**f**) The capsid protein packages the genomic RNA to assemble new virions. Newly assembled HEV particles are secreted by the cell across the apical membrane of the hepatocyte into the biliary canaliculus, from which they are passed into the bile and small intestine

proposed to serve as the subgenomic promoter. The subgenomic RNA can then be translated into the structural protein(s) (Fig. 40.3e). The capsid proteins package the viral genome to assemble progeny virions (Fig. 40.3f) that exit the cell through an undefined pathway. Direct experimental confirmation of this replication scheme is still awaited, but several findings support this model [4]. ln experimentally infected rhesus monkeys and pigs, HEV-positive and negative strand RNAs are observed in the liver. Since in vitro transcripts of full-length cDNA clones are infectious for nonhuman primates and pigs, the subgenomic RNAs are not required to initiate an infection and must be synthesized as part of the replication process. Replicons have shown mixed results with respect to detection of negative-stranded replicative intermediates [4, 35].

Genomic Variability of HEV

Although several classification schemes have been proposed, the most accepted scheme classifies HEV isolates into four major genotypes (1–4) [36]. HEV was discovered by IEM in 1983 [2]. Eight years after its discovery, the full genomic sequence of HEV was first determined for a strain from Myanmar (formerly Burma) [37], which had >88.2% nucleotide identity across the entire genome to isolates obtained from other developing countries in Asia (including China, India, Nepal, and Pakistan) and those in Africa (including Chad and Morocco). In 1992, a Mexican strain that was implicated in an outbreak that occurred in Mexico in 1986 was reported [38]. The Mexican strain is distinct from the Burmese variants and constitutes a second genotype. In 1997, an HEV isolate from a patient with sporadic acute hepatitis E in the United States who had no history of travel abroad was reported [39], and it constituted a third genotype, which was subsequently found to be widely distributed throughout the world [40]. In 1999, HEV isolates recovered from Chinese patients with acute hepatitis that were distinct from the original Chinese isolates of genotype 1 were reported and they constitute a fourth group [41]. HEV isolates classifiable into the fourth group have also been identified from sporadic cases of HEV infection, not only in China but also in Taiwan and Japan [40]. HEV genotypes are further classified into subtypes: genotype 1 into five subtypes (1a–e); genotype 2 into two subtypes (2a, b); genotype 3 into ten subtypes (3a–j); and genotype 4 into seven subtypes (4a–g) [40].

Distribution of HEV Genotypes

Genotype 1 is distributed in various countries including Bangladesh, Cambodia, China, India, Kyrgyzstan, Myanmar, Nepal, Pakistan, Uzbekistan, and Vietnam in Asia and Algeria, the Central African Republic (CAR), Chad, Djibouti, Morocco, Sudan, Tunisia, Namibia, Egypt, and South Africa in Africa. HEVs that are commonly found in Asia and Africa have been classified as the Asian and African subgenotypes of genotype 1, respectively [40]. Genotype 2 has been represented by the prototype sequence from an epidemic in Mexico [38] and new variants were recently identified from endemic cases in African countries including CAR, Chad, Democratic Republic of the Congo (DRC), Egypt, Namibia, and Nigeria [40]. HEVs of genotypes 1 and 2 have caused epidemics and outbreaks of hepatitis E in tropical and some subtropical regions usually due to transmission by fecal contamination of water supplies [40]. In contrast, HEVs of genotypes 3 and 4 were found in sporadic acute hepatitis E cases in the United States, European countries, China, and Japan, and these cases were most likely zoonotic in origin [40]. Genotype 3 accounts for the largest number of isolates among all HEV sequences archived in the GenBank/EMBL/DDBJ databases, and many of them were identified in the United States or Japan [40]. However, genotype 3 HEV is widely distributed and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in a many countries including Argentina, Australia, Austria, Cambodia, Canada,

France, Germany, Greece, Hungary, Italy, Japan, Korea, Kyrgyzstan, Mexico, the Netherlands, New Zealand, Russia, Spain, Taiwan, Thailand, the United Kingdom, and the United States. On the contrary, genotype 4 is restricted to Asian countries and contains strains from humans and/or domestic pigs in China, India, Indonesia, Japan, Taiwan, and Vietnam. Among 38 countries where HEV strains have been isolated from infected patients, HEVs of a single genotype were isolated from infected patients in 29 countries (genotype 1 in 12 countries, genotype 2 in 3 countries, genotype 3 in 12 countries, and genotype 4 in 1 country) and HEVs of two distinct genotypes were isolated from infected patients in eight countries (genotypes 1 and 2 in 4 countries, genotypes 1 and 3 in 2 countries, and genotypes 1 and 4 in 2 countries). Japan is unique in that three distinct genotypes (1, 3, and 4) of HEV strains have been identified in infected patients, although genotype 1 HEV is most likely imported [40].

Thus, Genotype 1 consists of epidemic strains in developing countries in Asia and Africa; genotype 2 has been described in Mexico and Africa; genotype 3 HEV is widely distributed and has been isolated from sporadic cases of acute hepatitis E and/or domestic pigs in many countries in the world, except for countries in Africa; and genotype 4 contains strains from humans and/or domestic pigs exclusively in Asian countries [40].

Quasispecies Nature and Evolution of HEV

A high degree of conservation of the amino acid sequence of the capsid protein among distinct genotypes is observed, which correlates with the little antigenic diversity; thus, there is only a single serotype of HEV. However, despite this limited amino acid heterogeneity, a significant degree of nucleic acid variability has been observed among different isolates from different regions of the world [40]. The molecular basis of this genetic variability may be the high error rate of the viral RNA-dependent RNA polymerase and the absence of proofreading mechanisms. Based on the assumption that JKK-Sap00 (isolation date: 10 November 2000), JYW-Sap02 (30 August 2002), and JTS-Sap02 (14 September 2002) are descendants of JSM-Sap95 (28 March 1995), all of which were isolated in Hokkaido and differed from each other by 0.056–1.050%, the mutation rate of HEV has been estimated to be $1.40–1.72 \times 10^{-3}$ base substitution per site per year [42].

Quasispecies have mainly been described in persistent virus infections such as those due to human immunodeficiency virus (HIV) type 1 and hepatitis C virus (HCV) during which virus populations develop a high degree of sequence variation within each infected individual. They are less common in viruses causing acute self-limited infections, such as dengue virus and HAV [40]. HEV epidemics are mainly caused by a common source of contamination, usually drinking water resources. Although the spread of HEV among humans is assumed to be clonal according to a "one outbreak, one strain" scheme, the quasispecies nature of epidemic HEV was demonstrated in a retrospective analysis of both inter- and intrapatient diversity using 23 serum samples collected during a water-borne outbreak that occurred in 1986–1987 in Algeria [43]. However, the extent of the sequence variation of HEV in vivo and its relationship to disease severity remain unknown. The reason why HEV strains of genotypes 1 and 2 have less genomic variability than HEV strains of genotypes 3 and 4 remains to be elucidated. HEV strains of genotypes 1 and 2 often cause outbreaks or epidemics of hepatitis as a result of efficient transmission via the fecal–oral route, usually by contaminated water or food supply [44]. In contrast, HEV variants of genotypes 3 and 4 are predominantly maintained among animal species such as domestic pigs and only occasionally infect humans; this is most likely due to inefficient cross-species transmission of these variants. Maintenance of HEV strains of genotypes 3 and 4 among animal species would contribute to the long-term circulation of HEV in particular geographic regions and independent evolution of the virus in specific animal species. Therefore, differences in the degree of viral divergence among genotypes of HEV may reflect different transmission patterns [40].

To investigate the genetic changes in HEV strains in the community, Shretha et al. compared the 412-nt sequence within ORF2 of HEV among HEV isolates recovered from 48 patients in 1997, 16 patients in 1999, 14 patients in 2000, and 38 patients in 2002 in Kathmandu valley of Nepal [45]. All 116 HEV-viremic samples were typed as genotype 1, and further as subgenotype 1a ($n=85$, 73%), 1c ($n=29$, 25%), and mixed infection of 1a and 1c ($n=2$, 2%): subgenotype 1c was detected only in 1997. Genetic variability was observed among HEV strains and even among HEV strains of the same subtype (1a) obtained each year in the years of 1997, 1999, 2000, and 2002. When phylogenetic analysis of the 87 subtype 1a isolates was performed, they further segregated into five clusters, with two predominant clusters of 1a–2 and 1a–3: the annual frequency of cluster 1a–2 isolates decreased from 63% in 1997, 50% in 1999, 7% in 2000, and no cases in 2002; cluster 1a–3 isolates were observed in all 4 years and its annual frequency increased from 5% in 1997 to 95% in 2002. Of the remaining three clusters, cluster 1a–1 was detectable only in 1997 and clusters 1a–4 and 1a–5 emerged in 2000 and 2002, respectively. These results indicate that the genetic changes and takeover of HEV strains may contribute to the genetic variability of HEV in the community. The fact that no significant amino acid substitutions were recognized in the HEV strains isolated during a 5-year period suggests that genomic mutations of HEV may occur naturally in infected individuals without immunological pressure from the host, and that selective forces that do not allow amino

acid substitutions may be involved in the observed pattern of divergence. Taking into account that partial sequencing of a selected genomic region was employed, a definitive picture of the biological significance of these and other possible changes in the entire genome needs to be obtained from more in-depth studies [40].

Serotypes and Antigenicity

Despite the presence of genetically different isolates of HEV, there appears to be only one serotype. Antigenic variations have important implications for the serological detection of HEV infection. Antibody responses to individual viral antigens are highly variable, due to both strain-specific differences in some epitopes and differences in response to single antigens between individual patients. For example, pORF3 varies greatly between strains, and many experimentally infected animals and some patients fail to develop antibodies to ORF3 protein. This variable reactivity contributes to the poor sensitivity and concordance of HEV-diagnostic tests based on such antigens [40]. Conversely, all isolates of HEV share some important cross-reactive antigens. Immunization of nonhuman primates with recombinant pORF2 proteins conferred immunity to both homologous and heterologous challenge, suggesting that major protection epitopes are common among HEV genotypes.

Animal Models and In Vitro Culture

HEV transmission studies have mostly been done in nonhuman primates such as cynomolgus, rhesus and owl monkeys, and chimpanzees [46, 47]. These have provided important information regarding the biology and pathogenesis of HEV and are indispensable tools for vaccine and drug testing. Experimental transmission studies have also been done in pigs, an established reservoir for HEV [48]. Recently, Mongolian gerbils and Balb/c nude mice have been found to be a useful animal model for studying the pathogenesis of HEV [49, 50].

There has been only limited success in generating suitable tissue culture replication systems for HEV. Early studies reported propagation of HEV in 2BS [38], A549 [51], and FRhK [52] cells. Infection of primary cynomolgus hepatocytes and PLC/PRF/5 cells has been shown, but replication was inefficient [53]. Recently, HEV genotype 3 from a high titer stool suspension was successfully passaged for multiple generations in PLC/PRF/5 cells [54] and these cells were used to assess the infectivity of HEV shed in patients' stools [55]. The replication of HEV has been observed in cell lines transfected with transcripts of infectious cDNA clones and with a replicon derived from it [35]. Monkeys inoculated with culture media or lysates of HEV replicon-transfected cells developed infection, but viral titers were low. Some species barrier for HEV replication might exist since replicons did not function in nonprimate cell lines. However, sufficient amounts of viral particles cannot be obtained for studies of the structure, life cycle, and pathogenesis of HEV [40].

Epidemiology

Incidence and Prevalence and Worldwide Disease Patterns

Worldwide, two geographic patterns can be differentiated: (1) areas of high HEV prevalence (endemic regions), in which major outbreaks and a substantial number of sporadic cases occur; and (2) nonendemic regions, in which HEV accounts for a few cases of acute viral hepatitis, mainly among travelers to endemic regions (Fig. 40.4).

HEV in Endemic Regions

Until the 1980s, epidemics of hepatitis in the developing world had been linked to HAV infections [1]. The subsequent development of serological assays showed HEV to be endemic throughout tropical and subtropical countries, with periodic epidemics reported from the Indian subcontinent [56–58], southeast Asia [59], Africa [60], and Mexico [61]. Although food-borne epidemics have been reported in China [62], most HEV-associated epidemics have been caused by contaminated water. Such epidemics usually follow heavy rainfall and can involve many thousands of cases [63].

Sporadic cases of HEV infection have also been reported, occurring at much higher rates in endemic regions than in nonendemic regions [64]. As expected, studies in endemic regions show high seroprevalence rates ranging from 15 to 60% [65, 66]. Notably, the age-specific seroprevalence profiles for HEV are found to differ from those reported for antibody to HAV, even though, in endemic countries, the transmission routes for these two viruses are similar. Whereas the anti-HAV seroprevalence rate reaches more than 95% in children by the age of 10 years, anti-HEV is rarely detected in children, increasing to 40% in young adults without substantial increases later in life [65].

During the outbreaks, overall attack rates range from 1 to 15%, being much higher among adults of 15–40 years (3–30%) than children (0.2–10%) [67]. The peak incidence in sporadic cases of hepatitis E in endemic regions also occurs in 15–35-year-olds [1]. The reason for this pattern of age distribution, which is unusual for an enteric infection, is

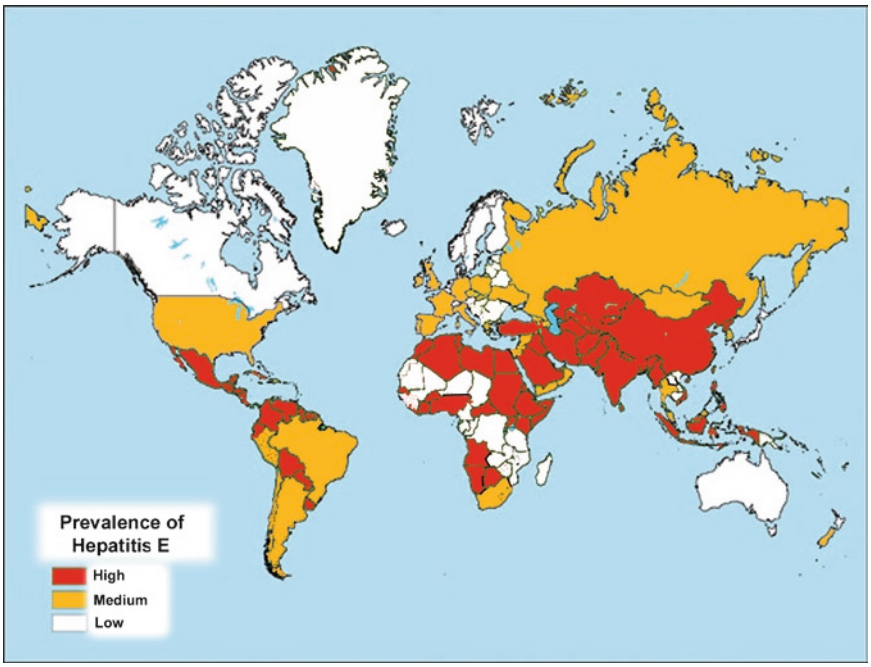

Fig. 40.4 Geographic distribution of hepatitis E. From Rose and Keystone [198]. Used with permission

unknown. It has been suggested that HEV runs a predominantly anicteric course in young age groups followed by gradual loss of immunity. It has also been speculated that HEV somehow has a selective tropism for liver cells of adults. Nevertheless, young children are susceptible to infection with HEV, because clinical disease has occurred with a similar frequency in all age groups in some epidemics [68] and sporadic clinical hepatitis E in children has been reported [69]. A male preponderance of cases has been observed in most reports (the male to female ratio varies from 1.5 to 3.5:1) [70]. It is unclear whether this reflects the greater involvement of men in professional and social activities and, accordingly, their greater exposure to risk factors, or a true difference in susceptibility. An outbreak may be single-peaked and short-lived, or multi-peaked and prolonged, lasting for more than a year.

The demographic and clinical features of patients with acute sporadic hepatitis E closely resemble those during epidemics of hepatitis E [71]. In endemic areas, outbreaks have a periodicity of 5–10 years, which in part reflects the patterns of heavy rainfall. The reservoir for HEV during interepidemic periods is unknown. Sporadic HEV infection in endemic areas may be sufficient to maintain the virus within the community during the interepidemic periods. Another possibility is that a nonhuman HEV reservoir exists. HEV has been isolated from swine, and antibodies to HEV have been detected in a number of animal species, including swine, sheep, cattle, chickens, rats, and captive monkeys. Moreover, viruses recovered from swine have been identified as variants related to human HEV strains found in the same geographic regions.

HEV in Nonendemic Regions

In developed countries, hepatitis E infections were traditionally thought to occur infrequently and only in individuals who had become infected while traveling in an area where the virus is endemic. However, cases of sporadic hepatitis E in people with no history of recent travel have been reported in developed regions such as North America, Europe, Japan, New Zealand, and Australia [72]. The reporting of such infections, together with the availability of more comprehensive molecular and serological data, has led to the reevaluation of HEV epidemiology and the acceptance that autochthonous (locally acquired) hepatitis E is a clinical problem in developed countries.

Rates of IgG positivity in endemic areas reflect the frequency of hepatitis E infections seen in these areas. The prevalence of HEV IgG antibodies in low-incidence populations in the developed world ranges from 3 to 20% [72, 73]. The reason for these observations has been the subject of debate. The presence of high rates of HEV IgG positivity in populations where acute infection is diagnosed rarely must mean that either subclinical infection is common, acute hepatitis E is unrecognized, or that IgG seropositivity is

nonspecific and reflects cross-reacting antibodies. Subclinical infections do occur [74]. Similarly, acute hepatitis E is not recognized in many cases either because serology is not done or because cases are assigned to other causes such as drug-induced hepatitis. Finally, there is the question of the specificity of the antibodies detected by HEV IgG assays in population studies. This is difficult to assess in the absence of a history of proven infection. However, sera tested in IgG assays based on a variety of HEV antigens give broadly concordant results which suggests that the antibodies are truly directed at HEV. Immunoblot assays have confirmed the reactivity of sera in seroprevalence studies and an interesting new development is the use of interferon-based assays of cell-mediated immunity to confirm previous exposure to HEV [72, 75]. Thus, it often seems that positive HEV IgG serology reflects previous exposure to HEV, but the seroprevalence data are dependent on the population tested and the assays used. High IgG rates in developing countries are a reflection of high rates of clinical infection; in developed countries much of the primary infection is unrecognized. The seroprevalence data from industrialized countries suggests that subclinical or unrecognized infection is common. However, the incidence of autochthonous hepatitis E is not known [72].

The number of documented cases in the developed countries has risen substantially over the past few years. This rise is almost certainly a result of increased and improved testing and case ascertainment, rather than any true increase in incidence. Moreover, recent data from few developed countries have shown that hepatitis E is more common than hepatitis A [72, 73, 76].

Modes of Transmission

HEV infection has four documented routes of transmission: water-borne transmission; consuming raw or undercooked meat of infected wild animals such as boar and deer and domestic animals such as pigs; parenteral transmission (blood-borne); and vertical transmission from mother to child.

HEV may be transmitted by the fecal–oral route. The most common vehicle of transmission during epidemics has been the ingestion of fecally contaminated water. Outbreaks in endemic areas occur most frequently during the rainy season, after floods and monsoons, or following recession of flood waters [64, 67]. These climatic conditions in conjunction with inadequate sanitation and poor personal hygiene lead to epidemics of HEV infection, when the sewage waters gain access to open-water reservoirs. In several regions of HEV-endemicity, a pattern of recurrent epidemics has been observed, which is probably related to the permanent existence of conditions in which drinking water is fecally contaminated. In Southeast Asian regions, the disposal of human excreta into rivers and the use of river water for drinking, cooking, and personal hygiene have been shown to be significantly associated with a high prevalence of HEV infection: the use of river water over years for various activities can lead to recurrent epidemics [77].

Both in epidemic and sporadic HEV, there is a low rate of clinical illness among household contacts of infected patients, an unexpected finding because the virus is transmitted by the fecal–oral route. Reported secondary attack rates in households of HEV-infected persons range from 0.7 to 2.2%, in contrast to secondary attack rates of 50–75% in households of HAV-infected individuals [78]. The reasons for this difference may be related to instability of HEV in the environment, differences in infectious dose needed to produce infection, or a higher frequency of subclinical disease among persons secondarily infected with HEV. Even when multiple cases occur among members of a family, such occurrence is related to exposure to a common source of contaminated water rather than to person-to-person spread [78]. The mode of transmission responsible for sporadic HEV infections is unclear. Contaminated water is probably responsible for most of the cases in this setting. However, food-borne hepatitis E infection after eating uncooked liver of pig or wild boar and meat of wild deer has been reported (see below).

More recent findings have led to speculation of an additional route of transmission for HEV. Higher HEV seroprevalence levels in specific groups such as paid blood donors positive for other blood-borne viruses and in repeatedly transfused hemodialysis patients have led to suggestions that HEV could be acquired parenterally (see below). There is also a risk of posttransfusion hepatitis E, and this should be considered in areas that are thought to be nonendemic (see below).

Presumed nosocomial spread of HEV has been reported in South Africa, where acute hepatitis developed in three healthcare workers 6 weeks after they treated a patient with fulminant hepatic failure (FHF) due to HEV infection [79]. In an experimental study, pregnant rhesus monkeys failed to transmit the virus to their offspring [80]. However, vertical transmission of HEV infection from mother to infant has been shown to occur. In one study, six of eight babies born to mothers who had either acute uncomplicated hepatitis or FHF due to HEV infection in the third trimester of pregnancy were found to have evidence of HEV infection. Of these, five had HEV RNA in samples of their blood taken at birth, suggesting that infection was transmitted transplacentally [81]. More recent studies have shown that mother-to-infant transmission occurred in 50–100% of HEV RNA-positive mothers during pregnancy [82, 83].

Specific Groups and Settings

Persons Having Contact with Swine and Untreated Waste Water

Recently, a high prevalence of antibodies to HEV was found among persons who work with swine. Human populations with occupational exposure to certain animals have an increased risk of HEV infection. However, whether infection with swine HEV leads to clinical illness is unclear (see below).

Recently, HEV RNA has been identified in a substantial proportion of untreated sewage samples in both nonendemic and endemic areas. Sewage does not appear to be a source of occupational infection by HEV in trained sewage workers with personal protective equipment working in a region with good sanitation [84]. However, this may not be true in other areas. One group found that 43.5% (20/46) of urban sewage samples collected in Barcelona, Spain, from 1994 to 2002 tested positive for HEV RNA [85]. In a study from India, anti-HEV IgG-positivity was significantly higher among staff members of a sewage treatment plant (56.5%) when compared with controls (18.9%). A sevenfold higher risk of hepatitis E infection was recorded in sewage workers working in close proximity to sewage and a 3.9-fold higher risk in staff members not coming into frequent contact with sewage [86]. In a study from Turkey, agricultural workers who use untreated waste water for irrigation had an anti-HEV positivity rate of 34.8% as compared to 4.4% in controls [87].

HIV-Infected Persons

An association between anti-HEV seropositivity and HIV infection has been suspected. Upto one third of HIV-infected homosexual men have IgG anti-HEV [88]. However, contrary observations have also been reported showing that HEV infection does not seem to be prevalent in the HIV population [89].

Transfusions and Other Health Care Settings

A small but significant proportion of blood donors even in developed countries with or without elevated alanine aminotransferase (ALT) levels are viremic and are potentially able to cause transfusion-associated hepatitis E [90, 91]. A few cases of transfusion transmission of HEV have been reported so far [92]. Thus, there is need for precautions against the potential risk of transfusion-transmitted HEV infection. The safety of plasma-derived products with respect to HEV may be an important issue and each product should be evaluated for safety against HEV contamination [93]. The sensitivity of HEV to heat has been shown to vary greatly depending on the heating conditions. On the other hand, the HEV particles are completely removed using 20-nm nanofilters. However, each inactivation/removal step should be carefully evaluated with respect to the HEV inactivation/removal capacity, which may be influenced by processing conditions such as the stabilizers used for blood products [94].

Anti-HEV IgG antibody prevalence has been found to be significantly higher in patients with hemophilia as compared to blood donors. HEV antibody was not detected in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors. Thus, parenteral transmission of HEV may occur in patients with hemophilia via nonvirus-inactivated coagulation factors [95]. A higher prevalence of IgG anti-HEV in various other groups has been found, including patients with sickle-cell anemia and beta-thalassemia major and persons working in emergency rooms or in operating rooms in a German study [96].

The prevalence rates of IgG anti-HEV are variably reported in hemodialysis patients and asymptomatic blood donors [97]. A significantly higher risk of HEV infection has been shown among patients on chronic HD in endemic regions [98, 99].

HEV Infection as a Zoonosis

In addition to human beings, virological evidence of mammalian HEV has been found in domestic pigs, wild boar, deer, mongoose, horse, and bivalves [100]. In all these animals – with the exception of a report from Cambodia of a pig with a genotype 1 virus [101], the HEV identified was either genotype 3 or 4. Antibodies to HEV (but not HEV RNA) have been detected in a wide range of domestic and feral mammals including cats, dogs, cattle, sheep, goats, macaques, donkeys, wild deer, rats, and mice [100].

Evidence for differences in genotype virulence is not abundant. Based on experimental cross-infection data [102], the genotype 3 strains are considered by some to be attenuated for human beings. There is little published evidence to date, however, of comparative assessments of the relative morbidity and mortality of travel-associated (genotype 1 or 2) and autochthonously acquired (genotype 3 or 4) hepatitis E. In reciprocal cross-infection trials using nonhuman primates and pigs, genotype 1 strains produced more severe pathology than genotype 3 strains [102]. There is also evidence from India that subtype differences might be responsible for the apparent inability of the genotype 4 HEV strains to infect human beings [103]. Investigators showed 26 amino acid substitutions of Indian HEV genotype 4 strains, compared

with genotype 4 strains found in pigs and people in China, Japan, and Taiwan.

The first evidence of a zoonotic source of autochthonous hepatitis E resulted from the observation in the USA that the partial nucleotide sequences of two pig and two human HEV strains were very closely related genotype 3 strains [104, 105]. In many developed countries, compared with travel-related cases, the human autochthonously acquired cases showed the closest genetic homology to pig strains from the same region [100]. Additionally, high seroprevalences of HEV were reported in the pig herds of many countries, both developed and developing [106, 107]. Occupational exposure to pigs was also identified as a risk factor for hepatitis E in human beings. This evidence was based not on clinical cases, since there are only a few documented, but on reports of veterinarians and other pig industry workers who presented with high HEV IgG seroprevalence [100, 106, 107]. The available data show that at any one time, more than 20% of pigs in pig production units are excreting HEV in feces [108], and large quantities of HEV most probably enter watercourses as a consequence of run-off from outdoor pig farms. HEV has been detected in slurry lagoons on pig farms, from urban sewage works, and from pig slaughterhouses [100]. The risks of spreading untreated slurry on farmland remain unknown. However, HEV recovered from sewage and slurry has been shown to infect rhesus monkeys [109–111].

The strongest evidence of zoonotic transmission of hepatitis E is from Japan, where consumption of uncooked or poorly cooked wild boar and deer meat resulted in hepatitis E infection, with identical viruses recovered from the meat and from the patients [112, 113]. Contamination of retail pig liver with HEV has been reported in many countries [100, 114]. HEV has been found to be infective after heating to 56°C for 1 h, but was inactivated at an internal liver temperature of 71°C for 5 min [115], which means that light cooking might not eliminate the risk of infection from contaminated meat. A provisional report using data from 1990 to 2000 shows that there is a relation between pork consumption and mortality from chronic liver disease in 18 developed countries [116]. Multivariate regression analysis showed that alcohol consumption, pork consumption, and hepatitis B virus (HBV) seroprevalence were all independent risk factors for death from chronic liver disease, but beef consumption was not. The reason for these observations is uncertain. It could be a result of factors in pig meat (e.g., pork fat) that cause cirrhosis. Another possible explanation is that an infectious agent found in pig meat causes increased mortality in patients with preexisting chronic liver disease [116]. A candidate for the latter hypothesis is HEV, since viable HEV has been found in pig meat in the human food chain [117], and HEV superinfection in patients with chronic liver disease carries a high mortality (see below).

Despite the serological, clinical, and molecular genetic evidence suggesting that autochthonous hepatitis E might be a porcine zoonosis, a direct connection between the disease and either consumption of pig meat or exposure to pigs is sparse [72]. Ingestion of infected animal tissue is one zoonotic transmission route, but the evidence indicates that several routes could be contributing to the burden of human HEV infections [72].

Pathogenesis

Incubation Period

The incubation period from exposure to the onset of clinical disease is approximately 28–40 days, based on analysis of water-borne epidemics in which the time of exposure was identified [70]. In experimental HEV transmission studies in humans, liver enzyme values peak 42–46 days after ingestion of the virus [2]. In experimental infection of pregnant rhesus monkeys, the incubation period varied from 1–2 to 4–5 weeks [80, 118].

Viral Replication

The knowledge of HEV replication is poor, due to the lack of practicable cell culture systems for the virus. Several strategies for experimental propagation and production of HEV to study the molecular biology have been reported, but their reproducibility and feasibility need confirmation (see above).

Because HEV does not replicate well in cell culture, the mechanisms of HEV pathogenesis and replication are not fully understood. Our understanding of the replication and expression of HEV is based largely on recognized conservative motives of nonstructural domains and analogies with other positive-stranded RNA viruses. Studies in rats and swine suggest that extrahepatic tissues such as peripheral blood monocytes, spleen, lymph nodes, and the small intestine are involved in the replication of HEV [48].

However, the main target cells are hepatocytes. The viral replication cycle has been explained above. A small amount of HEV is found in plasma during infection, consistent with the release of progeny virus through the basolateral domain of hepatocytes, leading to spread through the liver. However, most of the virus appears to be excreted through the biliary system to complete the replication cycle, consistent with the release of the virus through the apical domain of hepatocytes. Bile appears to be the principal source of HEV in the feces.

Pathogenesis

The incubation period in human volunteers after oral exposure is 4–5 weeks [119, 120]. HEV can first be detected in stools approximately 1 week before the onset of illness and persists for an initial few weeks, but in some patients positive RT-PCR results persist for as long as 52 days [121]. HEV RNA has regularly been found in serum by RT-PCR in virtually all patients in the first 2 weeks after the onset of illness. Periods of HEV RNA positivity in serum range from 4 to 16 weeks [121].

Exposure to HEV is thought to occur usually via the oral route, but the virus can be transmitted parenterally also. Infection of nonhuman primates via the oral route has been successful in some (but not all) studies. In one study in which quantitative data were available, the infectivity titer of HEV as measured by intravenous inoculation was at least 10,000-fold higher than when administered orally [122]. Infection transmitted by the intravenous route is more reproducible than the oral route. After the intravenous inoculation of HEV in cynomolgus macaques, the average incubation period for acute hepatitis is about 3 weeks. After exposure (regardless of route), the first evidence of infection with HEV is found in the liver. Shortly thereafter, virus is detected in the blood, bile, and feces. The expression of HEAg in hepatocytes, indicative of viral replication, first appears about day 7 after infection. HEAg can be detected simultaneously in hepatocytes, bile, and feces during the second or third week after inoculation, and before and concurrently with the onset of ALT elevation and histopathological changes in the liver [123]. The antigen can be detected in 70–90% of hepatocytes at peak expression and begins to decline after peak ALT activity has been reached. The peak shedding of virus into the blood and bile occurs before the onset of clinical disease. The onset of clinical disease usually coincides with first detection of the humoral immune response, diminished replication of the virus, and beginning resolution of the infection. Both IgG and IgM class anti-HEV can usually be detected by the time liver enzymes become elevated and hepatic pathology becomes detectable.

A site of replication in the intestinal tract has not been identified but is thought to exist. Nevertheless, most viruses detected in the intestinal tract are probably there by way of bile. HEV replication in the liver is the initial event and a rise in serum ALT level and the presence of mild histologic injury at this time would be consistent with a direct cytopathic effect of the virus or an early immune-mediated effect. Later, hepatic HEVAg becomes undetectable, indicating that viral replication has stopped, and during this time the histologic changes are more pronounced, suggesting that the injury at this time is primarily immune-mediated. In support of this idea is the finding of infiltrating lymphocytes in the liver that have a cytotoxic immunophenotype [124]. The delayed appearance of anti-HEV (if true and not the result of insensitivity of antibody testing) suggests that antibody is not essential for initiating hepatocyte injury, but may be important in perpetuating it. It is also possible that development of an antibody response occurs independent of hepatocyte injury. In summary, the mechanism of cell death is not known, but early in the course of infection the mechanism may be predominantly direct cytopathicity and later predominantly immune-mediated.

In experimental infections of nonhuman primates, the clinical presentation of hepatitis E is dose-dependent. Thus, the severity of infection is directly related to the infectivity titer of the challenge virus, and consistent demonstration of hepatitis in experimentally infected nonhuman primates has required challenge doses at least 1,000 times greater than the minimum dose needed for infection [125]. It is not known whether such a clinical-to-infectious dose relationship exists for naturally infected humans, but cycles of inapparent infection resulting from exposure to low doses of virus could explain how HEV can be maintained in a population with little or no clinical disease.

The disease is self-limiting and no chronic sequelae have been reported in general. However, recent reports present biochemical, histological, and genetic evidence of chronic HEV infection in transplant patients (see below). It would be interesting to test other immunosuppressed persons, such as those with HIV infection, for their ability to resolve acute hepatitis E.

Hepatitis E has a mortality rate of 0.2–1% in the general population. Increased morbidity and mortality is observed in chronic liver disease patients superinfected with HEV (See below). A unique clinical feature is its increased incidence and severity in pregnant women, with mortality rates of 15–20% (See below). A role of endotoxin-mediated hepatocyte injury was proposed [126], but the precise cellular/molecular mechanisms are not clear. A shift in the Th1/Th2 balance toward Th2 has been observed in pregnant women infected with HEV compared to nonpregnant women [127], but how this influences the severity of HEV infection is not clear. Pregnant women with jaundice and acute viral hepatitis due to HEV showed higher mortality rates and worse obstetric and fetal outcomes than those with other types of viral hepatitis [128]. There were increased levels of estrogen, progesterone, and βHCG in HEV-positive pregnant patients with fulminant hepatitis compared to HEV-negative patients and controls [129]. Selective suppression of nuclear factor kappa B (NFκB) p65 in pregnant compared to nonpregnant fulminant hepatitis patients has also been proposed to cause liver degeneration, severe immunodeficiency, and multiorgan failure [130].

Immune Response

Specific IgM and IgG immune responses to HEV occur early in the infection, usually by the onset of clinical illness. In this respect, hepatitis E resembles hepatitis A, and a serologic diagnosis can usually be made at the time of presentation of the patient. In patients with hepatitis E, IgM anti-HEV begins to develop just before the peak of ALT activity and reaches a maximal titer around the time of maximal ALT activity. IgM anti-HEV disappears about 4–5 months into the convalescent phase of the disease. Of samples of sera collected from patients during various outbreaks of hepatitis E at 1 and 40 days, at 3 and 4 months, and at 6 and 12 months after the onset of jaundice, 100, 50, and 40%, respectively, were positive for IgM anti-HEV [131]. IgG antibodies develop shortly after the IgM antibodies, and the titers increase throughout the acute phase into the convalescent phase, remaining high from 1 to 5 years after the resolution of the illness. IgG anti-HEV appears to diminish in titer at a more rapid rate than does antibody to hepatitis A, raising questions about the duration of immunity following acute hepatitis E. Anti-HEV has been detected as long as 13–14 years after infection; however, the possibility of repeated exposure cannot be ruled out [132].

Antibody responses to individual viral antigens are highly variable, due to both strain-specific differences in some epitopes and differences in response to single antigens between individual patients. For example, after reaching high levels during the acute phase, HEV pORF2-specific IgG declines rapidly over 6–12 months and might not persist at protective levels for life.

Conversely, the responses to pORF3 are highly variable, with a proportion of patients mounting no detectable response to the antigen while others maintain reactivity to pORF3 for many years. Anti-HEV of the IgA class (as a correlate of mucosal immunity) has also been detected in the serum of about 50% of naturally infected individuals. These antibodies rapidly decline to undetectable levels and the significance of such antibodies is unknown. Since passive immunization with IgG appears to be sufficient for protection, it is likely that IgA is not essential [133].

All isolates of HEV are serologically related, and convalescent antibody produced in response to infection with one strain of HEV probably protects against subsequent exposure to all other strains.

Little is known about the cell-mediated immune response to HEV in humans. Recent evidence suggests that cellular immune responses do occur in patients with acute HEV infection. Lymphocytes of patients with acute hepatitis E show sensitization to HEV peptides. The specific T cell response decreases along with convalescence and may play a role in the pathogenesis of acute HE and recovery [134].

A recent study assessed the frequency and activation status of natural killer (NK) and natural killer T (NKT) cells and cytotoxic activity of NK cells in the peripheral blood mononuclear cells (PBMCs) obtained from patients with hepatitis E ($n=41$) and healthy controls ($n=61$). In 14 patients, the studies were repeated during the convalescence period. Patients had fewer median (range) NK cells [8.9% (2.4–47.0) vs. 11.2% (2.6–35.4)] and NKT cells [8.7% (2.8–34.1) vs. 13.6% (2.3–36.9)] than controls ($P<0.05$ each). Activation markers were present on large proportion of NK cells [43.5% (11.2–58.6) vs. 15.5% (3.0–55.8)] and NKT cells [41.5% (17.4–71.1) vs. 12.8% (3.3–63.2); $P<0.05$ each] from patients. NK cell cytotoxicity was similar in patients and controls. During convalescence, all the parameters normalized. Thus, reversible alterations in NK and NKT cell number and activation status during acute hepatitis E suggest a role of these cells in the pathogenesis of this disease [135].

Pathology

Much of what is known about the pathology of acute HEV infection has been obtained from studies of ET-NANBH epidemics occurring in developing countries [136]. The morphologic findings are of two main types: (1) a typical acute hepatitis picture and (2) a cholestatic variant. In the latter, prominent features include bile stasis in canaliculi, gland-like transformation of hepatocytes, and extensive proliferation of small bile ductules. There is also prominent cholestasis in the centroacinar zone. Degenerative changes in hepatocytes and focal areas of necrosis are less frequent than in the noncholestatic type. Kupffer cells that contain lipofuscin granules are prominent. Portal tracts are expanded; polymorphonuclear leukocytes are conspicuous in the portal tract infiltrates, but lymphocytes predominate. Phlebitis of portal and central veins may be seen. Intralobular infiltrates consist mainly of polymorphonuclear leukocytes and macrophages. With the noncholestatic type of HEV infection, focal hepatocyte necrosis, ballooned hepatocytes, acidophilic degeneration of hepatocytes, and acidophilic body formation are frequent. An important morphologic feature is focal intralobular areas of hepatocyte necrosis with prominent accumulations of macrophages and activated Kupffer cells in the presence of lymphocytes. The histologic severity of the hepatitis is variable, but in one well-documented epidemic, 78% of biopsy specimens were graded as at least moderately severe. In fatal cases, severe acute hepatitis with submassive or massive hepatocyte necrosis is observed. No chronic histologic manifestations have been described in immunecompetent subjects.

In acute but nonfulminant cases of HEV infection, electron microscopy reveals considerable hepatocyte polymorphism.

Some hepatocytes show ballooning degeneration and vesiculation of the perinuclear envelope and rough ER, whereas other hepatocytes show shrinking and condensation of cytoplasm and cell organelles to form a web-like pattern. The bile canaliculi are dilated, and intracanalicular and intracytoplasmic bile stasis is seen. In fulminant cases of HEV infection, hepatocytes show extensive organelle damage.

Recently, some insight has been gained on pathology of autochthonous hepatitis E. Most patients with autochthonous hepatitis E do not require a liver biopsy since they have a self-limiting illness. A few patients will have more severe hepatitis with worsening liver blood tests, and therefore, a liver biopsy is sometimes helpful. There are few data on the hepatic histopathology of acute autochthonous hepatitis E, and such reports are limited to patients with severe disease. Liver histology of acute autochthonous hepatitis E in the non cirrhotic liver is similar to that seen in acute viral hepatitis, with lobular disarray with reticulin framework distortion. Portal tracts are expanded by a severe mixed polymorph and lymphocytic inflammatory infiltrate. Moderate to severe interface hepatitis and cholangiolitis are also present [137, 138]. In one study of three patients with autochthonous hepatitis E, polymorphs were shown to be concentrated at the periphery and interface of the liver, with lymphocytes – including aggregates – concentrated centrally [138]. These findings might be helpful in distinguishing autochthonous hepatitis E from other causes of hepatitis – for example, autoimmune hepatitis – but are based on a small number of cases and require confirmation. In patients with hepatitis E who have underlying cirrhosis, the liver histology is nonspecific and could easily be mistaken for alcoholic hepatitis in the context of established ethanolic cirrhosis [139]. These changes were much more marked than seen in patients with HEV infections from endemic areas. In the small number of immunosuppressed transplant patients who have developed chronic infection with HEV, the liver histology shows progressive fibrosis and portal hepatitis with lymphocytic infiltration and piecemeal necrosis, with progression to cirrhosis [140, 141].

Clinical Features

The incubation period from exposure to the onset of clinical disease is approximately 28–40 days, based on analysis of water-borne epidemics in which the time of exposure was identified.

Symptoms

Acute icteric hepatitis is the most common recognizable form of illness associated with HEV infection. Two phases of illness have been described: (1) a prodromal and preicteric phase and (2) an icteric phase. The prodomal phase, lasting about 1–4 days, is characterized by a variable combination of flu-like symptoms, including fever, chills, abdominal pain, anorexia, nausea, aversion to smoking, vomiting, diarrhea, arthralgias, asthenia, and urticarial rash. These symptoms are followed within a few days by the appearance of jaundice. The onset of the icteric phase is usually heralded by the appearance of darkening of urine and may be accompanied by itching or lightening of stool color. With the onset of jaundice, the fever and other prodomal symptoms tend to diminish and may entirely disappear. The exception is gastrointestinal symptoms which may persist for a longer time. The physical examination reveals jaundice and a mildly enlarged, soft, and slightly tender liver. Splenomegaly is seen in about one fourth of patients.

In acute hepatitis, classical biochemical abnormalities are seen. Laboratory test abnormalities include a variable rise in serum bilirubin (predominantly conjugated), markedly elevated aminotransferases, and γ-glutamyltransferase levels, and a mild rise in serum alkaline phosphatase activity. An elevated ALT often precedes the onset of symptoms by as much as 10 days and reaches a peak by the end of the first week, coinciding with the onset of the icteric phase. As the illness subsides, the ALT levels decrease significantly, followed by a decrease in serum bilirubin levels which are usually normal by 6 weeks [142] (Fig. 40.5).

HEV RNA can be detected in the stool for up to 10 days after the onset of the icteric phase, although viral shedding for up to 52 days after the onset of icterus has been described [121]. HEV RNA can be detected in the serum during the preicteric phase and before the detection of virus in stool, but becomes undetectable after the peak in serum aminotransferase activity. The detection of HEV RNA in the serum during the preicteric phase suggests that sporadic transmission of the virus may occur via the parenteral route.

The IgM antibody to HEV becomes detectable just before the peak ALT activity; peak antibody titers occur at

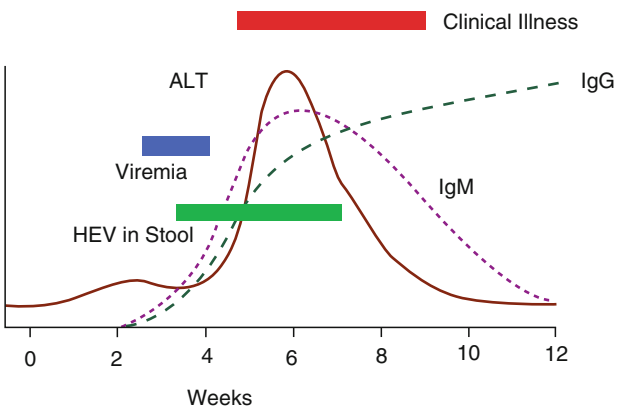

Fig. 40.5 Timeline of hepatitis E manifestations

approximately the same time as peak ALT levels and decline rapidly thereafter. In the majority of patients, IgM anti-HEV is undetectable 5–6 months after the onset of illness. IgG anti-HEV is detectable shortly after IgM anti-HEV becomes detectable, increases in titer throughout the acute and convalescent phases of infection, and remains detectable in most patients 1 year after acute infection. The duration of the IgG anti-HEV response is unknown, but high titers have been measured up to 14 years after acute infection [132]. The duration of protective immunity is not known; however, in the short term, there appears to be protection from reinfection. In nonfatal cases, acute hepatitis is followed by complete recovery without chronic sequelae. No evidence of chronic hepatitis or cirrhosis has been detected in immunecompetent patients who were followed clinically or underwent liver biopsy after acute hepatitis E [143].

Anicteric Hepatitis and Asymptomatic Infection

Nonspecific symptoms may develop in some infected persons, resembling those of an acute viral febrile illness without jaundice (anicteric hepatitis). Liver involvement in these patients is only recognized after laboratory studies are performed. In its most benign form, HEV infection is entirely inapparent and asymptomatic and passes unnoticed. The exact frequency of anicteric hepatitis and asymptomatic infection is unknown, although it probably far exceeds that of icteric hepatitis. In areas of endemicity, anti-HEV is present in a large proportion of the population; most of these seropositive individuals do not recall having had jaundice and may have had an asymptomatic or anicteric infection. In most of the outbreaks of hepatitis E, the highest rate of clinically evident disease is among persons of age 15–40 years – a pattern that contrasts with HAV infection, in which children have the highest attack rates. The lower rate of disease among children may be the result of a higher frequency of asymptomatic or anicteric HEV infection in this age group.

Chronicity

Until very recently, chronic infection with HEV was thought not to occur. The first indication of chronic infection with HEV was a recent report describing a Japanese patient with T cell lymphoma receiving chemotherapy who was found to have persistent HEV viremia [144]. Chronic hepatitis E infection has been documented in patients receiving immunosuppressive therapy following organ transplantation [140, 141]. In a French study of hepatitis E infection in solid organ transplant recipients, of the 14 patients who developed hepatitis E infection posttransplantation, eight patients went on to develop chronic liver disease with a persistently raised transaminase concentration, persistent viremia, and progressive inflammation and fibrosis on liver biopsy [141]. The patients who developed chronic infection with HEV had more profound immunosuppression (lower serum leucocytes, total lymphocytes, and CD4 lymphocytes) compared with the patients who did not progress to chronic infection. Several case reports have been reported on occurrence of chronic hepatitis and cirrhosis due to HEV infection in organ transplant recipients [140, 145]. Recent case reports have appeared about persistent infection of HEV in patients with lymphomas [146]. However, several issues deserve consideration before we add chronic hepatitis to the spectrum of disease associated with HEV infection. First, the persistent HEV infection was observed in immunosuppressed patients. Such patients are known to have persistence of viral agents which are associated with a self-limited course in otherwise healthy persons, such as Epstein-Barr virus and cytomegalovirus. Thus, occurrence of chronic HEV infection in immunosuppressed patients may reflect a similar phenomenon and may not occur in nonimmunosuppressed individuals. Also, finding persistent HEV viremia in such patients does not necessarily imply that HEV infection led to ALT elevation or chronic liver disease. Future studies on persistent HEV infection in immunosuppressed patients should not only include patients with ALT elevation and chronic liver injury, but also those with immunosuppression but normal ALT and no chronic liver injury, with the latter group serving as a comparison group to clarify the role of HEV infection in causing liver injury [147].

Complications

Prolonged Cholestatic Hepatitis

In a few patients, the course is prolonged, with marked cholestasis, persistent jaundice, and itching. In these cases, laboratory tests show a rise in alkaline phosphatase and a persistent rise in bilirubin, even after the transaminases have returned to normal. The prognosis is good because the jaundice resolves spontaneously after 2–6 months. Recurrent (bimodal) hepatitis E has not been reported, except in experimentally infected nonhuman primates [148]. In contrast, recurrent hepatitis A is relatively common.

Fulminant Hepatic Failure

The case-fatality rate in reports based on hospital data has ranged from 0.5 to 4%. However, the hospital-based data

may overestimate mortality. Studies based on data obtained from population surveys during outbreaks have reported lower mortality rates, ranging from 0.07 to 0.6% [149]. In a small proportion of patients, the disease is more severe and associated with subfulminant or FHF, which can be rapidly fatal. In regions of endemicity, HEV infection is an important cause of FHF.

Acute HEV Superinfection in Patients with Cirrhosis

Acute HEV superinfection in patients with cirrhosis produces decompensation and is associated with a high mortality rate. An Indian study [150] documented high rates of HEV superinfection in patients with cirrhosis and these infections were a common cause of hepatic decompensation of otherwise stable cirrhotic patients in endemic areas. The mortality in these patients was higher at 4 weeks and 12 months when compared with cirrhotic patients without HEV superinfection. In another study [151], HEV superinfection was a common cause of acute exacerbations in patients with otherwise stable and asymptomatic chronic hepatitis B carrier state. In another study ACLF occurred in 121 (3.75%) of 3,220 patients (mean age 36.3 ± 18.0 years; M:F 85:36) with liver cirrhosis admitted from January 2000 to June 2006. It was due to HEV in 80 (61.1%), HAV in 33 (27.2%), and both in 8 (6.1%). Three-month mortality was 54 (44.6%) [152]. In areas of high endemicity, the large majority of adult chronic liver disease patients are vulnerable to infection with HEV, but are protected against hepatitis A. This is a group that would be ideal candidates for an HEV vaccine.

In developed countries also, locally acquired hepatitis E infection in a cirrhotic patient can result in subacute liver failure and death [139].

Pregnant Women

Pregnant women, particularly those in the second and third trimesters, are more frequently affected during hepatitis E outbreaks and have a worse outcome. In fact, the major cause of mortality in epidemics is the high rate of FHF in pregnant women. In a recent study from India [128], pregnant women with HEV infection had higher maternal mortality rate and worse obstetric and fetal outcomes than did pregnant women with jaundice and acute hepatitis caused by other hepatitis viruses (Table 40.1). This confirmed earlier observations of increased incidence and severity of HEV infection in pregnancy, in which death rates approach 15–20%. In an earlier study [153], pregnant women with fulminant hepatitis caused by HEV had shorter duration of illness, lower serum bilirubin, shorter preencephalopathy period, lower serum bilirubin, and high occurrence of disseminated intravascular coagulation (DIC). Considering the explosive nature of HEV in pregnancy with DIC, it was postulated to represent a severe manifestation of a Schwartzman-like phenomenon. Several studies have documented high vertically transmitted intrauterine and newborn HEV infections causing high fetal

Table 40.1 Maternal mortality and medical complications in acute HEV-infected pregnant women

Variable	HEV-infected women ($n=132$), n/n (%)	Non-HEV-infected women ($n=88$), n/n (%)	Relative risk (95% CI)	P value
Maternal mortality rate				
• Overall	54/132 (41)	6/88 (7)	6.0 (2.7–13.3)	<0.001
• Patients with fulminant hepatic failure	54/73 (74)	6/18 (33)	2.2 (1.1–4.3)	0.001
– Second trimester	18/27 (66)	0/7 (0)	–	0.002
– Third trimester	36/46 (78)	6/11 (54)	1.4 (0.8–2.5)	0.11
• Patients without fulminant hepatic failure	0/59 (0)	0/70 (0)	–	1.00
Medical complications				
• Coagulation defect[a]	104/132 (9)	32/88 (36)	2.2 (1.6–2.9)	<0.001
• Nasal or gastrointestinal hemorrhage	25/132 (19)	4/88 (4)	4.2 (1.5–11.6)	0.002
• Leukocyte count $\geq 11 \times 10^9$ cells/l	8/132 (65)	31/88 (35)	1.8 (1.4–2.5)	<0.001
• Serum creatinine concentration ≥34 μmol/l (≥2 mg/dl)	39/132 (30)	4/88 (4)	6.5 (2.4–17.5)	<0.001
• Ascites	33/132 (25)	5/88 (6)	4.4 (1.8–10.8)	<0.001
• Clinical signs of increased intracranial tension	27/132 (20)	1/88 (1)	18.0 (2.5–130.1)	0.001

HEV hepatitis E virus

[a]International normalized ratio >2.0

From Patra et al. [128] Used with permission

mortality due to fulminant hepatitis. The disease in this age group presented with hypoglycemia, hypothermia, and death. In another study [154], data pointed to a relationship between severity of HEV infection in the mother and the fetus. It was postulated that severe fetal infections and fetal death may produce toxins that overload maternal circulation causing severe maternal disease. Mothers who delivered infected babies within the first few days of infection recovered from fulminant disease and those in whom pregnancy continued with fetal death often had fatal outcome.

Coinfection of HEV with Other Hepatotropic Viruses

Coinfection with multiple hepatotropic viruses occurs in various combinations in 7–24% of all patients of sporadic AVH. The most common combination is HAV and HEV coinfection [155, 156]. Simultaneous infection with multiple hepatotropic viruses in the disease states of both AVH and FHF does not adversely affect the outcome. Total serum bilirubin is significantly lower with multiple infections than with single infection for both AVH and FHF. Multiple hepatotropic viral infection thus seems to produce less cholestatic illness and equal outcome if not better as compared to single virus infection. Whether this represents a phenomenon of mutual viral suppression or viral restitution occurring in multiple virus infection requires further studies [156]. However, another study reported that dual infection with hepatotropic viruses was associated with greater elevation of aspartate and ALTs [157].

Other Complications

Other reported complications include: Guillain–Barré syndrome, transverse myelitis, Bell's palsy [158], hemolysis both immune and noniummune, thrombocytopenia [159], and acute pancreatitis, which can be severe.

Autochthonous HEV in Developed Countries

The clinical features of autochthonous hepatitis E infection range from asymptomatic infection to mild hepatitis to subacute liver failure [72]. In a UK hospital-based study of patients with unexplained hepatitis, 40 patients with autochthonous hepatitis E were identified, of whom 75% were icteric [160]. The incubation period of autochthonous hepatitis E infection ranges from 2 to 9 weeks. The presentation of HEV in individuals infected in developed countries seems to be similar to that from endemic regions; however, the mortality rate is higher, ranging from 8 to 11%. Peak viremia occurs during the incubation period and the early acute phase of disease. Immediately before the onset of clinical symptoms, HEV RNA can be detected in the blood and stool. The concentration of serum liver enzymes rises, with a predominant transaminitis, peaking at about 6 weeks' postexposure before falling to normal levels by week 10. The abnormality in serum liver enzyme concentrations at presentation is variable and, except in the few patients who go on to develop liver failure, the rise in serum transaminases and bilirubin usually peaks at presentation. A few days to weeks after the onset of clinical symptoms, HEV RNA is cleared from the blood; however, the virus continues to be shed in stool for another 2 weeks [72]. The period of viremia can be very brief in some patients. It is probable that most infections are asymptomatic or anicteric, or both. This would account for the discrepancy between the perceived rarity of clinically apparent infections and the relatively high anti-HEV IgG seroprevalence in some developed countries.

Compared with hepatitis E infections in the developing world, in developed countries most autochthonous hepatitis E infections are reported in middle-aged and elderly men [72]. As in endemic regions, secondary and intrafamilial spread has rarely been reported. Most autochthonous HEV infections are self-limiting; however, comprehensive follow-up studies showed that approx. 15% patients develop complications [161]. Moreover, 8–11% of HEV-infected patients develop fulminant hepatitis and liver failure. The outcome can be poor in those individuals with underlying chronic liver disease, with mortality approaching 70% [161, 162]. Autochthonous hepatitis E in developed regions is frequently misdiagnosed as drug-induced liver injury, a common problem that occurs with increased frequency in elderly people. A retrospective analysis showed that 21% of 28 patients who met the standard criteria for drug-induced liver injury did not have the condition, but instead had autochthonous hepatitis E [163].

Clinical Significance of HEV Genotype

It is generally thought that the severity of hepatitis E depends on host factors of the infected patients such as pregnancy, chronic liver disease, and aging. However, similar to other known hepatitis viruses, viral factors may play a role in the pathogenesis of HEV-associated fulminant hepatitis. Recent studies suggest that the severity of hepatitis E is affected by the genotype of HEV, based on the finding that patients infected with genotype 4 HEV tend to have more severe disease including FHF than those infected with genotype 3 HEV in Japan [164]. It has been suggested that genotype 4 had a higher HEV load in the circulation than genotype 3, based on a case with coinfection with both the

genotypes [165]. If this is common for HEV virions circulating in humans, it may explain why patients with genotype 4 appear to have a higher incidence of fulminant hepatitis than those with genotype 3. However, more data will be needed with studies conducted in other countries with other patients in order to draw a plausible conclusion regarding the relationship between the severity of hepatitis E and the genotype of HEV.

Diagnosis and Detection of HEV

Diagnosis of HEV infection in individual patients remains problematic. Although HEV particles have been visualized by electron microscopy in the stools of infected human beings and there has been recent progress with cell culture, the routine laboratory diagnosis of hepatitis E depends on serology and nucleic acid amplification techniques. Tests for the diagnosis of HEV infection are based on either the detection of virus or viral components or on serological tests determining a virus-specific immune response in the host.

Approaches to HEV Detection

Virus or Viral Component Detection

Virus isolation is not appropriate for HEV because it is largely refractory to routine isolation in cell culture. The first assay for detection of HEV infection used IEM to detect viral particles in feces [166]. This provides a specific diagnostic marker, but has very poor sensitivity and is technically demanding.

Conventional and real-time RT-PCR assays have been used to detect HEV RNA in clinical specimens (mainly blood) and seem to be more sensitive than serology for hepatitis E diagnosis [167]. Assuming that contamination can be excluded, a positive result proves HEV infection and allows for further study including sequencing and genotyping of infecting viruses. However, the window of detectable HEV viremia is narrow, continuing for a mean of 28 days (range 17–48 days) after the onset of symptoms [55]. Since patients might not present until sometime after the onset of illness, a negative result does not exclude infection. Two highly sensitive and specific real-time PCR assays (TaqMan chemistry and Primer-Probe Energy Transfer-PriProEttechnique) have been developed to detect a wide range of HEV variants and simultaneous detection of all known four genotypes [168].

HEV antigen may have a role for diagnosis during the window period prior to sero-conversion to anti-HEV [169].

Serological Assays

Most primary serological testing uses an EIA format, although rapid immunochromatographic assays have been developed [170], which make near-patient and field testing feasible. A small number of commercial EIA assays are in general use and several "in-house" EIA assays have been developed. These assays use recombinant antigens derived from different strains of HEV. This diversity of strains should not affect the accuracy of the assays because it seems that HEV viruses of different genotypes constitute a single serotype.

Enzyme-linked immunosorbent assay (ELISA) kits based on ORF2 of HEV have been reported to have broad activity and reproducible results and are better than kits using a combination of ORF2 and ORF3. Two HEV assays are available worldwide commercially named Abbott immunoglobulin G (IgG) assay (Abbott Laboratories, Wiesbaden, Germany) and Genelabs IgG and immunoglobulin M (IgM) assay (Genelabs Diagnostics, Singapore). IgM serology is often used to identify acute cases, but it is not always detectable [171] and false-positive results occur [172]. The duration of IgM positivity varies between patients and also depends on the assays used [173], but strongly positive results are rare after 3 months [174]. Specific IgG is usually produced early in infection and concentrations rise rapidly afterwards [174]. Estimates of the duration of the IgG response and immunity to subsequent infection vary, but antibody has been detected for at least 12–14 years after acute infection [132]. This variation might result in part from differences in the assays used and from the extent of continued exposure to HEV. The negative seroconversion rate has been estimated at 1.4% per year in China [175] and secondary infections have been documented [176], implying that protection is not lifelong. IgG assays have been adapted to quantify the antibody response [177] and, although there is a WHO standard for measuring anti-HEV IgG [178], the protective levels of antibody have not yet been established. The ability to determine protective levels of vaccine-induced antibody will be useful if immunization against HEV is to be developed. Determination of antibody avidity in the early posthepatitis period can be used to distinguish between IgG produced following a recent or distant infection [174], but this technique requires further validation.

Detection of anti-HEV IgA can be a useful supplement for diagnosis of acute HEV infection, especially in patients negative for anti-HEV IgM [133].

Laboratory Diagnosis

The diagnosis of acute hepatitis E infection thus rests on the demonstration of specific IgM, rising levels of IgG, or detection of HEV RNA. A pragmatic case definition of acute

hepatitis E is a patient who has one or more of these features and a substantially raised serum transaminase concentration [72]. This definition will miss some cases, but is easy to apply and is rarely falsely positive. Other strategies under evaluation include IgA serology [133] and confirming positive EIA serology with an immunoblot assay [179], although this produces some equivocal results.

Differential Diagnosis

Acute hepatitis E cannot be clinically distinguished from other forms of acute viral hepatitis. Due to their identical clinical presentation and modes of transmission, hepatitis E may be suspected in the same circumstances as hepatitis A. While hepatitis A is more common than hepatitis E in the developed countries, in endemic areas, HEV is often the most common cause of acute hepatitis. In a patient with symptoms and biochemical evidence of acute hepatitis, serologic tests for excluding acute hepatitis A (IgM anti-HAV), B (HBsAg, IgM anti-HBc), C (anti-HCV), cytomegalovirus, and Epstein-Barr virus are obtained. In nonendemic areas, suspicion of acute HEV should be heightened by a history of travel to areas endemic for HEV. In endemic areas, an outbreak may be associated with a common contaminated water source, and such information should be sought. In endemic areas, infection with HEV may be seen in association with other hepatotropic viruses (A, B, and C), and in the absence of specific anti-HEV testing, the diagnosis of acute HEV coinfection or superinfection may be missed.

Natural History

In nonfatal cases, acute hepatitis is followed by complete recovery without chronic sequelae. Under certain circumstances, chronicity can occur (See above). There appears to be protection from reinfection for a time, but the duration of this protection is unknown. Long-term serologic studies will be needed to determine the duration of protective immunity and the nature of the anamnestic response.

Therapy and General Management

Like other forms of acute viral hepatitis, the mainstay of therapy is to monitor for the development of complications and provide good nutrition. The HEV infection is usually self-limited, and no specific therapeutic interventions are required. Rarely, if fulminant hepatitis develops, intensive management and options of liver transplantation should be considered.

Prevention

General Measures

Improved sanitation is important in controlling diseases that have oro-fecal transmission as a prominent part of their epidemiology. Industrialized countries with a generally high level of public sanitation do not experience epidemics of water-borne hepatitis E. Effective prevention relies primarily on maintaining a clean drinking water supply and paying strict attention to sewage disposal, because immunoprophylaxis is not currently available. Many epidemics in developing countries have occurred due to leakage of sewage pipes into municipal water supply pipes laid in the same or adjacent trenches. A barrier between these two supplies is essential for long-term prevention. During an epidemic, steps to improve water quality can lead to rapid abatement of the occurrence of new cases. During an epidemic in India, failure to chlorinate water was followed by a rapid rise in the number of cases, and reinstitution led to rapid abatement of the epidemic [67]. Since the virus is transmitted by the fecal–oral route, it must withstand exposure to bile salts during excretion and low pH during ingestion, but it is generally considered to be more labile than hepatitis A. Boiling water appears to inactivate HEV effectively. Chlorination may be ineffective in the presence of large amounts of organic matter, for example, in water contaminated with feces.

Travelers to endemic areas must take precautions against the consumption of contaminated water. They should be advised to avoid drinking water or beverages from sources of unknown purity. Only boiled or bottled water should be used. Although infection via food is much less common for hepatitis E than for hepatitis A, it is important to maintain caution about contaminated food and avoid eating uncooked shellfish, or eating uncooked and unpeeled fruits and vegetables. Women should avoid unnecessary travel to endemic areas during pregnancy. Isolation of affected persons is not indicated as person-to-person transmission is uncommon. However, infected persons should not be involved in food preparation or handling until their symptoms have fully resolved [12].

Passive Immunoprophylaxis

Although four genotypes of HEV have been identified, only one serotype has been described. In experimental studies in primates, passive transfer of anti-HEV has been shown significantly to reduce virus-shedding in feces and abrogate disease when given to nonhuman primates challenged with a high dose of homologous HEV [180]. Thus, immunoglobulin

(IG) preparations similar to those used for protection against hepatitis A would be efficacious against hepatitis E. However, no reduction in disease rates could be shown in pre- or postexposure prophylaxis studies among recipients of IG preparations manufactured in hepatitis E-endemic areas [181]. Failure of IG preparations to protect against HEV in humans may reflect the low titers of IgG anti-HEV in IG preparations derived from persons living in endemic areas. IG prepared in nonendemic countries would be expected to have even lower levels of anti-HEV and hence would be of little benefit as preexposure prophylaxis for travelers to endemic areas. Therefore, pooled normal human IG is unlikely to be useful as an immunoprophylactic agent against HEV and the protective role of anti-HEV antibodies in humans requires further study. The occurrence of large hepatitis E epidemics among adults in disease-endemic areas suggests either that anti-HEV antibody may not be fully protective or that antibody levels decline with time and gradually reach an unprotective level.

As an alternative, an IG preparation consisting of HEV-neutralizing monoclonal antibodies (MAbs) might protect against hepatitis E [182]. Clinical trials of MAbs have not been done and further work is necessary before a definitive role of immune serum globulin can be discerned.

Active Immunoprophylaxis

Although four HEV genotypes and significant geographic genome variability have been described, all HEV subtypes share major cross-reactive epitopes, prompting the development of a recombinant HEV (rHEV) vaccine based on the capsid protein [183]. The HEV genome contains three ORFs. The ORF1 encodes nonstructural protein(s) and, therefore, ORF1 protein(s) would not be a target for humoral immunity. ORF3 overlaps with ORFs 1 and 2 and encodes a small protein of unknown function but significant antigenicity. However, antibodies to ORF3 do not neutralize virus in in vitro assays, whereas antibody to ORF2 does. ORF2 encodes the capsid protein and, since the ORF2 protein is the major protein in the virion, it has been the focus of vaccine development.

The various options for a vaccine include attenuated or killed virus vaccine, recombinant protein-based vaccine, and nucleic acid-based vaccines. The development of an attenuated or killed virus vaccine is not currently feasible, as an efficient cell culture system for HEV does not exist. Therefore, either a recombinant protein-based or nucleic acid-based vaccine is needed. Most work has focused on recombinant vaccines.

The different recombinant vaccines include prokaryote (*E. coli*) or eukaryote (insect cell-derived and plant-derived) vaccines. Two candidate HEV vaccines produced with truncated structural proteins have reached clinical trials. One of the vaccines (aa 112–607) is expressed in insect cells [184]. A genotype 1 HEV recombinant protein (rHEV) vaccine, which provided protection in nonhuman primates [125], was found to be immunogenic in humans [185]. These results prompted a phase II clinical trial of the vaccine's efficacy in volunteers from the Nepalese Army, a population at high risk for hepatitis E. A total of 1,794 subjects (898 in the vaccine group and 896 in the placebo group) received three vaccine doses; the total vaccinated cohort was followed for a median of 804 days. After three vaccine doses, hepatitis E developed in 69 subjects, of whom 66 were in the placebo group. The vaccine efficacy was 95.5% (95% confidence interval [CI], 85.6–98.6). In an intention-to-treat analysis that included all 87 subjects in whom hepatitis E developed after the first vaccine dose, nine subjects were in the vaccine group, with a vaccine efficacy of 88.5% (95% CI, 77.1–94.2). Thus, this vaccine was found to be safe and immunogenic for humans and could also protect against hepatitis E [186]. Yet, some questions about the "true" efficacy remain unanswered. Studies from rhesus monkeys revealed that rHEV may protect from clinical disease, but not from HEV infection [187, 188]. As in the study by Shrestha et al. only symptomatic subjects were screened for HEV infection, the potential number of asymptomatic cases, who could potentially carry and spread the virus, is unknown [189]. The duration of protection by the rHEV vaccine is also unclear; whereas 100% of screened rHEV-vaccinated subjects had high antibody titer 1 month after the third vaccination (81% after the second vaccination), only about 56% had HEV antibodies at the end of the study, about 1.5 years after the third dose [190].

The other vaccine is expressed in bacteria (HEV 239 vaccine) and it is more extensively truncated (aa 376–606) than the insect cell expressed vaccine [191]. The vaccine consists of 23 nm virus-like particles, which are strongly reactive with acute and convalescent sera from hepatitis E patients and are recognized by a panel of HEV-specific murine MAbs, including at least two neutralizing antibodies [192–194]. It evoked a vigorous T cell-dependent antibody response in mice, and this was partly attributed to its particulate nature and at least two T cell epitopes locating to aa 533–552 [195]. The vaccine was found to confer protection on nonhuman primates against infection by genotypes 1 and 4 HEV and sera from vaccinated animals were found to neutralize infectivity of the virus for the animals [196]. A randomized controlled phase II clinical trial of the vaccine was conducted in southern China and it was found that the vaccine is safe and immunogenic for humans. A course consisting three 20 μg doses of the vaccine induced 100% seroconversion. The mean antibody level of 15.9 U/ml induced by the vaccine was lower than that of 43.4 U/ml determined for the pooled serum from hepatitis E patients, but markedly higher than

that of 0.76 U/ml in the pooled sera from seropositive subjects. The results reflect the response by subjects, who had not been previously infected by HEV and had not been significantly confounded by new HEV infection occurring during the course of the study. The significant reduction in frequency of new infection occurring after receipt of the second vaccine dose suggests that vaccination could prevent HEV infection. A phase 3 trial using this recombinant vaccine (HEV 239) from China, assessed the efficacy and safety of the vaccine in a general population of healthy men and women (aged 16–65 years). 48 693 participants in the vaccine group and 48 663 participants in the placebo group received three vaccine doses and were included in the primary efficacy analysis. During the 12 months after 30 days from receipt of the third dose 15 per-protocol participants in the placebo group developed overt hepatitis E compared with none in the vaccine group. Vaccine efficacy after three doses was 100%. [Zhu F-C, Zhang J, Zhang X-F, et al. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomized, double-blind placebo-controlled, phase 3 trial. *Lancet* 2010; 376: 895–902.]. However, the duration of protection afforded by this vaccine remains unknown. Also whether this vaccine protects from HEV infection also remains unknown.[197]

It is generally encouraging that rHEV has been effective in preventing hepatitis E disease in a high-risk population. Who could and who should benefit from this vaccine? The contribution of HEV to overall morbidity is still a matter of debate, because the vast majority of HEV-related diseases are benign. However, HEV superinfection in patients with chronic liver disease can cause severe hepatic decompensation. Therefore, vaccination of these patients would be desirable. Also, international travelers to endemic regions and especially development aid volunteers would likely benefit from the vaccine. Other groups at risk of HEV infection include residents of areas with extended community outbreaks, refugees residing in overcrowded temporary camps following catastrophies in endemic areas, or pregnant women without prior infection in these regions. The cost of rHEV, whenever available, will be a crucial determinant for the use of rHEV in endemic countries.

Summary, Conclusions, and Directions for the Future

HEV remains an important cause of both sporadic and epidemic viral hepatitis in poor developing and underdeveloped countries with poor sanitation and other civic amenities. It is also an important cause of maternal mortality in those countries. The incidence of autochthonous hepatitis E in developed countries is unknown, as is the mode of infection. Recent studies have revealed that hepatitis E is a zoonosis and multiple HEV strains with significant sequence divergence are circulating in both humans and animals throughout the world. Hepatitis E in developed regions is far more common than previously recognized and could have a zoonotic source. In certain areas like Japan where people have peculiar habits of ingesting undercooked, or even raw, meat or viscera of animals, it is suggested that zoonotic food-borne transmission of HEV plays an important role in the occurrence of cryptic hepatitis E. Molecular epidemiological studies suggested that the genetic changes and takeover of particular HEV strains may contribute to the genetic variability of HEV in the community. However, further clinical, epidemiological, and virological studies are needed to elucidate the extent of genomic heterogeneity of HEV strains circulating in the world, the various and possibly region-dependent modes of HEV transmission, and the association of HEV genotype with disease severity and its underlying mechanism.

Significant research is being done to develop an HEV vaccine and is likely to be available soon. It would be a boon for select groups of individuals like pregnant women, patients with preexisting liver disease, and for travelers to endemic areas.

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Chapter 41
Autoimmune Hepatitis

Albert J. Czaja

Introduction

Autoimmune hepatitis is the result of an immune response that is misdirected against normal self-antigens or foreign antigens that resemble self-antigens [1–6]. Its onset requires multiple deficiencies in a highly complex and interactive counter-regulatory network, and it is the consequence of genetic factors [7], molecular mimicry [2, 8], cytokine imbalances [9], and imprecise (or promiscuous) targeting of activated immunocytes (Fig. 41.1) [10, 11]. Single disturbances in a highly coordinated interactive system can be disruptive, and subtle perturbations can magnify into a disease state as defects in counter-regulatory homeostatic mechanisms cascade [4]. These concepts are derived from "chaos theory," [12] and they apply to autoimmune hepatitis.

The etiology of autoimmune hepatitis is unknown, and even the predominant mechanisms of liver cell injury are debated [1–6]. Multiple infectious [13–18] and toxic [19–25] agents have been implicated in its production, and these observations support the concept that autoimmune hepatitis is caused by a short epitope that is commonly found or mimicked in the environment [2, 4]. The rarity of the condition, despite the ubiquitous nature of its presumed triggering antigen, has suggested that host predisposition is crucial for its occurrence (Fig. 41.1) [7, 26]. The diverse clinical phenotypes of autoimmune hepatitis in different geographical regions and ethnic groups have implicated indigenous etiological agents, region-specific socioeconomic conditions, and race-predominant genetic traits as factors that modify and individualize the disease [27–31].

Cross-reactivities between the antibodies expressed in autoimmune hepatitis and those in acute and chronic viral hepatitis have supported the hypothesis that molecular mimicries between infectious agents and self-antigens can sensitize the host-derived immunocytes [8, 32]. Repeated contact with the same or similar antigen by infection or environmental exposure could eventually override self-tolerance and result in autoimmune hepatitis and other concurrent immune diseases in anatomically distant organs [2, 6, 8]. Disturbances in both the humoral and cellular mechanisms of immune homeostasis are necessary to account for the clinical, laboratory, and histological manifestations of the disease (Fig. 41.1) [2, 4, 6, 33, 34].

Autoantibodies are the diagnostic hallmarks of autoimmune hepatitis, but they are not pathogenic or disease-specific [35, 36]. Their expression in autoimmune hepatitis may simply reflect immune reactivity and the secondary host response to self-antigens released during severe liver injury [37]. Alternatively, they may be imprints of a critical pathogenic mechanism that is contributing to liver cell destruction [38]. An antibody-dependent cell-mediated form of cytotoxicity has been implicated in autoimmune hepatitis in which the clonal expansion of plasma cells results in excess production of immunoglobulins (Fig. 41.1) [2, 33, 34]. The immunoglobulins then bind to normal membrane constituents of the hepatocyte membrane, and the antibody–antigen complex attracts natural killer cells that provoke cytolysis [39, 40]. This humoral mechanism of liver cell injury fluctuates in intensity during the course of the disease, possibly due to shifts in the cytokine milieu, and it is insufficient to explain autoimmune hepatitis [2, 6, 9, 34].

A cell-mediated cytotoxicity has also been described in autoimmune hepatitis in which disturbances in CD4 T helper cell activation, differentiation, and proliferation result in the clonal expansion of liver-infiltrating cytotoxic T lymphocytes [1, 2, 4, 33]. Please see Chap. 27 for role of inflammation in liver injury. These activated CD8 cells are antigen-specific, and they release lymphokines within the liver that promote hepatocyte death. Molecular mimicry between foreign and self-antigens has been invoked as a basis for the cellular mechanism of autoimmunity [41, 42], but cross-reactivity between activated immunocytes in viral and autoimmune diseases has been more difficult to demonstrate than cross-reactivity between autoantibodies [43–45]. The relative importance of antibody-dependent and cell-mediated forms of cytotoxicity may vary during the course of the disease and its treatment [2, 6, 33, 34].

A.J. Czaja (✉)
Mayo Clinic College of Medicine,
200 First Street SW Rochester, Minnesota 55905
e-mail: czaja.albert@mayo.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_41, © Springer Science+Business Media, LLC 2011

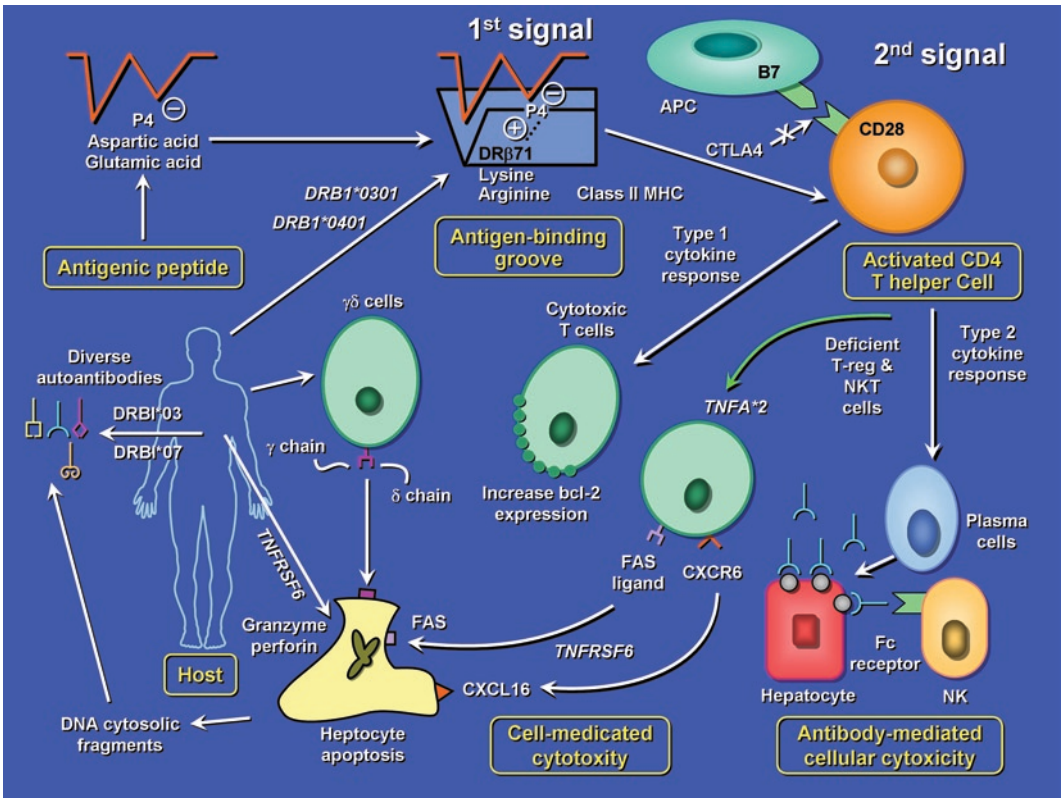

Fig. 41.1 Interactive genetic, cellular and molecular mechanisms for type 1 autoimmune hepatitis in White North American and northern European adults. The genetic predisposition of the host (DRB1*0301 and DRB1*0401) favors the selection of a negatively charged peptide with aspartic acid or glutamic acid at position P4. The favored peptide is displayed in the antigen-binding groove of a class II molecule of the major histocompatibility complex (MHC) where a salt bridge juxtaposes position P4 against a positively charged lysine or arginine at position DRβ71. Two costimulatory signals must then be completed for activation of the CD4 T helper cell. The first signal requires ligation of the antigen-presenting cell (APC) and the autoantigen, and the second signal requires ligation of CD28 on the CD4 T helper cell with B7 on the APC. Cytotoxic T lymphocyte antigen-4 (CTLA-4) may be deficient in function or quantity, and completion of the second costimulatory signal may be enhanced. Natural killer (NK) cells contribute to antibody-mediated cellular toxicity, and deficiencies in the number and function of T-regulatory (T-reg) cells and natural killer T (NKT) cells promote a type 1 cytokine response and a cell-mediated cytotoxicity. Polymorphisms of the tumor necrosis factor alpha gene (TNFA*2) and tumor necrosis factor receptor super family gene (TNFRSF6) facilitate the type 1 cytokine pathway and apoptosis of hepatocytes by stimulating the FAS receptor-mediated pathway of programmed cell death. The chemokine, CXCL16, within the liver enhances the trafficking of immunocytes bearing the chemokine receptor, CXCR6, to the target organ. The increased surface expression of the antiapoptotic protein, bcl-2, protects the cytotoxic T cell from programmed cell death, and gamma delta (γδ) cells pursue antigenic targets on the hepatocyte without prior sensitization or after presentation by nonclassical MHC

The goal of this review is to describe the genetic, cellular, and molecular factors that influence the antibody-dependent and cell-mediated forms of cytotoxicity in autoimmune hepatitis. In this fashion, the fragments of scientific evidence can be sorted and a composite picture of pathogenesis developed.

Types of Autoimmune Hepatitis

Two types of autoimmune hepatitis have been described based on their serological markers [46]. These types have not been established as valid pathological entities, but the designations have been useful as clinical descriptors and research labels. Their continued separation is justified in this review because each has been ascribed distinctive genetic predispositions and antigenic targets.

Type 1 autoimmune hepatitis is the most common form worldwide, and it is characterized by the presence of antinuclear antibodies (ANA) and/or smooth muscle antibodies (SMA) [46]. Type 1 autoimmune hepatitis has a global distribution, and it affects all age groups, including infants and the elderly [47]. Its genetic predispositions have been described, but its triggering antigen has not been identified [7].

Type 2 autoimmune hepatitis is characterized by the presence of antibodies to liver/kidney microsome type 1 (anti-LKM1) [48]. It has also been associated with antibodies

to liver cytosol type 1 (anti-LC1) [49]. Type 2 autoimmune hepatitis affects mainly children, and it is most common in Northern Europe and Italy [48, 50, 51]. Only 4% of adults with autoimmune hepatitis in the United States express anti-LKM1 [52, 53]. Children with type 2 autoimmune hepatitis may have cholangiographic abnormalities ("autoimmune sclerosing cholangitis") [54], which may affect their outcome [55].

Genetic predispositions for type 2 autoimmune hepatitis have been described [56–58], and the target autoantigen has been identified as the cytochrome monooxygenase, CYP2D6 [59–61]. Five epitopes have been found on recombinant CYP2D6 which are recognized by anti-LKM1 [62], and the amino acid sequence between positions 193–212 predominates [63]. Formiminotransferase cyclodeaminase is the target of anti-LC1 [64–66], and a murine model of autoimmune hepatitis has been developed using the antigenic components of human CYP2D6 and human formiminotransferase cyclodeaminase [67].

Autoimmune hepatitis can also be part of a syndrome characterized by multiple endocrine organ failure, mucocutaneous candidiasis, and ectodermal dystrophy [68]. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is caused by a single-gene mutation located on chromosome 21q22.3. This mutation affects the generation of the autoimmune regulator (AIRE) [68, 69]. AIRE is a transcription factor that is expressed in epithelial and dendritic cells within the thymus, and it regulates the negative selection of autoreactive T cells. APECED has an autosomal recessive pattern of inheritance; its autoantigens are CYP1A2 and CYP2A6; and antibodies to liver membrane (anti-LM) typify the syndrome [70–72]. Autoimmune hepatitis in this context constitutes a separate category of autoimmune hepatitis that will not be considered further in this chapter [73].

Determinants of Antigen Selection and Recognition

The professional antigen-presenting cells (dendritic cells, macrophages, and B lymphocytes) are specialized to internalize, process, and present antigens to uncommitted CD4 T helper cells [26, 74, 75]. The antigenic peptides are presented by molecules of the major histocompatibility complex (MHC) in antigen-binding grooves expressed on the cell surface [11, 76, 77]. These binding grooves are genetically encoded, and their structural and electrostatic properties affect antigen selection and presentation [26, 78, 79]. The nature and density of the complexes of antigen and MHC on the surface of the antigen-presenting cells in turn influence immunocyte activation [75]. The alleles encoding the antigen-binding grooves thereby determine the nature and vigor of the immune response (Table 41.1) [79].

Table 41.1 Genetic factors in autoimmune hepatitis inside and outside the MHC

Genetic factor	Attributes	Consequences
*DRB1*0301*	Susceptibility alleles in USA, Britain Linked with *DRB3*0101* Encodes LLEQKR at DRβ67–72	Associated with two alleles encoding lysine at DRβ71
*DRB1*0401*	Susceptibility alleles in USA, Britain Linked with *DRB4*0103* Encodes LLEQKR at DRβ67–72	Associated with one allele encoding lysine at DRβ71
*DRB1*0404*	Susceptibility alleles in Mexico, Japan, China	Arginine replaces lysine at DRβ71 and attenuates disease
*DRB1*0405*	Encode LLEQRR at DRβ67–72	
*DRB1*1501*	Protective allele in USA, Britain Encodes ILEQAR at DRβ67–72	Protects against type 1 AIH
*DRB1*1301*	Susceptibility allele in South America Encodes ILEDER at DRβ67–72	Type 1 AIH, mainly children Impairs clearance of HAV
*DRB1*07*	Linked to *DQB1*0201* and *DRB1*03*	Anti-LKM1 in type 2 AIH and chronic hepatitis C
*DQB1*0201*	Susceptibility allele associated with *DRB1*07* and *DRB1*03*	Type 2 AIH
CTLA-4	Polymorphism with guanine for adenine at position 49 of first exon	Enhances second costimulatory signal
*TNFA*2*	Polymorphism with adenine for guanine at position −308 Found in USA and Britain	Increase TNF-α and strengthen type 1 cytokine pathway
TNFRSF6	Polymorphism with adenosine to guanine substitution at position −670 Found in USA, Britain	Impaired apoptosis of immunocytes Early progression to cirrhosis

AIH autoimmune hepatitis, *CTLA-4* cytotoxic T lymphocyte antigen 4 gene, *HAV* hepatitis A virus, *LKM1* liver/kidney microsome type 1, *MHC* major histocompatibility complex, *TNFA* tumor necrosis factor alpha gene, *TNF-α* tumor necrosis factor alpha, *TNFRSF* tumor necrosis factor receptor super family gene, *USA* United States of America

Nature of the Antigen-Binding Groove

Susceptibility to autoimmune hepatitis is related in part to the nature of the MHC class II molecules that display peptide for immunocyte recognition [11, 26, 78, 80, 81]. These molecules are heterodimeric glycoproteins that have an invariant DRα chain and a polymorphic DRβ chain [26]. Three hypervariable regions in the DRβ chain affect the nature of the antigen that can be displayed [80, 81]. Hypervariable region 3 (HVR3), constituting residue sequences at positions 67–74 on the DRβ polypeptide chain, is located on the α-helical portion of the DRβ polypeptide, and it is at a critical contact point between the cradled antigen and the T cell antigen receptor (TCR) of the uncommitted CD4 T helper cell. Variations in the amino acid sequence in HVR3 (DRβ 67–74) are allele-specific and associated with susceptibility to autoimmune hepatitis [26].

Genetic Determinants of the Antigen-Binding Groove

High resolution DNA techniques have demonstrated that susceptibility to type 1 autoimmune hepatitis in White North American and northern European adults is related to HLA DRB1*03 and DRB1*04 [82, 83]. The susceptibility alleles reside on the *DBR1* gene, and they are *DRB1*0301* and *DRB1*0401* (Table 41.1) [84, 85]. Analyses of the variations in amino sequences encoded by these susceptibility alleles have indicated that type 1 autoimmune hepatitis in the United States and Britain is associated with the six amino acid sequence; encoded as L (leucine), L (leucine), E (glutamic acid), Q (glutamine), K (lysine), and R (arginine), at positions 67–72 in the HVR3 region [78, 84]. The *DRB1*0301* and *DRB1*0401* alleles each encode the same LLEQKR sequence in this region (Table 41.1). Lysine (K) at position DRβ71 is at the critical contact point between the antigen, class II MHC molecule, and TCR, and it is the key residue that affects susceptibility to type 1 autoimmune hepatitis in White North American and northern European adults [75, 79].

Antigen Selection and Presentation

The six amino acid sequence between DRβ67 and 72 can affect the nature of the antigen selected for presentation [26, 76]. The class II MHC molecules have strong biases for those amino acids in the antigenic peptide at positions P1, P2, P6, P7, and P9 [80, 81]. Amino acid residues at these positions that complement the amino acid residues encoded in the antigen-binding groove will optimize display of the antigen and activation of CD4 T helper T cells. The amino acid sequence of LLEQKR at the DRβ67–72 position in HVR3 restricts the range of peptides that can be optimally presented to those containing the negatively charged residues, aspartic acid or glutamic acid, at position P4 [26]. The antigenic trigger for type 1 autoimmune hepatitis can be modeled by understanding the amino acid components that must be present for optimal display in the antigen-binding groove (Fig. 41.1) [76, 80, 81].

Multiple peptides with variable binding affinities can be presented by the same class II MHC molecule [26]. Optimal presentation implies an ideal structural and electrostatic fit of the peptide within the antigen-binding groove. The vigor of immunocyte activation can be enhanced by the creation of dimers that contain the antigen–MHC complex on the surface of the antigen-presenting cells [75]. These dimers facilitate simultaneous stimulation of the immunocyte at multiple sites, and they may intensify the immune response [79, 86].

Diverse Susceptibility Alleles for Type 1 Autoimmune Hepatitis

Type 1 autoimmune hepatitis in different geographical regions has different susceptibility alleles (Table 41.1). In some regions, the allelic differences result in only subtle changes in the structural and electrostatic properties of the antigen-binding groove, and these changes would be unlikely to greatly affect the nature of the antigenic trigger [7, 26, 75, 79]. In other geographical regions, the susceptibility alleles encode markedly different antigen-binding grooves [79, 86]. Individuals in these regions may be naturally selected to develop autoimmune hepatitis after exposure to an indigenous or unique antigenic trigger.

The susceptibility alleles for type 1 autoimmune hepatitis in Japan [87–89], mainland China [90], and Mexico [91] are *DRB1*0404* and *DRB1*0405,* which encode an arginine (R) for lysine (K) at the DRβ71 position (Table 41.1). Arginine (R) is a polar amino acid that is structurally similar to lysine (K), and its substitution for lysine at position DRβ71 would not greatly alter the steric and electrostatic properties of the class II MHC molecule. Consequently, the susceptibility alleles associated with type 1 autoimmune hepatitis in Japan, mainland China, and Mexico encode a six amino acid motif (LLEQRR) that is similar to the sequence (LLEQKR) encoded by the *DRB1*0301* and *DRB1*0401* alleles in White North American and British patients. These similar antigen-presenting capabilities suggest that the type 1 autoimmune

hepatitis among adults in these geographical regions is triggered by a similar antigenic peptide.

In contrast, type 1 autoimmune hepatitis in Argentina (especially among children) [92, 93], Brazil [57, 94–96], and Venezuela [97] is associated with the *DRB1*1301* allele (Table 41.1). This allele encodes the amino acid sequence ILEDER at positions DRβ67–71 where isoleucine (I) replaces leucine (L) at position DRβ67, aspartic acid (D) replaces glutamine (Q) at position DRβ70, and glutamic acid (E) replaces lysine (K) at position DRβ71 within the antigen-binding groove. These negatively charged amino acid residues within the ILEDER motif favor presentation of antigens different from those accommodated by the LLEQKR motif.

The hepatitis A virus (HAV) has been implicated as a cause of autoimmune hepatitis [15–18], and HAV infection is endemic in South America, especially among children [98]. The *DRB1*1301* allele has been associated with protracted HAV infection [99], and prolonged exposure to viral and liver antigens released during HAV infection might promote the loss of self-tolerance and the development of type 1 autoimmune hepatitis in individuals with *DRB1*1301*. The disparity between the susceptibility alleles in the children and the adults with type 1 autoimmune hepatitis in Argentina supports this hypothesis [92, 93].

Other genetic susceptibility factors outside HLA DRB1*03 and DRB1*04 have been associated with the type 1 autoimmune hepatitis of North America [100–102], Germany [103], India [104, 105], Italy [106], Taiwan [107], and Turkey [108], and these findings illustrate the complexity of the genetic bases for autoimmune hepatitis and the diversity of antigens that are likely to trigger it. Genetic analyses have focused mainly on alleles of the MHC, but a variety of polymorphisms and point mutations outside the MHC have also been implicated [7]. Furthermore, the mapping of the human genome [109] and the emergence of cDNA microarray technology [110] and genome-wide DNA microsatellite techniques [111] have already increased the number of candidate alleles and disease-modifying genetic factors associated with type 1 autoimmune hepatitis.

Protective Genetic Factors for Type 1 Autoimmune Hepatitis

Genetic factors can also protect against the occurrence of type 1 autoimmune hepatitis in certain populations by encoding an antigen-binding groove that does not accommodate the presentation of the prevalent triggering antigen. *DRB1*1501* confers resistance to type 1 autoimmune hepatitis in White adult patients from Britain and the United States (Table 41.1) [78, 84]. This allele encodes the sequence, ILEQAR, at DRβ positions 67–72, and the substitution of an isoleucine (I) at DRβ67 and alanine (A) at DRβ71 changes the "susceptibility motif" into a "resistance motif." The substitution of isoleucine (I) for leucine (L) at DRβ67 is a nondisruptive replacement since each amino acid is similarly charged. However, the substitution of a neutral nonpolar amino acid, alanine (A), for the highly charged polar amino acid, lysine (K) or arginine (R), at DRβ71 could impact on antigen-binding, TCR recognition, and CD4 T helper cell activation. These findings indicate that propensity for type 1 autoimmune hepatitis can be sensitive to single amino acid substitutions at a critical site of immune recognition.

The protective alleles for type 1 autoimmune hepatitis can differ among different geographical regions and ethnic groups. In Turkey, HLA B8 may protect against the disease [108], whereas in Germany HLA DQ2 may be protective [103]. The burgeoning number of susceptibility and protective alleles of the MHC associated with type 1 autoimmune hepatitis attests to the diversity of antigenic triggers for the disease and the inability of any one gene to explain its occurrence. Type 1 autoimmune hepatitis is a complex polygenic disorder in which genetic predisposition colors, but does not cause the disease. The implicated genetic factors are present in the normal population [7, 82], and the disease has a low familial occurrence [112–114].

Genetic Associations with Type 2 Autoimmune Hepatitis

Type 2 autoimmune hepatitis has been associated with *DRB1*07* in Brazil [57], Britain [115], and Germany [56], and with *DRB1*03* in Spain [116]. *DQB1*0201* is in strong linkage disequilibrium with *DRB1*07* and *DRB1*03*, and it has been proposed as the principal genetic determinant of the disease [58, 117]. The association of a single disease with multiple alleles suggests that various aspects of the condition are modified by individual alleles (Table 41.1).

*DRB1*07* has been associated with the production of anti-LKM1 in children with type 2 autoimmune hepatitis [115] and in Italians with chronic hepatitis C [50], whereas the production of anti-LC1 has been associated with *DRB1*03* [58]. The genetic factors that influence the serological manifestations of the disease may be different from the principal determinant that affects the selection, presentation, and recognition of the triggering antigen. In type 2 autoimmune hepatitis, this principal factor may be *DQB1*0201* [58, 117]. The low occurrence of anti-LKM1 in North American patients with autoimmune hepatitis [52] and in chronic hepatitis C [50, 53] may reflect the lower frequency of HLA DRB1*07 in the normal population of North America than in Europe [50].

Antigen Recognition

The triggering antigen for autoimmune hepatitis is recognized by the CD4 T helper cell when contact is made between the TCR and the antigen–MHC molecular complex [118]. The ligation involves residues of the antigenic peptide, the α-helical region of the class II MHC molecule, and the complementarity-determining regions (CDR) of the α- and β-chains of the TCR [11, 26, 119–121]. The three variable CDR loops of the α-chain (Vα) and CDR3 of the β-chain (Vβ) contact the α-helix of the MHC molecule, and the CDR1 and CDR3 loops of both the α- and β-chains of the TCR contact the bound peptide. Molecular modeling suggests that the α- and β-chains of the TCR fit diagonally over the peptide–MHC complex and that CDR3 contacts the amino acid residue at position DRβ71 in the antigen-binding groove and the amino acid residue at position P4 in the antigen [119–121]. This model supports the relevance of the polar, positively charged amino acid residue (lysine or arginine) at position DRβ71 and the negatively charged residue (aspartic acid or glutamic acid) at position P4 in the development of type 1 autoimmune hepatitis [122, 123].

The CDR of the TCR is genetically encoded [124], and patients with type 1 autoimmune hepatitis have different CDR than normal individuals [125]. Homozygosity for the genetic polymorphism encoding the TCR constant β (Cβ) region (*Bgl II*) occurs more commonly in patients with autoimmune hepatitis than in normal individuals (42% versus 21%, $P<0.0075$), and heterozygosity for the TCR Cβ polymorphism (*Bgl II*) protects patients aged <30 years with HLA DRB1*03 from developing early-onset disease [124].

Assessments of the nucleic acids of liver tissue and peripheral blood-derived T cells from untreated patients with autoimmune hepatitis and normal individuals have demonstrated overexpression of TCR Vβ3 in autoimmune hepatitis and enrichment of the liver tissue with this gene product [125]. Furthermore, determinations of the nucleotide and amino acid sequences of the Vβ chain of CDR3 have disclosed a fixed amino acid motif, aspartic acid (D)-arginine (R)-proline (P), at the junctional region 1.2 of the Vβ3 chain of the TCR in Japanese patients with HLA DRB1*04 [126].

The liver-infiltrating lymphocytes associated with type 1 autoimmune hepatitis in Japan can be characterized by overexpression of Vβ3 of the TCR and the presence of the three amino acid motif, D-R-P, at the junctional region 1.2 of Vβ3 [126]. These findings illustrate another mechanism by which antigen recognition and immunocyte activation can be affected in autoimmune hepatitis, and they indicate an additional pathway by which the genetic predisposition of the patient can influence the occurrence and severity of the disease. Further characterization of the lymphocytes that have been compartmentalized in the liver tissue may help identify the molecular target of the autoimmune response.

Candidate Autoantigens

The cytochrome monooxygenase, CYP2D6, is expressed within the endoplasmic reticulum [59] and on the plasma membrane of hepatocytes [127, 128]. Antibodies to LKM1 inhibit its function in vitro [129], and CD4 T helper cells in the blood [130] and liver tissue [131] have specificity against it. The recombinant molecule has 5 epitopes that react with anti-LKM1, and the epitope between 193 and 212 is targeted in 93% of patients with anti-LKM1 [63]. Furthermore, a murine model that resembles human autoimmune hepatitis is based on human CYP2D6 and formiminotransferase cyclodeaminase [67]. These observations have justified the recognition of CYP2D6 as the antigenic target in type 2 autoimmune hepatitis.

Homologies exist between recombinant CYP2D6 and the peptide sequences within the hepatitis C virus, cytomegalovirus, and herpes simplex type 1 virus [62, 115], and these molecular mimicries can result in cross-reacting antibodies. Antibodies to LKM1 are found in 10% of patients with chronic hepatitis C in Europe [62, 132, 133], but they are virtually absent in similar patients from North America [53]. The epitopes of CYP2D6 that are predominantly targeted by anti-LKM1 in chronic hepatitis C differ from those targeted by anti-LKM1 in type 2 autoimmune hepatitis [132]. Furthermore, the structure of the hepatitis C virus associated with anti-LKM1 is similar to that of the structure of the virus infecting patients without anti-LKM1 [134, 135]. The genetic predisposition of the host rather than the nature of the virus or liver disease may be the determining factor for anti-LKM1 expression [135], and HLA DRB1*07 has been associated with its detection [56–58].

The homologies between recombinant CYP2D6 and the structures of different viruses support the hypothesis that repeated viral infection may be a mechanism by which to break self-tolerance and induce type 2 autoimmune hepatitis [2, 6, 8]. Cross-reactivity has also been demonstrated between HCV antigens and host-derived smooth muscle and nuclear antigens [136], and HLA B51 has been associated with cross-reactive immune responses between viral and microsomal antigens [32]. These latter observations support speculation that viral infection in a genetically predisposed host may also induce type 1 autoimmune hepatitis. Chronic hepatitis B and C frequently have concurrent autoantibodies and immune-mediated diseases that resemble those found in type 1 autoimmune hepatitis [13, 137–141], and the hepatitis A virus has been implicated as an etiological agent in this form of autoimmune hepatitis [15–18]. Pertinent homologies, however, between self-antigens and viral components have not yet been described in type 1 disease.

Various other cytosolic enzymes have been implicated as autoantigens, including uridine triphosphate glucuronosyltransferases [142], glutathione S-transferases [143],

formiminotransferase cyclodeaminase [65, 67], and the transfer ribonucleoprotein complex, $tRNP^{(Ser)Sec}$ [144]. Furthermore, asialoglycoprotein receptor (ASGPR) remains a candidate autoantigen as it is expressed on the hepatocyte membrane and active in the uptake, transport, and export of diverse peptides [145–147]. Antibodies to ASGPR correlate closely with the histological activity of the disease [148, 149]; they are present in type 1 and type 2 autoimmune hepatitis [146, 147]; and their absence [150] or disappearance [148] during therapy is associated with quiescent disease. Each candidate antigen has the potential to generate an autoreactive response that may overcome self-tolerance.

Disturbances in Immunocyte Activation

CD4 T helper cells must be sensitized to the autoantigen before autoimmune hepatitis can develop, and multiple counter-regulatory control mechanisms must be breached before the autoreactive response can mature [4]. Two costimulatory signals must be completed for activation of the immunocyte, and each signal can be altered in autoimmune hepatitis. Signal I requires recognition of the antigen–MHC molecular complex by the TCR of the CD4 T helper cell, and signal II requires ligation of the CD28 molecule on the surface of the CD4 T helper cell with one of the B7 molecules on the surface of the antigen-presenting cell (Fig. 41.1).

Completion of signal I can be affected by the structural and electrostatic properties of the antigen-binding groove of the class II MHC molecule and the nature of the TCR, each of which is encoded by host-dependent alleles [4]. A vigorous immune response can be anticipated if the autoantigen is optimally presented; dimers of the antigen-presenting complex form across the surface of the antigen-presenting cell; and the structure of the Vβ3-chain in the TCR contains an amino acid motif that complements the residues of the displayed antigen.

Completion of signal II requires ligation of the CD28 molecule on the surface of the CD4 T helper cell with B7–1 (CD80) or B7–2 (CD86) on the surface of the antigen-presenting cell (Fig. 41.1) [4]. Cytotoxic T lymphocyte antigen-4 (CTLA-4) competes with CD28 for the B7 ligands. The expression of CTLA-4 is genetically determined, and this gene product can dampen the activation of CD4 T helper cells by inhibiting the second costimulatory signal [151–154].

In White North American and northern Europeans with autoimmune hepatitis, a polymorphism of the *CTLA-4 gene* is strongly associated with *DRB1*0301*, and it may favor autoreactivity (Table 41.1) [155, 156]. The substitution of a guanine for an adenine at position 49 in the first exon of the *CTLA-4 gene* results in a threonine for alanine substitution in the expressed protein, and this variation in the gene product may be sufficient to impair its inhibitory effects on immunocyte activation.

The same polymorphism of the *CTLA-4 gene* described in autoimmune hepatitis has been found in British [157] and North American [158, 159] patients with primary biliary cirrhosis, but not in Brazilian [160] or Japanese [161, 162] patients with type 1 autoimmune hepatitis. The *CTLA-4 gene* may be one of many genetic modifiers outside the MHC that lack disease specificity and that manifest ethnic variability. Other perturbations in the two costimulatory signals are likely since the activation sequence involves multiple structural, electrostatic, and genetic interactions.

Disturbances in Immunocyte Differentiation

The differentiation and proliferation of activated CD4 T helper cells are accomplished along cytokine pathways that result in their clonal expansion into liver-infiltrating cytotoxic T cells (the type 1 cytokine pathway) or their differentiation into dedicated immunoglobulin-producing plasma cells (the type 2 cytokine pathway) (Fig. 41.2) [163–166]. Interleukin (IL)-2, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) are the principal components of the type 1 (Th1) cytokine pathway, and IL-4, IL-5, IL-6, IL-8, IL-10, and IL-13 are the principal components of the type 2 (Th2) cytokine pathway. The cytokine pathways are counter-regulatory, and autoimmune hepatitis may represent the net effect of various redundant and inhibitory actions that cross cytokine pathways in response to the deficiencies or excesses of other components.

The predominant cytokine pathway can change during the course of autoimmune hepatitis as counter-regulatory measures respond to the activity of the disease, and in children it can differ between type 1 autoimmune hepatitis (where a mixed or Th1 cytokine pathway can predominate) [167, 168] and type 2 autoimmune hepatitis (where the cytokine pathways are undisturbed) [167]. Serum determinations of the cytokine profile in White North American and northern European adults with type 1 autoimmune hepatitis show mixed patterns with components of the type 2 cytokine pathway predominating [9, 169–171]. In contrast, the type 1 cytokine pathway predominates in Japanese children [168].

Type 1 cytokines prevail during periods of active inflammation, and type 2 cytokines prevail during periods of quiescence [2, 9]. HLA DRB1*03 and the A1-B8-DRB1*03 phenotype occur more commonly in White North American adults with mixed type 1 and type 2 cytokine profiles than in counterparts with a pure type 2 cytokine response [9], and a genetic predisposition for cytokine imbalance may be

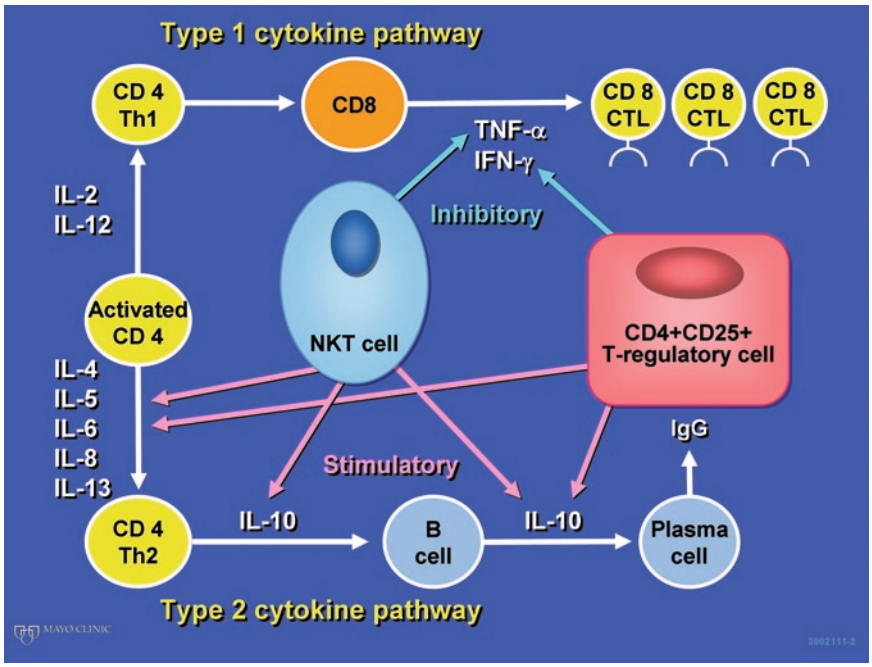

Fig. 41.2 Differentiation of activated CD4 T helper cells along type 1 (Th1) and type 2 (Th2) cytokine pathways. Natural killer T (NKT) cells and CD4+ CD25+ regulatory T cells modulate the cytokine milieu with inhibitory and stimulatory actions that affect the interleukins (IL) that modulate the direction of differentiation. Interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) favor differentiation into liver-infiltrating CD8 cytotoxic T lymphocytes (CTL), and interleukin-10 (IL-10) favors differentiation into plasma cells and the production of immunoglobulin G (IgG)

associated with HLA DRB1*03. Alternatively, patients with HLA DRB1*03 may have a more active immune response than patients with other HLA, and this heightened immune reactivity may in turn drive the Th1 pathway.

Polymorphisms of genes responsible for cytokine production may modify the cytokine milieu and favor a particular cytokine pathway [172]. The *tumor necrosis factor-α* allele (*TNFA*2*) is carried on the 8.1 ancestral haplotype of White northern Europeans, and this linkage disequilibrium with HLA DRB1*03 may modulate the cytokine milieu to favor a Th1 pathway (Table 41.1) [173]. The substitution of an adenine for a guanine at position −308 of the *TNFA* gene may increase the inducible and constitutive levels of TNF-α [173]. In White North American and northern European patients with type 1 autoimmune hepatitis, the polymorphism in which an adenine is substituted for a guanine at position −308 of the *TNFA* gene occurs more commonly than in normal individuals [173], and it is associated with HLA DRB1*0301 and higher frequencies of a poor response to corticosteroid therapy [174]. Other cytokines, such as *IL-2*, *IL-4*, *IL-6*, *IFN-γ* [172], and transforming growth factor-β (*TGF-β*) [175], are also under genetic control, and their occurrence and functional consequences in autoimmune hepatitis have yet to be fully defined. The absence of the *TNFA*2* polymorphism in Brazilian patients [176] again emphasizes the regional differences that can occur in the genetic factors that influence the disease.

Factors Influencing the Intensity of the Immune Response

The intensity of the immune response reflects the number of immunocytes that are activated, the effectiveness of intrinsic protective mechanisms to blunt the response, the integrity of signaling mechanisms to target the attack, and the duration that the activated effectors survive. Genetic, cellular, and molecular interactions are responsible for the net effect, and they involve the number of genes optimizing antigen presentation ("gene dosing"), the actions of regulatory cell populations, the density of chemo-attractant molecules, and the genetic determinants of programmed cell death (apoptosis).

"Gene Dosing"

The number of alleles encoding the same or similar amino acid sequence within the antigen-binding groove of the class II MHC molecules ("gene dose") determines the number of antigen-presenting complexes that can activate uncommitted CD4 T helper cells [84, 85, 177]. The density of the antigenic display, especially if the P4-HVR3 units form dimers, in turn influences the vigor of immunocyte activation [26].

The susceptibility alleles, *DRB1*0301* and *DRB1*0401*, for type 1 autoimmune hepatitis in White North American and northern European adults encode identical amino acid sequences at positions DRβ67–72 (LLEQKR) with lysine (K) at DRβ71 (Table 41.1) [84, 178]. *DRB1*0301* is in strong linkage disequilibrium with *DRB3*0101* which also encodes lysine at DRβ71. In contrast, *DRB1*0401* is in strong linkage disequilibrium with *DRB4*0103*, which encodes arginine (R) at DRβ71 [178]. Consequently, the *DRB1*0301* allele, because of its strong linkage with *DRB3*0101*, is usually associated with twice the number of antigen-binding motifs that contain lysine at DRβ71 than the *DRB1*0401* allele. The "double dose" of lysine at DRβ71 may account for the stronger association of *DRB1*0301* with the occurrence and severity of type 1 autoimmune hepatitis [84, 85].

Multiple alleles other than *DRB1*0301* and *DRB1*0401* can encode lysine at the DRβ71 position, and they may each contribute to disease susceptibility and severity in White North American and northern European patients with type 1 autoimmune hepatitis [7]. *DRB1*0302*, *DRB1*0303*, *DRB1*0409*, *DRB1*0413*, *DRB1*0416*, and *DRB1*1303* are the other *DRB1* alleles with this attribute, and *DRB3*0101*, *DRB3*0201*, *DRB3*0202*, and *DRB3*0301* are *DRB3* alleles with the same capability [178]. The maximum number of alleles encoding for lysine at the DRβ71 position by a given *DRB1-DRB3* haplotype is four, but the diversity of possible combinations indicates that the genetic predisposition for type 1 autoimmune hepatitis is broad [177].

Since the substitution of arginine (R) for lysine (K) at position DRβ71 is neutral, haplotypes that combine alleles encoding for these amino acids may also influence the occurrence and severity of the disease [7]. The diversity of alleles capable of encoding the same or similar susceptibility motif may account for the development of type 1 autoimmune hepatitis in individuals from the same geographical region who lack *DRB1*0301* and *DRB1*0401* [101, 177]. It may also explain variations in disease severity among these individuals [85]. A hierarchy of haplotypes based on the number of alleles encoding lysine or arginine at DRβ71 has been described, ranging from low risk when alanine is at DRβ71, medium risk when arginine is at DRβ71, and high risk when lysine is at DRβ71 [26, 78, 84].

In type 2 autoimmune hepatitis, the proliferative T cell response to CYP2D6 is against multiple antigenic regions, and these antigenic regions differ in patients with and without *DRB1*07* [115]. The same overlapping antigenic sequences within the CYP2D6 molecule can stimulate both T and B cell responses, and the *DRB1*07* status appears to influence this reactivity [115]. The known redundancies within the genetic network predict that other alleles within and outside the MHC can influence the occurrence and severity of type 2 autoimmune hepatitis by similarly affecting the density of displayed antigen. Polymorphisms and point mutations outside the MHC [7] and genes in different regions of the MHC (such as the C locus) [179] may also exert "dosing effects."

Modifying Cell Populations

The immune response is also modulated by regulatory CD4+ CD25+ T cells (T-reg cells), natural killer T (NKT) cells, and γδ T cells (Table 41.2). T-reg cells have a direct suppressive effect on the production of interferon (IFN)-γ and a stimulatory effect on the secretion of IL-4, IL-10, and TGF-β (Fig. 41.2) [180, 181]. By altering Th1 and Th2 cytokine responses, T-reg cells can modulate CD8 T cell proliferation and the intensity of cell-mediated cytotoxicity.

T-reg cells are decreased in number and function in autoimmune hepatitis, and this reduction in their suppressive action favors the clonal expansion of liver-infiltrating cytotoxic T cells (Table 41.2) [180, 181]. Deficiencies in T-reg function have been recognized in children and siblings of patients with primary biliary cirrhosis, and this finding suggests that genetic factors may affect the integrity of this cell population [182]. Corticosteroid therapy can restore T-reg function, and the improvements described in autoimmune hepatitis during such therapy may in part reflect this action [180, 181]. Functioning T-reg cell populations can be expanded or freshly generated in culture, and future treatments of autoimmune hepatitis might include adoptive transfer of these cells [183].

NKT cells coexpress a lectin (NK1.1) that characterizes natural killer (NK) cells and a TCR that typifies lymphocytes (Table 41.2) [184, 185]. They reside mainly in the liver, spleen, and bone marrow, and they can exhibit pro- or anti-inflammatory actions by producing either IFN-γ or IL-4 (Fig. 41.2) [185]. NKT cells are also constitutively cytotoxic as they contain granzymes and perforin that can promote apoptosis (Fig. 41.1). NKT cells do not express antigen-specific TCRs; they are not MHC-restricted; and they are activated mainly by glycolipids on antigen-presenting cells and hepatocytes [185]. The multiplicity of their actions suggests that NKT cells can moderate destructive and reparative processes.

The exact role of NKT cells in autoimmune hepatitis is uncertain. Their number is reduced in liver tissue from patients with autoimmune hepatitis compared to patients with primary biliary cirrhosis, and the absence of intrahepatic NKT cells has been associated with severe liver inflammation [186]. In autoimmune hepatitis, NKT cells may function mainly as immune suppressors. They typically target cells that lack indicators of self, and this intrinsic protective function may be blunted or accentuated at different stages of autoimmune

Table 41.2 Cellular and molecular factors in autoimmune hepatitis

Factors	Attributes	Consequences
Cellular factors		
CD4+ CD25+ regulatory T cells (T-reg cells)	Suppress production of interferon-γ Stimulate production of IL-4, IL-10, and transforming growth factor-β Reduced number and function in AIH Restored by corticosteroid therapy	Deficiencies favor type 1 cytokine pathway to CTL proliferation and severe AIH
Natural killer T cells (NKT cells)	Mainly immune suppression in AIH Inhibits tumor necrosis factor-α Stimulates type 2 cytokine pathway Reduced numbers in AIH	Impaired immune suppression and severe AIH
Gamma-delta (γδ) T cells	Recognizes antigens on nonclassical MHC or without MHC display Innate and adaptive immune features Increased in blood and liver in AIH	Support liver inflammation Associated with immune-mediated diseases
Molecular factors		
CXCL16	Chemokine within liver	Attracts activated CD4 and CD8 T lymphocytes to liver
CXCR6	Obligatory ligand of CXCL16 that is expressed on CD4 and CD8 T lymphocytes	Directs activated immunocytes to liver
CCR5	Chemkine receptor coexpressed with CXCR6 on activated T cells	Complex and uncertain
	High intrahepatic levels during inflammation May increase apoptosis of liver-infiltrating T cells	Deficiencies associated with severe liver inflammation in animal models

AIH autoimmune hepatitis, *CCR5* chemokine (C-C motif) receptor 5, *CTL* cytotoxic T lymphocytes, *CXCL16* chemokine (C-X-C motif) ligand 16, *CXCR6* chemokine (C-X-C motif) receptor 6, *IL* interleukin, *TCR* T cell antigen receptor

hepatitis. Disease-specific cofactors, such as the nonselective release of membrane glycolipids during the cytodestructive process, may influence their reactivity.

Gamma-delta (γδ) T cells are typically $CD4^-$ and $CD8^-$ cells whose TCRs are composed of one γ-chain and one δ-chain (Table 41.2) [187]. The TCR configuration distinguishes them from the majority of T cells whose TCRs are composed of α- and β-chains. Gamma-delta T cells straddle the boundary between an innate immune response that can proceed rapidly without prior antigenic sensitization [188] and an adaptive immune response that has antigen-specificity and a memory phenotype (Table 41.2) [189, 190]. Gamma-delta T cells can recognize antigens presented by nonclassical MHC molecules (class IB molecules), and they may exhibit promiscuous activity against self-antigens [191]. They may also be activated without the need for antigen processing or the MHC presentation of peptides, and they may supplement an antigen-directed immune response [192, 193].

The percentage and absolute number of γδ T cells are increased in the peripheral blood and the portal areas of patients with autoimmune hepatitis compared to normal individuals [194], and they have been associated with other immune-mediated diseases, including primary biliary cirrhosis, primary sclerosing cholangitis, rheumatoid arthritis, celiac disease, and multiple sclerosis [194]. Gamma-delta T cell clones derived from the peripheral blood and liver tissue of children with autoimmune hepatitis have more cytolytic activity against hepatocytes than αβ T cell clones [195, 196]. They may be recruited with the αβ T cells to the sites of liver inflammation by the same chemokines, and they may support or modify the activity of the αβ T cells. Variability (V) genes encode the heterodimer of the TCR, and the genetic predisposition of the host may affect the immune response and the antigenic targets by modifying the number of the TCR subsets [124]. Individual patients with autoimmune hepatitis may have different constellations of T-reg cells, NKT cells, and γδ T cells, and they may exhibit different disease activities depending on these constellations (Fig. 41.2).

Chemo-Attractant Molecules

Chemokines and their receptors regulate cell trafficking, and they help sustain and intensify organ injury by directing immunocytes and inflammatory cells to their target [197]. The chemokine, CXCL16, is highly expressed in liver tissue, and it supports lymphocyte adhesion to cholangiocytes, sinusoidal endothelial cells, and hepatocytes by activation of β1 integrins and binding to VCAM-1 (Table 41.2) [198]. CXCL16 is the only known ligand for the receptor, CXCR6, which is found on human CD4 and CD8 lymphocytes, NK cells, and NKT cells. Activation of these immunocytes enhances their expression of CXCR6 and accelerates

trafficking to the obligate ligand within the liver [197]. The interaction of CXCR6 and CXCL16 favors the attraction of T cells that are polarized along a type 1 cytokine pathway [198]. The lymphocytes expressing CXCR6 are associated with CXCL16 in the bile ducts of the portal tracts and at the sites of interface hepatitis in patients with autoimmune liver disease [198].

The cytokine receptor, CCR5, is expressed coordinately with CXCR6 as T cells are activated (Table 41.2) [197]. CCR5 is another chemokine receptor that is expressed on NKT cells, CD4 T cells, CD8 T cells, macrophages, and dendritic cells in murine models [199, 200], and high levels have been demonstrated in the liver-infiltrating lymphocytes of inflammatory liver disease [197]. CCR5 may have complex and diverse actions as CCR5 deficiency states in murine models have been associated with severe hepatitis [199] and fulminant hepatic failure [200], possibly because of impaired apoptosis of the liver-infiltrating T cells. The chemokine, CXCL16, expressed by the hepatocytes, and the chemokine receptors, CXCR6 and CCR5, expressed by the immunocytes constitute another regulatory network that can affect the occurrence and severity of immune-mediated liver disease.

Programmed Cell Death (Apoptosis)

The intensity and duration of the immune response are regulated in part by factors that hasten or delay the programmed cell death (apoptosis) of activated T lymphocytes and plasma cells [201]. Apoptosis is also the principal mechanism of hepatocyte loss in autoimmune hepatitis, and it can directly affect the severity of the disease. Two overlapping signal pathways stimulate apoptosis of immunocytes and hepatocytes in autoimmune hepatitis, and they may each contribute to the disease process [202].

An intrinsic pathway is associated with mitochondrial dysfunction, the release of cytochrome c and apoptosis inducing factor (AIF), and the activation of caspases that cleave chromosomal DNA (Fig. 41.1) [202]. An extrinsic pathway has the same outcome after caspase activation, but it is receptor-mediated. It depends on the ligation of the receptors, Fas and TNF-α receptor-1 (TNF-R1), with FasL (Fas ligand) and TNF-α, respectively, to activate the death signals [202]. The apoptotic pathways are modulated by counter-regulatory proteins that prevent apoptosis (bcl-2) or enhance it (bax, bad), and these proteins can be variably expressed depending on the cytokine milieu [202].

Activated lymphocytes in autoimmune hepatitis that express CD95 (Fas/APO-1) fail to downregulate the expression of the antiapoptotic protein, bcl-2 [203]. High concentrations of bcl-2 in these liver-infiltrating lymphocytes may in turn protect them from programmed cell death and thereby extend the disease process (Fig. 41.1). The persistence of activated lymphocytes expressing CD95 (Fas/APO-1) in autoimmune hepatitis suggests that the intrinsic apoptotic pathway for immunocyte deletion is defective [203, 204].

Polymorphisms of the human *Fas* gene on chromosome 10q24.1 may also affect the duration and severity of the autoreactive response by affecting the extrinsic or receptor-mediated pathway of apoptosis [205]. Four polymorphisms have been associated with the occurrence of autoimmune hepatitis in Japan [205], and in White North American and northern European patients with autoimmune hepatitis, an adenosine to guanine single nucleotide polymorphism in the *Fas gene* promoter at position −670 (*tumor necrosis factor receptor super family 6 gene* or *TNFRSF6*) has been associated with the early development of cirrhosis compared to patients with the guanine/guanine genotype (29% versus 6%, $p = 0.006$; odds ratio = 6.4) (Table 41.1) [206]. Genetic factors that affect the integrity of receptor-mediated programmed cell death can influence disease progression in autoimmune hepatitis by perpetuating the survival of activated immunocytes or increasing the vulnerability of targeted hepatocytes to accelerated apoptosis.

Nonspecific Factors Influencing the Clinical Phenotype

Polymorphisms of the *vitamin D receptor* (*VDR*) gene [207], *Fas* gene (*tumor necrosis factor receptor super family*) [205, 206], and *IL-1*, *IL-6*, *and IL-10 promoter genes* [172], and a point mutation of the *tyrosine phosphatase CD45* gene [208] are part of a burgeoning catalog of genetic promoters that can alter the clinical phenotype of autoimmune hepatitis. The *TGF-β1 gene* has been a focus of attention since polymorphisms have been associated with increased fibrosis in chronic hepatitis C [209], and the *TGF-β1 gene* induces expression of the transcription factor, forkhead box P3 (Foxp3), which is a key molecule in the development and activation of T-reg cells [210]. Deficiencies in the production of TGF-β1 are associated with impaired function of T-reg cells and expansion of intrahepatic cytolytic T cells [211]. These genetic modifiers of the immune response are outside the MHC, and they can work alone or in synergy (epistasis) with the principal drivers of the disease to vary its manifestations.

Gender can also modify the intensity and nature of the immune response [212–215]. High estrogen levels, as in pregnancy, inhibit a type 1 cytokine response that favors the proliferation of cytotoxic T cells, and they promote a type 2 cytokine response that has anti-inflammatory actions [216]. In contrast, low estrogen levels, as in the postpartum state, favor a type 1 cytokine response and promote cell-mediated pathogenic pathways [216]. These shifts in cytokine

predominance during and after pregnancy can contribute to quiescence of the disease during pregnancy and abrupt exacerbations after it [217–219]. Immunocytes have two estrogen-receptors, and this sensitivity to estrogen levels may contribute to the female propensity for increased immune reactivity [216].

Acquired inactivation of the X chromosome may also contribute to the female predilection for immune disease [220]. The X chromosome is large, and it contains numerous genes implicated in the maintenance of self-tolerance. One X chromosome is normally inactivated in a random fashion to achieve equivalent X-linked gene products in both sexes. In primary biliary cirrhosis, the X chromosome is inactivated more frequently than in normal women and disease controls, and this chromosomal loss may weaken the ability of these women to maintain self-tolerance [220]. Inactivation of the X chromosome appears to be acquired and random, and it may be a consequence rather than a cause of the disease.

White North American women with autoimmune hepatitis have HLA DRB1*04 more commonly than men with the same disease [83, 85], and they have a greater diversity of HLA DRB1*04 alleles [213]. This diversity of HLA DRB1*04 alleles in women may increase their ability to present diverse antigens to CD4 T helper cells. This propensity may enhance their ability to trigger an autoimmune response as well as develop concurrent immune diseases [213]. Many genetic and environmental factors that lack disease specificity have the ability to alter the immune response in an unpredictable fashion.

Conclusions

Autoimmune hepatitis is a polygenic disease that exhibits heightened immune reactivity and loss of self-tolerance because diverse genetic predispositions and disturbances in counter-regulatory molecular and cellular networks alter immune homeostasis. Diverse antigens may trigger the disease, and molecular mimicry between homologous epitopes in self and foreign antigens may be an etiological mechanism. Polymorphisms of various immune regulatory genes influence immunocyte activation, and T-reg cells, NKT cells, and γδ T cells modulate disease activity by affecting the cytokine milieu. Chemo-attractant molecules affect the trafficking of the activated immunocytes, and polymorphisms of the Fas gene (*TNFRSF gene*) may dampen the programmed cell death (apoptosis) of effector cells. The highly interconnected, redundant, and counter-regulatory actions of genes, molecules, cells, and hormones prevent the identification of a predominant flaw that initiates or perpetuates the disease. A small disturbance in any area of the network can create a ripple that cascades into a torrent.

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Chapter 42
Toxicant-Induced Liver Injury

Hartmut Jaeschke

Introduction

Chemical- and drug-induced liver injury is a significant problem in clinical practice and during drug development. Many drugs fail during development due to acute hepatotoxicity. However, a substantial number of drugs currently on the market or already withdrawn have the potential to cause liver injury [1, 2]. Most of these drug-induced hepatotoxicities are idiosyncratic and the mechanisms of cell injury are not well understood [1, 2]. However, currently the most frequent cause of drug-induced liver failure in the United States and many other countries is acetaminophen (APAP) overdose [3]. In contrast to many other drugs on the market, acetaminophen causes acute liver injury in a dose-dependent manner. Relevant animal models are available and many aspects of the pathophysiology have been studied [4]. In addition to its clinical relevance, APAP is widely used as a model liver toxin to evaluate potential hepatoprotective compounds and therapeutic strategies. Therefore, this review will focus on mechanisms of drug-induced liver injury using mainly APAP as the best studied example. However, there are reviews focusing on other compounds not covered in this chapter including many environmental hepatotoxicants, for example, microcystins [5], herbal extracts [6], metals (e.g., iron, copper, cadmium) [7, 8], and specific chemicals such as carbon tetrachloride [9].

Mode of Cell Death During Drug Hepatotoxicity

Historically, it was thought that cell death mediated by an external insult leads to oncotic necrosis, which is characterized by cell and organelle swelling, release of cell contents, karyorhexis, and karyolysis [10]. However, after the recognition and emerging popularity of apoptotic cell death, an increasing number of studies reported evidence for apoptotic cell death for various toxicant-induced liver injury. Apoptosis is characterized by cell shrinkage, chromatin margination, nuclear condensation, and formation of apoptotic bodies with mostly intact cell organelles [10, 11]. Although the signaling mechanisms of apoptosis are complex, a central role for caspases in this process is well established [12]. However, the clear morphological distinction between these modes of cell death in their classical form is not always maintained throughout a pathophysiology. First, apoptotic cell death during a pathological process is rarely completed with the phagocytosis of apoptotic bodies as it is the case when individual cells are undergoing apoptosis during cell turnover. If too many cells are undergoing apoptosis at the same time and/or the proapoptotic signals are too strong, cellular ATP levels decline and the cell switches to secondary necrosis involving release of cell contents [10]. Second, many signaling pathways originally thought to be only involved in apoptosis can also be part of an oncotic necrosis process [13]. Third, cells in culture are under substantial stress, which can easily be enhanced by a chemical and thus triggering apoptosis. An example would be superoxide formation, which clearly induces apoptosis in cultured rat hepatocytes in vitro [14]. However, the same oxidant stress in rat livers in vivo is easily detoxified and only an excessive oxidant stress results in oncotic necrosis [15]. Fourth, if the toxicity of the chemical is dependent on metabolic activation by the cytochrome P450 system, use of a metabolically incompetent cell line may result in apoptosis [16]. An example would be APAP hepatotoxicity, which involves oncotic necrosis in vivo in primary cells, but clearly triggers apoptosis in hepatoma cell lines [17]. In this case, the apoptotic mechanism has no relevance for the in vivo pathophysiology. Taken together, if the mode of cell death is in question, it is imperative to use experimental conditions and systems relevant for in vivo, measure multiple complementary parameters to detect apoptosis, and use a positive control as a reference point.

H. Jaeschke (✉)
Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA
e-mail: hjaeschke@kumc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_42, © Springer Science+Business Media, LLC 2011

Apoptotic cell death has been suggested to be involved in the in vivo hepatotoxicity of a number of chemicals and drugs including carbon tetrachloride [18], APAP [19, 20], galactosamine [21], galactosamine with endotoxin [22, 23], dimethylnitrosamine [24], and microcystin [25]. Although not all examples can be discussed, cell death induced by these chemicals can be mainly apoptotic (galactosamine/endotoxin) [22], a mixture of apoptotic and necrotic cell death (e.g., galactosamine in rat liver) [21], or mainly oncotic necrosis (APAP) [19]. Which type of cell death predominates may also be influenced by the experimental conditions. Furthermore, interpretation of individual parameters may vary leading to conflicting conclusions. Thus, even in relatively straightforward examples such as APAP hepatotoxicity, opposing results may be found.

Based on morphological data, which include early cell and organelle swelling and cell contents release affecting large number of cells around the centrilobular area, cell death during APAP hepatotoxicity is mainly an oncotic necrotic process [19, 26]. There is nuclear DNA damage indicated by nuclei that stain positive with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, DNA ladders, and the appearance of DNA fragments in the cytosol [27–29]. Although some of these parameters are indistinguishable from apoptosis (DNA Ladder) [29], others are clearly different (TUNEL) [10, 19]. In addition, there is no relevant activation of caspases during APAP-induced liver injury and potent caspase inhibitors do not protect [28, 30], suggesting that DNA fragmentation was not caused by caspase-activated DNase (CAD). Despite this overwhelming evidence for an oncotic necrotic process during APAP hepatotoxicity, there are claims of apoptosis [31, 32]. Most recently, it was suggested that high doses of APAP in fed CD1 mice cause caspase-dependent apoptosis during very early time points (3–5 h), but at later stages, the cell death mode was exclusively necrosis [20]. Interestingly, a caspase inhibitor eliminated evidence of apoptosis (DNA ladder, caspase processing) and aggravated necrosis (ALT release) [20]. These findings are somewhat surprising as it has never been shown that caspase inhibitors eliminate apoptosis and then aggravate necrosis in the liver during APAP hepatotoxicity [28, 30] or during TNF- [23, 33] or Fas receptor-mediated apoptosis [34, 35]. Furthermore, assessment of apoptotic morphology in fed C3HeFe/J mice [19] and in C57Bl/6 mice (Williams and Jaeschke, unpublished) did not show the same early apoptotic morphology as reported for CD1 mice [20]. Further studies will need to assess if this is a strain-specific effect and if the observation is of relevance in humans. At this point, the massive liver enzyme release observed during APAP overdose in patients also suggests mainly an oncotic necrotic cell death in human livers.

Intracellular Signaling Mechanisms of Drug-Induced Cell Death

Most drug- or chemical-induced liver injury depends on the formation of a reactive metabolite generated frequently during the metabolism by the cytochrome P450 system [36]. Metabolic activation of the drug is then associated with covalent modifications of proteins and other cellular macromolecules and/or the formation of reactive oxygen species (ROS) with subsequent lipid peroxidation. However, during the last decade it became increasingly clear that some of the early mechanisms (protein binding, lipid peroxidation) as they occur under realistic in vivo conditions are not sufficient to cause cell death. APAP, quantitatively the most relevant hepatotoxicant and also the most studied chemical, provides a perfect example for the evolution of drug-induced cell death mechanisms.

Protein Binding Hypothesis

Shortly after the first reports on APAP hepatotoxicity in humans [37], a mouse model was developed that showed that APAP-induced liver injury depends on the formation of a reactive metabolite generated by cytochrome P450 enzymes [38]. This metabolite was identified as *N*-acetyl-*p*-benzoquinone imine (NAPQI) [39]. NAPQI is initially detoxified by glutathione, which reacts with this electrophile and forms a stable APAP-glutathione adduct [40] (Fig. 42.1). These adducts can be excreted from hepatocytes through the canalicular transporter multidrug resistance-associated protein2 (mrp2) and its basolateral counterparts mrp3 and mrp4 [41]. However, if after an overdose of APAP the amount of NAPQI formed exceeds the capacity of the available GSH to detoxify it, NAPQI covalently binds to sulfhydryl groups of proteins and presumably modifies their activity leading to cell death [42] (Fig. 42.1). Although this early mechanistic insight was a major advance, which led to the introduction of N-acetylcysteine as antidote against APAP toxicity in humans [43], there was also some concerns if the somewhat limited overall binding of APAP to proteins (<4–6% of the administered dose) can actually explain its hepatotoxicity. In a refinement of the original hypothesis, it was postulated that maybe not the overall protein binding but modifications of specific proteins may be critical to cell death. For example, APAP overdose caused inhibition of the Ca2+-ATPase, an enzyme important for the cellular Ca2+ homeostasis [44]. An increase in intracellular Ca2+ could lead to activation of phospholipases and DNases and can cause mitochondrial dysfunction, all of which may contribute to cell death [45]. Unfortunately, no covalent adducts for Ca2+-ATPase were found. In contrast, a

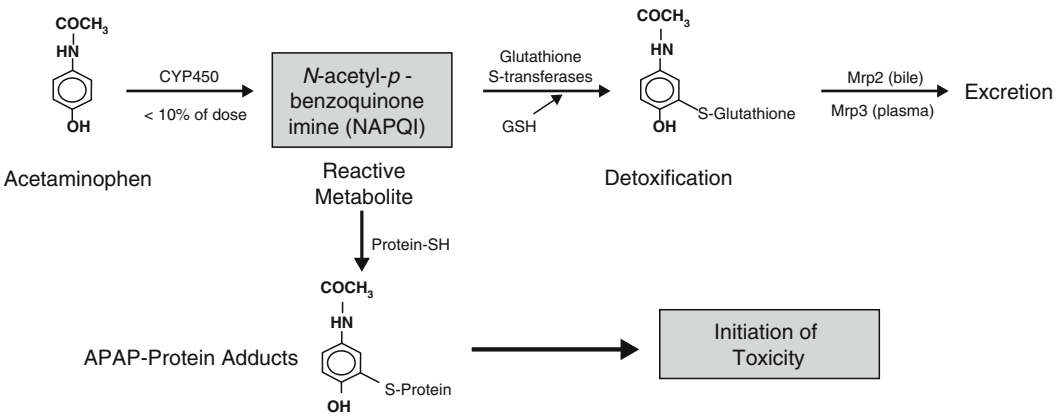

Fig. 42.1 Metabolic activation of acetaminophen in hepatocytes and initiation of toxicity (see text for details)

number of other APAP-proteins were identified over the years [46, 47]. However, the activity of most of these proteins was only moderately affected and could not explain the rapid cell death.

Lipid Peroxidation

An alternative hypothesis to protein binding was that reactive oxygen generated during the metabolism of APAP by P450 induces lipid peroxidation as the main mechanism of cell injury [48, 49]. However, this mechanism was favored by the use of vitamin E-deficient mice [50]. In animals on a regular diet, APAP induces only very limited lipid peroxidation and various forms of vitamin E do not protect, suggesting that lipid peroxidation, despite the occurrence of an oxidant stress, is not a relevant mechanism of cell injury after APAP overdose [51]. Furthermore, the formation of ROS during the metabolism phase was not confirmed [52, 53]. Nevertheless, numerous papers have been published where presumed antioxidants protect and reduce the limited lipid peroxidation in the liver. However, in most cases it is not clear if the reduced lipid peroxidation is not just a consequence rather than the cause of reduced injury. In addition, none of these investigations documented that quantitatively lipid peroxidation induced during APAP hepatotoxicity is sufficient to cause cell injury [50]. Thus, with the exception of vitamin E-deficient mice, lipid peroxidation does not play a role in the mechanism of APAP-induced liver injury. Although lipid peroxidation can be observed during many other drug toxicities [54], in most cases, due to the effective endogenous antioxidant defense mechanisms, the importance of this mechanism to the overall pathology is limited.

Mitochondrial Dysfunction

Mitochondria are the power plants of the cell. Mitochondrial dysfunction with formation of the membrane permeability transition (MPT) pore and collapse of the membrane potential is a terminal step in cell death mechanisms [55] (Fig. 42.2). Inhibition of the mitochondrial respiration and declining ATP levels have been observed early after APAP overdose [56–59]. These effects could be reproduced when isolated mitochondria were directly exposed to NAPQI suggesting that binding to mitochondrial protein by NAPQI could be responsible for these effects [60]. Furthermore, the comparison of APAP with its nontoxic isomer *N*-acetyl-*m*-amino-phenol (AMAP) showed that exposure to both compounds resulted in similar total liver protein binding, but only APAP triggered binding to mitochondrial proteins [60, 61]. These findings are consistent with the hypothesis that NAPQI generated during metabolism of APAP is first detoxified by GSH, but then covalently binds to cellular proteins. However, only binding to mitochondrial proteins results in a severe enough disturbance of the cellular homeostasis to cause cell death. Thus, the early metabolic activation and mitochondrial protein binding is an indispensible initiating event for the toxicity. However, for the cells to actually die, this initial insult requires amplification and propagation [13].

Mitochondrial Oxidant Stress

The main function of mitochondria is to transfer electrons from NADH to oxygen and generate water, an energy-liberating process that is utilized to synthesize ATP (Fig. 42.2).

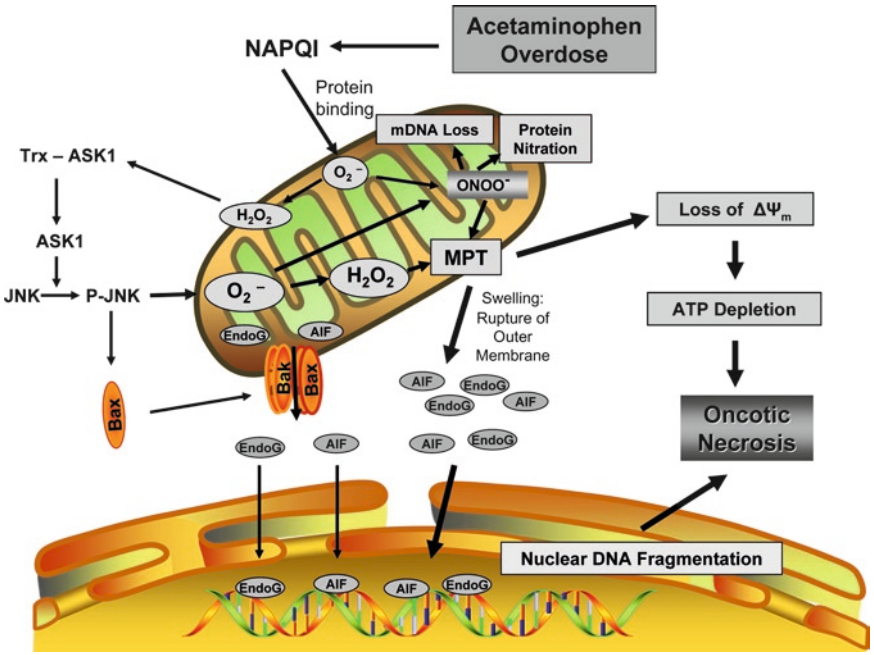

Fig. 42.2 Proposed scheme of the amplification and propagation of the initial cellular disturbance (metabolic activation and protein binding, especially to mitochondrial proteins). The early mitochondrial dysfunction triggers an initial mitochondrial oxidant stress, which activates apoptosis signal-regulating kinase 1 (ASK1) and c-jun N-terminal kinase (JNK). JNK activation (P-JNK) leads to mitochondrial Bax translocation and enhanced mitochondrial oxidant stress, which eventually triggers the formation of the mitochondrial membrane permeability transition (MPT) pore. Both the Bax pore and the later rupture of the outer mitochondrial membrane due to organelle swelling (MPT) cause the release of intermembrane proteins including apoptosis-inducing factor (AIF) and endonuclease G, which are mainly responsible for nuclear DNA fragmentation. Loss of the mitochondrial membrane potential, ATP depletion and nuclear DNA fragmentation, results in oncotic necrotic cell death

Formation of partially reduced oxygen species, for example, superoxide, occurs on a regular basis in mitochondria. However, formation of ROS can substantially increase when the electron transport chain is inhibited [62]. During APAP hepatotoxicity, a mitochondrial oxidant stress has been identified by the selective increase of glutathione disulfide inside mitochondria [58, 59]. In addition, the superoxide radical can react with the nitric oxide (NO) radical to form the very potent oxidant and nitrating species peroxynitrite [63, 64]. Because the superoxide anion cannot diffuse through membranes, peroxynitrite is also generated mainly in mitochondria [29]. Although it is well established that mitochondria can be an important generator of superoxide in the cell, the source of NO is controversial. Both inducible nitric oxide synthase (iNOS) and the constitutive endothelial nitric oxide synthase (eNOS) have been suggested based on beneficial effects in the respective gene deficient mice [65, 66], but some of these results could not be verified by others [67] (Ramachandran and Jaeschke, unpublished). It also remains unclear if NO is generated in hepatocytes or actually in adjacent endothelial cells. Nevertheless, the formation of peroxynitrite is well established based on immunohistochemistry, ELISA assays, and mass spectrometric analysis of hepatic proteins [63, 64, 68, 69]. The impact of peroxynitrite is facilitated by the massive depletion of mitochondrial glutathione [64]. Thus, if the recovery of mitochondrial glutathione levels is accelerated by treatment with GSH or NAC, peroxynitrite is effectively detoxified, mitochondrial protein nitration is prevented, and APAP-induced liver injury is significantly reduced [68, 70] and regeneration is enhanced [71]. These data strongly support the conclusion that ROS and peroxynitrite in mitochondria are important mediators of APAP-induced liver injury.

Although many potential mitochondrial targets for peroxynitrite or reactive oxygen remain to be defined, loss of protein sulfhydryl groups in mitochondria appears to be limited during APAP hepatotoxicity [72]. However, mitochondrial DNA damage was observed [29]. Because mitochondrial DNA loss was prevented by scavenging peroxynitrite with GSH, this effect is most likely caused by a direct oxidation of DNA by peroxynitrite or ROS [29]. Other targets of these oxidants need to be evaluated. However, the most severe overall consequence of this mitochondrial oxidant stress is the opening of the mitochondrial MPT pore [73]. In cultured cells, the MPT inhibitor cyclosporine A significantly delayed, but ultimately did not prevent cell necrosis [73]. These findings suggest that initially a regulated MPT pore is affected, but due to the severity of the insult, an unregulated pore is

formed at later time points [73]. However, the relevance of this in vitro observation has to be verified in vivo. At this point, cyclosporine A was shown to be protective against APAP-induced liver injury in vivo [74].

Nuclear DNA Damage

A characteristic of both apoptotic and oncotic necrotic cell death is nuclear DNA fragmentation (Fig. 42.2). In most cases this is not caused by direct interactions of the toxicant or its metabolites with DNA, but is caused by DNases, which are activated during the cell death process. The difference between the two modes of cell death is mainly related to specific DNase(s) being activated. DNA fragmentation during apoptosis is caused by CAD, which generates small DNA fragments that can be visualized by gel electrophoreses as DNA ladder [75, 76]. During necrotic cell death, frequently a complete disintegration of the nucleus is observed [19]. This process generates DNA fragments of various sizes including large fragments [76]. During APAP toxicity, evidence for early DNA fragmentation was provided by DNA ladder [27], the TUNEL assay [19, 28], and a histone ELISA [19, 28]. Because there was no caspase activation and caspase inhibitors, which eliminated apoptosis-induced DNA fragmentation, did not affect APAP-mediated DNA damage or cell injury, it was unlikely that the typical CAD was responsible for this effect [19, 28]. However, it was noted that the DNA damage after APAP could be prevented by protecting mitochondria [29]. This suggested a connection between mitochondrial dysfunction and nuclear DNA fragmentation. In fact, nuclear translocation of endonuclease G and apoptosis-inducing factor (AIF), which are proteins located in the intermembrane space of mitochondria, has been demonstrated after APAP overdose [77]. Both AIF and endonuclease G can cause DNA fragmentation [78, 79]. Because interventions that prevented the movement of AIF and endonuclease G to the nucleus effectively prevented nuclear DNA damage, it is likely that these endonucleases are mainly responsible for nuclear DNA fragmentation during the early phase of APAP hepatotoxicity [77, 80]. The release of AIF and endonuclease G occurs initially through a Bax pore in the outer mitochondrial membrane, but at later time points the release is caused by mitochondrial swelling during the MPT and rupture of the outer membrane [80]. In addition to AIF and endonuclease G, there is also evidence for involvement of the lysosomal endonuclease DNase1 in APAP-induced DNA damage [26, 81]. The fact that interventions that prevent nuclear DNA damage reduce liver injury suggests that this effect contributes to cell death [26, 80, 82]. However, the molecular mechanism remains unclear.

One potential mechanism by which DNA damage can trigger cell necrosis is through the activation of poly(ADP-ribose) polymerase-1 (PARP-1). This enzyme is critically involved in DNA damage repair by synthesizing poly(ADP-ribose) residues derived from NAD to DNA strandbreaks [83]. A drawback of excessive activation of PARP-1 is massive depletion of NAD, which can severely impair transfer of reducing equivalents to mitochondria, cause ATP depletion, and contribute to cell death [84]. During APAP hepatotoxicity, extensive PARP-1 activation and NAD depletion have been observed [26, 85]. However, the initially reported protective effect of PARP-1 inhibitors such as 3-aminobenzamide [86] could not be confirmed in PARP-1 deficient mice [85]. In fact, the protective effect of 3-aminobenzamide in PARP-1-deficient mice suggests off-target effects of this chemical [85]. In contrast, absence of PARP-1 slightly increased APAP-induced liver injury suggesting that initiation of DNA repair mechanisms may actually be beneficial [85].

Initiation of Mitochondrial Dysfunction

Although it is well established that APAP overdose results in mitochondrial dysfunction, the initiating events are less clear. Because toxicity correlates with mitochondrial protein adduct formation [60, 61], it was hypothesized that this mechanism might be responsible for the mitochondrial dysfunction and oxidant stress [13]. However, recent data suggest that some of the mitochondrial dysfunction can be reversed by scavenging of peroxynitrite and ROS [70]. This would indicate that adduct formation with mitochondrial protein may only be a very early event generating a moderate oxidant stress, which could lead to activation of the c-jun N-terminal kinase (JNK) [87] possibly through activation of the upstream mitogen-activated protein kinase apoptosis signal-regulating kinase 1 (ASK1) [88]. The initial oxidant stress may lead to thioredoxin-ASK1 dissociation, which results in the activation of ASK-1 and subsequently JNK [88]. A critical role for ASK and for JNK in APAP hepatotoxicity has been demonstrated [88–90]. Activation of JNK promotes the mitochondrial translocation of Bax [89], which is involved in the early release of intermembrane proteins resulting in nuclear DNA fragmentation and cell injury [80]. However, JNK inhibition is more effective in reducing APAP hepatotoxicity than elimination of Bax suggesting additional beneficial effects [80, 91]. Recently, it was shown that inhibition of JNK eliminates mitochondrial peroxynitrite formation and oxidant stress [91], which would explain the high efficacy of the JNK inhibitor as well as the fact that it prevents the MPT [87]. The mechanism of this effect is not yet established, but may be related to the phosphorylation of

mitochondrial proteins as activated JNK translocates to the mitochondria during APAP hepatotoxicity [87]. Given the effect of JNK activation on the mitochondrial oxidant stress, it is unlikely that JNK directly promotes the MPT [91]. Taken together, these findings demonstrate that hepatotoxic drugs such as APAP can trigger an extensive network of signaling pathways that trigger mitochondrial dysfunction and nuclear DNA damage, which ultimately leads to necrotic cell death (Fig. 42.2).

Drug Hepatotoxicity and Innate Immunity

Cells of the innate immune system including Kupffer cells (resident macrophages), newly recruited tissue macrophages, neutrophils, nature killer (NK) cells, NK T cells and others have been implicated in drug hepatotoxicity [92–94].

Kupffer Cells

Evidence for activation of Kupffer cells has been provided for a number of drugs and chemicals including APAP [95, 96], carbon tetrachloride [97], allyl alcohol [98], 1,2-dichlorobenzene [99], and others. In most of the studies the conclusion of Kupffer cell involvement was based on the protective effect of gadolinium chloride treatment. However, gadolinium chloride effectively inhibits reactive oxygen formation, but does not suppress cytokine generation [100]. Thus, effects with gadolinium chloride on drug hepatotoxicity are not always reproducible [101–103]. In fact, if Kupffer cells are actually eliminated by liposomal chlodronate rather than just in part functionally inactivated, APAP hepatotoxicity is enhanced [103]. This effect is caused by the elimination of anti-inflammatory cytokine formation, for example, IL-10, which can suppress iNOS induction and thus limit intracellular peroxynitrite formation [104]. In contrast, elimination of the capacity to generate ROS by deletion of components of the NADPH oxidase (NOX-2) [105] or by use of inhibitors of NOX-2 [106] does not affect the oxidant stress or the injury suggesting a limited relevance of Kupffer cell-derived ROS and peroxynitrite formation for the pathophysiology of APAP hepatotoxicity.

Natural Killer and NKT Cells

Hepatic NK and NKT cells are critical for antitumor and antiviral defense, but have also been implicated in the pathogenesis of various liver disease processes by producing proinflammatory cytokines and potentially cell killing [107]. Depletion of NK and NKT cells was initially shown to be beneficial in APAP-induced liver injury [108]. It was hypothesized that these cells generate interferon-γ (IFN-γ), which may contribute to liver injury by recruiting and activating neutrophils and enhancing Fas receptor expression in the liver [108]. Although mice with IFN-γ deficiency show reduced injury and inflammation after APAP overdose [108, 109], the role of the Fas receptor and of neutrophils in the pathophysiology remains unclear. Some studies have been done in the presence of substantial amounts of the solvent dimethyl sulfoxide (DMSO), which is a potent inhibitor of APAP activation [30] and can recruit activated NKT cells into the liver [110]. Thus, APAP hepatotoxicity in the absence of DMSO may not involve NK or NKT cells [110].

Polymorphonuclear Leukocytes (Neutrophils)

As one of the most aggressive phagocytes of the innate immune systems, neutrophils have the potential to cause additional tissue damage when inappropriately activated [111–113]. A neutrophil-induced injury phase has been most clearly established in hepatic ischemia-reperfusion [114, 115], obstructive cholestasis [116, 117], and endotoxemia [118]. However, direct evidence for neutrophil involvement in drug- or chemical-induced liver injury is limited. Halothane [119], alpha-naphthyl isothiocyanate [120, 121], and concanavalin A [122] are some of examples where neutropenia proved to be protective. On the other hand, results for APAP hepatotoxicity are controversial. Earlier data suggest that neutrophils accumulate in the liver in response to APAP-induced liver injury [123] and do not contribute to the injury in murine or rat models of APAP hepatotoxicity [123, 124]. Subsequent studies using a 24 h pretreatment with a neutropenia-inducing antibody showed protection against APAP hepatotoxicity [125, 126]. A caveat of these studies is the prolonged pretreatment period. Neutropenia leads to neutrophil accumulation in sinusoids where Kupffer cells remove these cells. A consequence of this massive pagocytosis of cellular debris is the activation of Kupffer cells [127], which leads to induction of various stress genes in hepatocytes [128]. This may cause a preconditioning effect leading to higher resistance against the intracellular events in APAP hepatotoxicity [128]. Consistent with these conclusions are further findings that the neutropenia antibody given after APAP was not protective [106]. Moreover, mice deficient in intercellular adhesion molecule-1 (ICAM-1) [106], CD18 [129], or NOX-2 [105] showed similar injury as the respective wildtype animals. Likewise, inhibitors of NOX-2 did not protect [106]. Since neutrophil-induced cell killing depends

on the effective migration from sinusoids into the parenchyma facilitated by CD18/ICAM-1 interactions [130] and the formation of hydrogen peroxide [131] and hypochlorite [132], the lack of efficacy of any of these interventions in APAP hepatotoxicity is inconsistent with a role of neutrophils in the pathogenesis. This is also supported by the fact that administering a low dose of endotoxin, which induces neutrophil activation and recruitment into the liver, does not aggravate APAP-induced liver injury [129]. Taken together, the vast majority of data support the conclusion that neutrophils do not actively contribute to APAP hepatotoxicity in the murine model.

Neutrophils and Kupffer Cells in Regeneration

The early initiation of regeneration is important in limiting the extent of liver injury after APAP overdose and is critical for repair of the damaged tissue [71, 133, 134]. A major function of the innate immune response is the removal of necrotic cell debris. Neutrophils and monocyte-derived macrophages are recruited into the liver where they localize around the area of necrosis especially at later time points [123, 135]. Monocyte chemoattractant protein 1 (MCP-1), which is generated by hepatocytes in the damaged centrilobular, region of the liver contributes to the recruitment of new macrophages into the necrotic areas [136]. The newly recruited macrophages are distinct from the resident macrophages (Kupffer cells) and are characterized by an increased capacity for phagocytosis [136, 137]. These new macrophages express the C-C chemokine receptor-2 (CCR2), which binds MCP-1. Thus, CCR2-deficient mice show reduced macrophage recruitment and delayed tissue repair, but no difference in the initial APAP-induced tissue injury [136, 137]. These data support the concept that new macrophages derived from blood monocytes are recruited into the necrotic areas through MCP-1 where they participate in the removal of cellular debris. Similar to macrophages, neutrophils are also recruited into the necrotic areas. Proteins released by necrotic cells (see below) are involved in proinflammatory mediator formation which can lead to hepatic neutrophil recruitment [111]. However, in contrast to macrophages, a pivotal role of neutrophils in tissue repair after APAP overdose has not been demonstrated. If these phagocytes participate, they do not need elastase or NADPH oxidase (ROS formation) as mice deficient in either enzyme activity did not show any impairment of tissue repair [138]. This is an interesting observation as both macrophages and neutrophils depend on NADPH oxidase (NOX-2) activity for cell killing [111].

Sterile Inflammation and Drug Hepatotoxicity

Despite the variable role of inflammatory cells in drug hepatotoxicity and regeneration, the mechanisms of activation and recruitment into the liver remained unclear. More recently, it was recognized that necrotic cells release a number of cellular proteins including the nuclear protein high mobility group box 1 (HMGB1), heat shock proteins (HSP70), and others as well as DNA fragments. These endogenous compounds are now collectively named "danger associated molecular patterns (DAMPs)" as opposed to "pathogen-associated molecular pattern (PAMPs)" derived from exogenous pathogens [139]. The important point is that DAMPs are also recognized by pattern recognition receptors (toll-like receptors [TLRs]) present on most liver cells especially on phagocytes [140]. Important TLRs are TLR4, which can be activated by lipopolysaccharide (LPS) (PAMP) and HMGB1, HSPs (DAMPs), and TLR9, which recognizes endogenous and exogenous DNA fragments [141]. Upon binding of ligands, most TLRs activate signaling events through their main adapter molecule Myd88, leading to activation of the transcription factor NF-κB and expression of cytokines and chemokines [140]. These proinflammatory mediators can then activate and recruit inflammatory cells including neutrophils and macrophages into the liver [142]. Thus, necrotic cell death can trigger an inflammatory response in the absence of pathogens/endotoxin. This sterile inflammation process is responsible for most of the inflammatory liver injury during reperfusion after hepatic ischemia [111].

In addition to the transcriptional activation of cytokine formation, DAMPs have also been shown to activate the inflammasome, a cytosolic protein complex including a NLR (nucleotide-binding domain, leucine-rich repeat containing) family member and the adapter molecule ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 [143]. The activation of caspase-1 is important for the processing of pro-IL-1β and pro-IL-18, two inflammatory cytokines. The importance of TLR signaling and the inflammasome has been investigated in APAP hepatotoxicity [144]. In this study, TLR9-deficient mice are protected and show reduced IL-1β mRNA levels during APAP hepatotoxicity [144]. In addition, the authors reported reduced injury in mice deficient in various proteins of the inflammasome [144]. Together with data that IL-1 receptor-deficient mice are protected against APAP-induced liver injury [145], these observations appear to support an important role for TLRs and the inflammasome in producing mature IL-1β, which triggers a potent inflammatory response and cell injury after APAP overdose. However, there are a number of concerns. First, pan-caspase inhibitors, which effectively prevent mature IL-1β formation, do not protect against APAP hepatotoxicity [28, 30, 146]. Second, IL-1 receptor-deficient mice are not

protected in our hands [146]. Third, administration of high doses of IL-1β after APAP activated neutrophils and enhanced recruitment of these phagocytes into the liver, but did not aggravate the injury [146]. Fourth, as discussed previously, numerous interventions against neutrophils do not reduce APAP-induced liver injury [105, 106, 129]. However, DAMPs like HMGB1, HSPs, etc. are released into the plasma during the early phase of APAP hepatotoxicity [20, 147, 148] and can induce proinflammatory cytokine formation in Kupffer cells and sinusoidal endothelial cells [147]. However, during APAP overdose HMGB1 also activates the CD24-Siglec pathway, which reduces cytokine formation selectively in response to DAMPs, but not to PAMPs [149]. This appears to prevent an overreaction to tissue damage. However, antibodies against HMGB1 reduced hepatic neutrophil accumulation and did not reduce injury in one study [148], but showed the opposite in a different experiment [149]. Such conflicting results with respect to the role of innate immune mechanisms are increasingly reported in the literature. A concern with the majority of such studies is that a potential effect of the gene manipulations or interventions on intracellular signaling mechanisms or metabolic activation of APAP is rarely considered. Thus, many reported observations that are not reproducible or do not fit into established mechanisms of toxicity may be caused by unrecognized off-target effects.

Taken together, during APAP hepatotoxicity caused by intracellular signaling events, necrotic cells release DAMPs, which trigger inflammatory mediator production and activate and recruit inflammatory cells into the liver (Fig. 42.3). Some of the mediators may affect the intracellular mechanisms of cell death. However, injury induced by inflammatory cells does not appear to be a relevant factor in the overall pathogenesis. How much of these findings with APAP are applicable to other drug toxicities remain to be investigated.

Idiosyncratic Drug-Induced Liver Injury

In addition to intrinsic toxicants, which directly cause toxicity in animals and humans in a clear dose-dependent fashion, idiosyncratic hepatotoxicants affect generally only very few individuals (less than 1 in 10,000), do not appear to cause a dose-dependent toxicity, and the liver injury is generally not

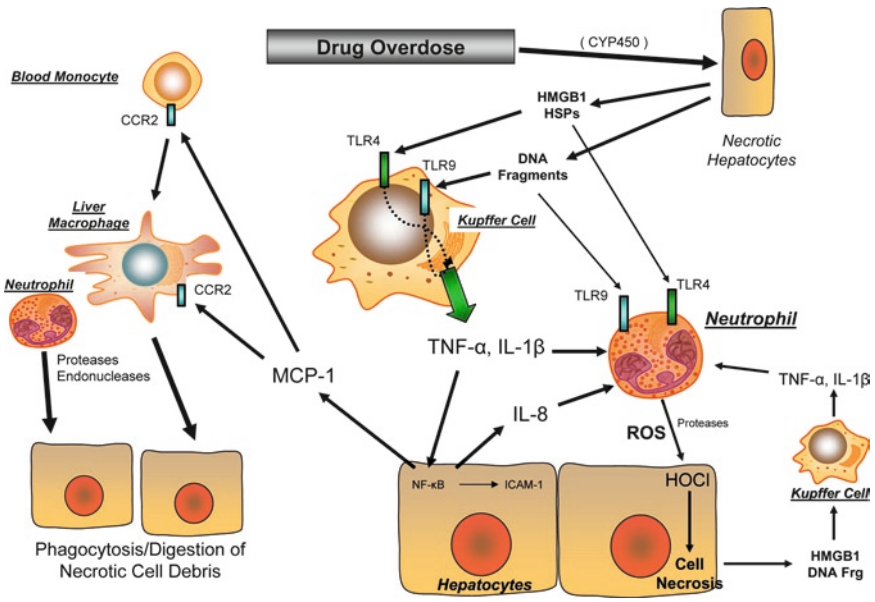

Fig. 42.3 Sterile inflammatory response and drug hepatotoxicity. Cell necrosis caused by exposure to hepatotoxic drugs or chemicals results in the release of cellular contents including DNA, RNA, and proteins such as high mobility group box-1 (HMGB1) and heat shock proteins (HSPs). These danger-associated molecular patterns (DAMPs) bind to toll-like receptors (TLRs) on neutrophils and resident macrophages of the liver (Kupffer cells) and trigger cytokine and chemokine formation. These proinflammatory mediators can activate neutrophils and monocytes in blood and recruit them into the liver vasculature. After extravasation into the parenchyma and adherence to target cells, neutrophils can generate potent reactive oxygen species, which can trigger cell death (cytotoxicity). This enhanced injury will further amplify the inflammatory response by enhanced release of DAMPs. However, both macrophages and neutrophils can phagocytose and digest cell debris. The dominant effect of the inflammatory response (cytotoxicity vs. removal of dead cells) depends on the individual drug. If the drug itself effectively causes cell death, for example, acetaminophen, a cytotoxic effect of neutrophils is less relevant. However, if the drug induces significant stress in many cells but is able to kill only a few, the recruited neutrophils may aggravate the injury by attacking and killing some of the stressed cells. *CCR2* C-C chemokine receptor-2; *MCP-1* monocyte chemoattractant protein-1; *HNE* hydroxynonenal; *HOCl* hypochlorous acid; *ICAM-1* intercellular adhesion molecule-1; *IL-1β* interleukin-1β; *ROS* reactive oxygen species; *TLRs* toll-like receptors; *TNF-α* tumor necrosis factor-α

predictable by animal testing. The mechanisms of idiosyncratic drug-induced liver injury are unknown, but are thought to be mainly immune-mediated [92, 150]. Most likely a reactive metabolite of the drug (hapten) binds to cellular proteins and generates an immune response, which can be directed against the hapten or any protein bound by the hapten [150]. The immune response is expected to be very complex and in most cases leads to development of tolerance [150]. Thus, the rare event when exposure to the drug causes liver injury may involve certain susceptibility factors within the individual in combination with a specific immune response. Risk factors for idiosyncratic drug-induced liver injury include age, sex, and polymorphism of drug metabolizing enzymes [151]. However, none of these risk factors have been conclusively linked to idiosyncratic drug-induced liver injury [150].

A severe limitation in understanding of mechanisms of idiosyncratic drug-induced liver injury is the lack of suitable animal models. Recently, it was hypothesized that a drug-induced subclinical stress on the mitochondria in patients with silent mitochondrial dysfunction (genetic or acquired) may trigger idiosyncratic liver injury [152]. This hypothesis is based on the first documentation of liver injury by chronic troglitazone treatment in heterozygous SOD2-deficient mice [153]. A limitation of this approach is that only moderate liver injury, but no liver failure, was produced in these animals [154]. Thus, this model could be useful to uncover potential mitochondrial liabilities of a new drug, but is unlikely to explain idiosyncratic drug reactions. In addition, the model has not been reproduced [155]. Another approach in modeling idiosyncratic liver injury is to use a combination of LPS and the drug [156]. The hypothesis is that a random infection/inflammation episode during drug exposure may shift the dose-response of the drug to the left and uncover liver toxicity at doses that are not lethal [156]. This toxicity is caused by a direct potentiation of the individual subclinical toxicities or the addition of an innate immune toxicity. The LPS/drug combination has been used repeatedly to induce liver injury when the drug alone or LPS alone did not cause any pathology [156]. However, there are several concerns with this approach. First, the clinical characteristics of idiosyncratic drug-induced liver injury in humans do not fit this model [150]. Second, the dose of LPS used is generally at the threshold of liver toxicity and therefore it is not surprising that a second insult (drug) can trigger liver injury. Third, the mechanisms of liver injury for all these combination models irrespective of the drug appear to be very similar, for example, seem to involve always the coagulation cascade, cytokines, and neutrophils [156], which is very similar to LPS alone [156]. Thus, this model appears to mainly show that a combination of two subclinical insults can induce trigger liver injury. As such, it may be useful to detect some hidden toxic liabilities of a drug, but is unlikely to reveal the potential for idiosyncratic liver injury.

Summary

Liver injury caused by drugs and chemicals involves complex intracellular signaling mechanisms, most of them are not well understood. Even for the most studied and clinically important drug, acetaminophen, many aspects of the pathophysiology are still unclear. However, a central role for mitochondria in determining cell death or survival is emerging for acetaminophen hepatotoxicity and many others. The intracellular signaling mechanisms can be modulated by mediators generated by nonparenchymal cells and newly recruited inflammatory cells, which are also involved in the removal of necrotic cells and tissue repair. In addition, the mechanisms can be affected by individual variations in drug metabolism gene expression, antioxidant defense systems, and innate immune responses. In contrast to these more predictive drug toxicities, idiosyncratic drug-induced liver injury, which is a significant problem for many formulations already on the market, is even less understood. Immune mechanisms in combination with hypothesized individual susceptibility are currently the most favored view. Thus, the challenges ahead are formidable. A more accurate prediction of drug hepatotoxicity as well as improved therapeutic approaches is clearly needed. Progress in both of these areas depends on the better understanding of the mechanisms of known hepatotoxicants in animals and in humans.

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Chapter 43
Wilson's Disease

Michael L. Schilsky and Kisha Mitchell

Introduction

New advances in our understanding of the molecular pathogenesis of Wilson disease profoundly impact our ability to diagnose and treat this disorder. This autosomal recessive disorder of copper metabolism has evolved from a descriptive syndrome over a century ago [1] to a treatable disorder where the pathogenesis of the copper accumulation is known at the cellular and molecular level [2]. In the decades after the initial description of Wilson disease, it was recognized that the disorder is inherited in an autosomal fashion, but diagnostic tools were limited and treatments unavailable. Over the last five decades, treatments have been proven effective and the diagnostics have changed as well [3].

More recently, the gene responsible for Wilson disease was identified [4–7] on chromosome 13. This enabled further understanding of this disorder at the tissue and cellular level. We have learned that the basis of the reduced biliary copper excretion from livers of afflicted patients that is the hallmark of this disorder, as well as the altered incorporation of copper into the glycoprotein ceruloplasmin, result from a functional failure of the copper transporting ATPase, ATP7B in hepatocytes. The reduced biliary excretion of copper leads to accumulation of this metal in the liver, and over time, in other sites in the body, most notably the central nervous system. The excess copper is the basis for the pathological injury that results in clinical liver and neurological disease. Treatments that remove copper, prevent its further accumulation, or improve cellular antioxidant capacity, may reverse the copper induced cellular injury. Though we understand the basis of Wilson disease, some challenges still remain. Diagnosing this disorder may be difficult in some patients due to the variability of clinical presentation and histological findings. Clinical manifestations range from asymptomatic patients, to those with liver disease, to those with neuropsychiatric disease. Similarly, histological features are wide ranging as well, and overlap with other causes of chronic hepatitis and cirrhosis. The variation in clinical and histological presentation is likely due to both epigenetic and environmental phenomenon. It is important for clinicians and pathologists to recognize this variability and to establish a diagnosis of Wilson disease by currently available clinical and biochemical testing. While the arrival of molecular diagnostics has added to our ability to diagnose patients with Wilson disease, limitations to its utility remain. New mutations in this ~80 kb stretch of DNA that encode ATP7B are still being identified, and two mutant alleles are not always identified in subjects with phenotypic disease. Further technological advances and a careful exploration of the intricacies of copper metabolism at the cellular and whole organism levels will continue to help evolve our understanding and treatment of Wilson disease.

Copper Metabolism and the Pathogenesis of Wilson Disease

Copper homeostasis is maintained through the efforts of several organs – the intestine, liver, and kidneys. Each tissue has specialized proteins that are involved in copper transport. Given the essentiality of copper to cellular enzymatic function, every cell, and not just tissues involved in copper homeostasis, must have the ability to take up copper and utilize it properly. The mechanisms involved in this process are now better understood following the identification of many of the individual proteins involved.

To maintain homeostasis, the daily dietary intake of copper must meet losses. The daily dietary intake of copper is typically between 2–10 mg. Only about 10% of the ingested copper is absorbed by duodenal enterocytes [8–10]. Daily losses of copper arise from cellular turnover and from the enteral secretion of fluids (bile, saliva, and pancreatic secretions) and urinary excretion. The renal excretion of copper is only a small fraction of the total daily excretion, typically about 10–20 μg daily [8, 9]. Excess dietary copper absorbed from the gut is excreted into bile while urine copper excretion is

M.L. Schilsky (✉)
Division of Digestive Diseases and Section of Transplantation and Immunology, Yale New Haven Medical Center, New Haven, CT, USA
e-mail: michael.schilsky@yale.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_43, © Springer Science+Business Media, LLC 2011

maintained more or less constant in amount. Only in states of copper overload, acute liver injury with release of hepatocellular stores of copper, or after treatment with chelating agents does urinary copper rise significantly [8, 9].

Dietary copper is transformed from Cu^{+2} to Cu^{+} for transport across the enterocyte plasma membrane by the ubiquitous copper transporting protein, Ctr1 by one or more cell-surface metalloreductases [11]. The uptake and efflux of copper from cells is accomplished via the ubiquitously expressed cellular-copper transporter, Ctr1, an integral membrane protein that is structurally preserved throughout evolution from yeast to man [12–14]. Once inside cells, copper transport is facilitated by small peptides known as metallochaperones, peptides that bind and move copper preferentially to other copper transporters, compartments, or to copper-dependent enzymes. Atox1 was identified as the metal chaperone that directs copper to the copper transporting ATPase ATP7A in enterocytes [15, 16]. Copper in the enterocyte is bound by Atox1, the peptides metallothionein (MT) and glutathione or, alternatively, is transferred out of cells to the bloodstream by ATP7A at the basolateral membrane.

Copper that enters the bloodstream in the portal circulation is thought to be bound by albumin at its amino-terminal metal binding domain, or to histidine or other copper ligands that function in copper delivery to the periphery [17]. Albumin or histidine bound copper is rapidly extracted by hepatocytes. The membrane transporter for uptake into hepatocytes is likely to be Ctr1, as Ctr1 is strongly expressed in liver. Cu^{+} is transported into the hepatocytes and is bound to the metallochaperone Atox1 that delivers copper to ATP7B, the Wilson disease gene product [18, 19]. The ATP7B protein is typical of metal transporting ATPases that are evolutionarily conserved [20]. ATP7B resides mainly in the trans-Golgi as a membrane bound protein with a cytosolic amino terminus that contains six repeats of cysteine rich metal-binding domains that accept copper from Atox1 [21, 22]. Copper is moved from this cytosolic site via transfer through a membrane channel lined with a critical cysteine-proline-cysteine motif. The process of transport is driven by conformational changes of the protein that are dependent upon cycles of phosphorylation and dephosphorylation [23]. This dependence of ATP7B function on its phosphorylation state and the importance of the ATP binding region of ATP7B are underscored by the frequency of disease specific *ATP7B* gene mutations affecting the phosphorylation or ATP binding sites. The carboxyl terminus of ATP7B contains signals that drive the proteins' localization to and from the Golgi to other intracellular sites, vesicles or to the apical region, where it participates in copper export into bile [24].

ATP7B is located mainly in the trans Golgi and also in endosomal or pre-lysosomal vesicles participating in biliary export via the bile canaliculus [10, 24]. ATP7B cycles its location between the trans Golgi and the endosomal vesicles depending upon the amount of copper present. When copper is abundant, there is an increased presence of ATP7B in the vesicular compartment and when copper is not present in excess, ATP7B is mostly localized to the trans Golgi network. Some investigations have suggested that ATP7B reaches the apical membrane directly [25, 26]; however, this remains a point of controversy. Mutation of ATP7B that is the underlying cause of Wilson disease leads to a reduced biliary copper excretion and a reduced incorporation of copper into ceruloplasmin peptide.

Ceruloplasmin is a serum glycoprotein synthesized mainly, but not exclusively by hepatocytes. Ceruloplasmin functions in iron distribution through its actions as a ferroxidase along with the tissue protein hephaestin. Ceruloplasmin contains six copper atoms per molecule of protein that are acquired biosynthetically in the trans Golgi secretory network [27]. In Wilson disease, where the transport of copper into the trans Golgi is defective, ceruloplasmin peptide is synthesized, but does not acquire copper properly. Apo-ceruloplasmin (the peptide without copper) has a different conformation from the holoprotein with copper that results in its more rapid degradation that in turn leads to a reduced steady state of ceruloplasmin in the circulation of most patients with Wilson disease [28].

Copper Toxicity

When present in excess, copper is toxic to cells via generation of free radicals and hydroxyl radicals. In this manner, excess copper may promote oxidative injury to membranes, DNA, and to the cellular machinery for protein synthesis [29, 30]. Cellular injury with necrosis results from copper accumulation in hepatocytes, but apoptotic injury may predominate in patients that have acute liver failure (ALF) due to Wilson disease [31]. A connection between copper accumulation and apoptosis is the protein XIAP (X-linked inhibitor of apoptosis). With increased cellular copper content, XIAP function is reduced thereby lowering the threshold to copper-induced injury [32].

Glutathione, a cysteine containing tri-peptide that is present in high concentration in the cytosol of liver cells provides intracellular reducing potential that protects proteins. Lower levels of glutathione cause liver cells to be more susceptible to copper injury, and elevations in tissue copper in Wilson disease is associated with a lower glutathione content as well as an increase in oxidized glutathione [33]. Any injurious process to the liver that lowers glutathione levels will lower the threshold for copper induced-liver injury. Copper can also be removed from liver cells by the transport of copper glutathione complexes by the canalicular multi-organic anion transporter (cMOAT) [34]. As occurs in patients with Wilson

disease, this cMOAT function does not appear adequate for eliminating all of the excess copper, and cellular damage ensues over time.

Pathology

The pathology of Wilson disease, though heterogenous, has been observed to often progress sequentially [9]. A schematic diagram of the natural history of the pathology of liver disease in Wilson disease is outlined in Fig. 43.1. The pathologic changes are related to the toxic accumulation of copper in the liver and other affected organs such as brain, kidney, and cornea. These changes are variable, and in all stages, may mimic non-Wilsonian liver disease.

Typically, the acute changes of Wilson disease are characterized by hepatic steatosis, commonly initially microvesicular, with increasingly larger fat droplets and resulting macrovesicular or mixed steatosis. Other early changes include glycogenated nuclei, focal hepatocellular necrosis, and hepatocyte swelling, which may be associated with a sparse lymphocytic infiltrate and with cholestasis [9]. Some patients may have significant interface hepatitis with a significant plasmacytic component, with hepatocellular ballooning, and are therefore morphologically difficult to distinguish from autoimmune hepatitis. Autoantibodies to cryptic proteins have been found in an animal model of Wilson disease, the LEC rat [35] and overlap features have been reported in some pediatric patients [36]. Fibrosis may be mild in this acute phase, but progressively increases to cirrhosis, when morphologic changes that are seen in the non-cirrhotic liver may also be present. Often, in younger patients with minimal changes, liver copper concentrations are significantly increased [9].

During this early stage, ultrastructural mitochondrial changes are most helpful and include pleomorphic, large mitochondria with intermembranous spaces, dilated cristae, and an increased number of dense inclusions [9, 37, 38]. Copper stains, though helpful when positive, do not exclude the diagnosis when negative, since at this stage, the diffuse cytoplasmic distribution of copper is detectable only by very sensitive stains such as Timm's silver sulfide stain. The p-dimethylaminobenzylidene rhodanine stain has been observed to be the most reproducible [39], and as is the rubeanic acid stain, is described as specific for copper [40]. However, both may be negative in early Wilson disease. Orcein, aldehyde fuschin, and Victoria blue may also be used; however, these stains detect copper binding protein and may not be positive at variable stages of the disease [41].

The progressive copper induced damage, in the absence of therapy, leads to cirrhosis that is usually macronodular, but may also be mixed or micronodular. This cirrhosis is frequently present in many patients by their second decade of life. In some, this progression may take decades more so that cirrhosis is not universally present in all patients at the time of their disease presentation as once thought [42]. However, advanced fibrosis and cirrhosis are common findings in untreated disease, particularly in those with neurological presentations. The cirrhosis may appear morphologically indistinguishable from other forms of chronic hepatitis, with variably thin or thick fibrous septa, with or without morphologic features observed in earlier stages of disease. Mallory bodies, binucleated and trinucleated hepatocytes, and increased pigment deposition (lipofuscin, iron, copper associated protein, and acid phosphatase) may be seen [9]. Copper deposition is often more readily detectable by less sensitive methods, since during this stage, lysosomal accumulation is characteristic. However, deposition is variable and may be diffuse in some nodules, while absent in others. In contrast to many other types of cholestatic copper deposition, such as primary biliary disorders, the copper is commonly seen throughout nodules, rather than largely located in a

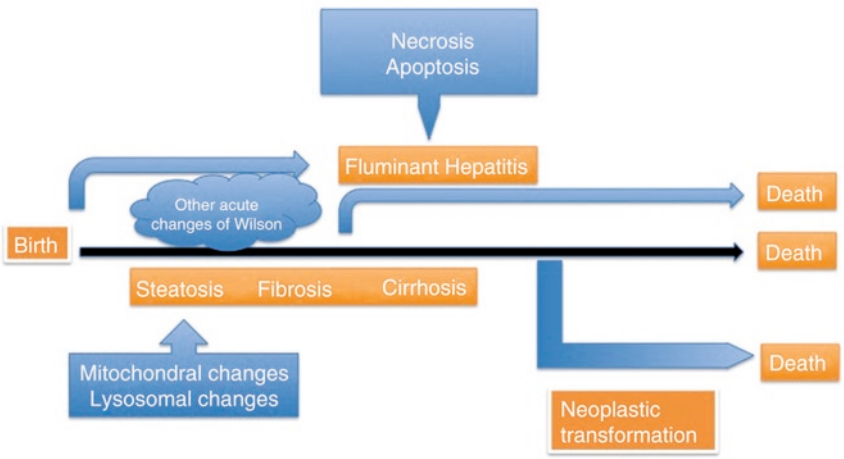

Fig. 43.1 Schematic diagram of natural history of Wilson disease

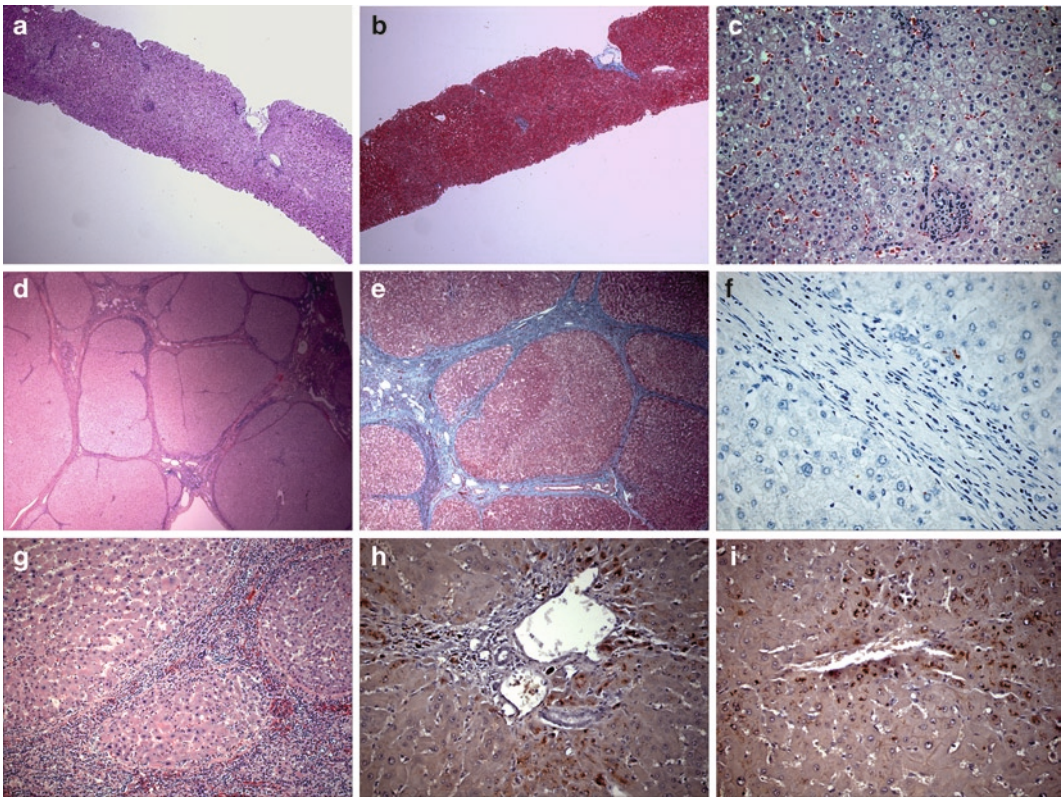

Fig. 43.2 Histological characteristics of Wilson Disease. (**a**) (H&E-low power), (**b**) (Trichrome-low power) and (**c**) (H&E-low power): mild, nonspecific mainly portal inflammation with only very early fibrosis and scattered glycogenation of hepatocyte nuclei. (**d**) (H&E-low power), (**e**) (Trichrome-low power), and (**f**) (Rhodanine stain-medium power): non-descript cirrhosis, indistinguishable from other forms of cirrhosis vwith mainly macronodules and very rare, patchy copper accumulation. (**g**) (H&E), (**h**) and (**i**) (Rhodanine stains): well developed, mainly macronodular cirrhosis with moderate septal inflammation and conspicuous perivenular (**h**) and periportal (**i**) copper accumulation

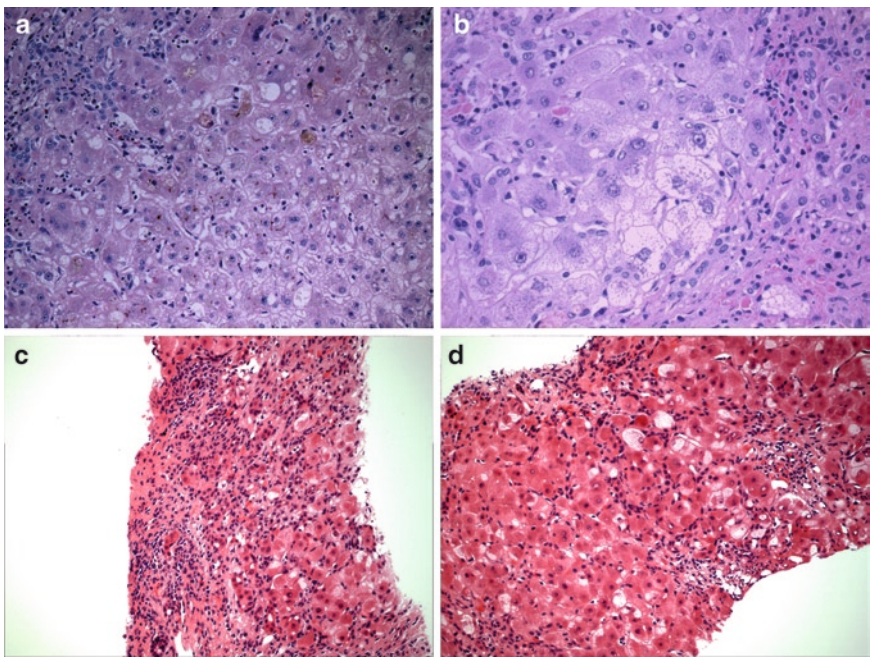

Fig. 43.3 Histological characteristics of Wilson disease (H&E images). (**a**) Septal and portal mixed inflammation with interface hepatitis. (**b**) Ballooning degeneration with acidophil bodies in setting of cirrhosis. (**c**) Bridging fibrosis with septal and interface inflammation. (**d**) Lobular inflammation and balloon degeneration associated with glycogenated nuclei without cirrhosis

periseptal/periportal distribution at this stage. Examples of morphologic features of Wilson disease are demonstrated in Figs. 43.2 and 43.3.

Infrequently, the liver in Wilson disease can undergo neoplastic change (Fig. 43.4). Association with hepatocellular carcinoma (HCC), cholangiocarcinoma, and carcinomas of unknown origin have been described [43–46], including progression to HCC in children [44]. Copper stains may be positive in such tumors, as well as may show the typical regional positivity in non-neoplastic nodular parenchyma.

Patients with Wilson disease may present with ALF, which histologically is represented by fulminant hepatitis. This is characterized fairly commonly by microvesicular steatosis, marked hepatocellular balloon degeneration, hepatocyte apoptosis, large pigment-laden Kupffer cells [9], and parenchymal dropout with lobular collapse (Fig. 43.5). Mallory bodies are often prominent and when not seen, may be highlighted by cytokeratin (CK8/18), ubiquitin, or p62 stains [47]. This fulminant liver failure occurs typically on the background of advanced fibrosis or cirrhosis and corresponds to the clinical picture of "acute on chronic" liver injury, where there is little hepatic reserve due to the chronic injury that is usually uncovered only after the acute presentation. It is important that Wilson disease is always considered a potential etiology of advanced fibrosis/cirrhosis with an initial presentation of ALF. In patients with severe hemolysis, cholestasis may be marked and pathologic features often include hepatocellular bile, canalicular bile plugs, periportal feathery degeneration, and marked cholangiolar reaction and proliferation.

With treatment, there is restoration of liver function in many patients that is accompanied by histological improvement, even in those with cirrhosis and active hepatitis at the outset [48, 49]. Ultrastructural changes in mitochondria and the light microscopic correlate of steatosis also improve, and fibrosis may also regress with prolonged treatment.

Epidemiology

Wilson disease is present in ~1 in 30,000 individuals in almost all populations [9]. Patients present mainly with clinical liver disease at an earlier age in their first and second decades of life, and with neurologic or psychiatric symptoms later in their late second or third decades [3, 9]. Rarely, patients present later in life [42]. Treatments specific for Wilson disease can arrest and prevent disease progression and prevent development of symptoms if begun while the patient is asymptomatic.

Regions of the world where there are higher gene frequencies for Wilson disease include those with closed populations and consanguinity. The consequence of this limited gene pool is the appearance of dominant *ATP7B* mutations in these populations. Examples include patients from the Canary Islands and areas in Sardinia [50]. The study of an isolated population from the Middle East helped the initial identification of the chromosomal localization of the Wilson disease gene to chromosome 13 due to recognition of its linkage to deficiencies of esterase D in this population [51]. We can even learn about population migrations by following specific *ATP7B* mutations. An example of this is the presence of the Sardinian mutations in Costa Rica that occurred due to the colonization of Costa Rica by individuals from Sardinia [50]. A summary of common mutations is shown in Table 43.1 [52, 53], but a more complete compendium of mutations may be found on the web site maintained by the laboratory of Dr. Diane Cox at the University of Alberta [53].

About 5% of all patients with Wilson disease present with ALF, accounting for ~2–4% of all patients with ALF [54]. These patients typically present in their second decade of life and the majority are women [55]. A clinical picture identical to this early presentation of ALF can occur in older patients previously treated that stop their medications.

Diagnosis

The diagnosis of Wilson disease must be considered in family members of affected individuals and in those with unexplained liver or neuropsychiatric disease [3, 9]. Clinical and biochemical testing for Wilson disease are required for testing this disorder for most patients. Clinical findings include the presence or absence of corneal Kayser-Fleischer (KF) rings on slit lamp examination, signs of liver or neurological disease, psychiatric illness (ranging from a mood disorder to depression or psychosis), and rarely blue lunulae or sunflower cataracts. Biochemical findings include a lower serum ceruloplasmin and serum copper in ~95% of patients presenting with chronic liver disease, and KF rings in nearly 98% of those with neuropsychiatric forms of Wilson disease. Other laboratory findings include evidence of hepatic insufficiency and portal hypertension including a low albumin, elevated bilirubin and prothrombin time, or international normalized ratio (INR), and on thrombocytopenia as a surrogate marker of portal hypertension. In the patient with ALF due to Wilson disease, a non-immune mediated hemolytic anemia is typically present in addition to a low alkaline phosphatase and elevated bilirubin [55]. This causes the ratio of bilirubin to alkaline phosphatase to be <4, and in addition, the ratio of AST to ALT is >2. Serum and urine copper is high in patients with liver failure due to Wilson disease, typically above 200 μg/dL for serum copper and 100 μg/24 h for urine copper. In children, some authors have suggested

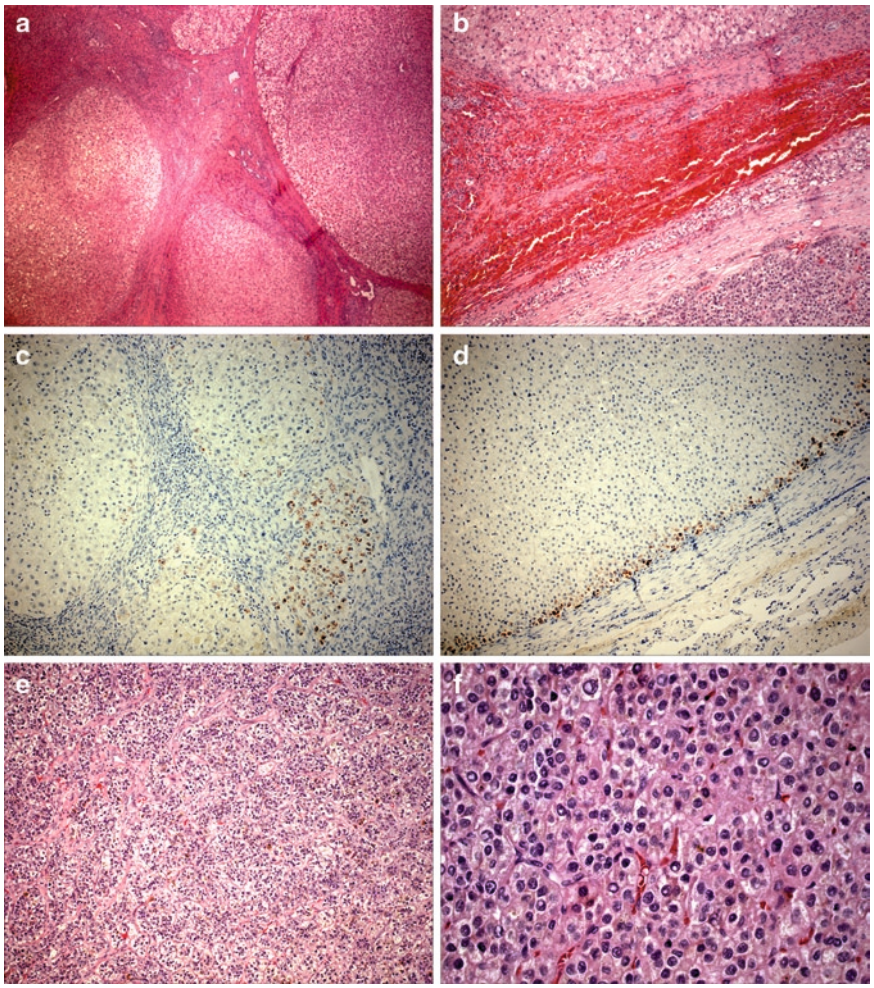

Fig. 43.4 Histological characteristics of Wilson disease. (**a**) (H&E image) Well developed cirrhosis with neoplastic nodule to top left of image. (**b**) (H&E image) nodule of hepatocellular carcinoma (HCC) (*upper right*) adjacent to non-neoplastic nodule. (**c**) and (**d**) (Rhodanine stains) prominent copper in cirrhotic nodules (**c**) and at the periphery of HCC. (**e**, **f**) Solid arrangement of neoplastic hepatocytes indicative of HCC. (**e**) Low-power H&E image; (**f**) high-power H&E image

decreasing the threshold of urine copper to 75 μg/24 h to increase the sensitivity of diagnosis [56].

Establishing the diagnosis of Wilson disease may be difficult in some patients. The use of molecular testing can provide additional evidence for the diagnosis or exclusion of Wilson disease by the presence or absence of mutations of *ATP7B*. Molecular testing is useful when routine testing is equivocal for Wilson disease and for family screening once the genotype for the proband is established. The limitation to molecular testing is the ability to identify all the affected alleles in suspect individuals. In prior studies only ~65% of all affected alleles were identified in patients with an appropriate phenotype [52] though further improvements are increasing the frequency of detecting mutant alleles. Continued cataloguing of the results of mutation analyses will help the interpretation of the results of molecular diagnosis by *ATP7B* mutation detection [53].

Treatment

Once the diagnosis of Wilson disease is established, lifelong therapy with copper chelators or zinc salts is necessary. Chelating agents include trientine and penicillamine that are used for initial and maintenance therapy, while zinc salts are useful for maintenance therapy [3, 9]. Tetrathiomolybdate is another copper chelator that is still investigational in the USA and has undergone testing for treatment of patients presenting with neurological Wilson disease [57]. In afflicted patients, impaired hepatic function and neurological symptomatic improvement may occur over a 1–4-year period of time.

Liver transplantation is curative for Wilson disease and amounts to a form of gene therapy for this disorder by replacing the main site of defective ATP7B and changing the phenotype of the patient [58]. Those with Wilson disease who should be considered for liver transplantation include those with

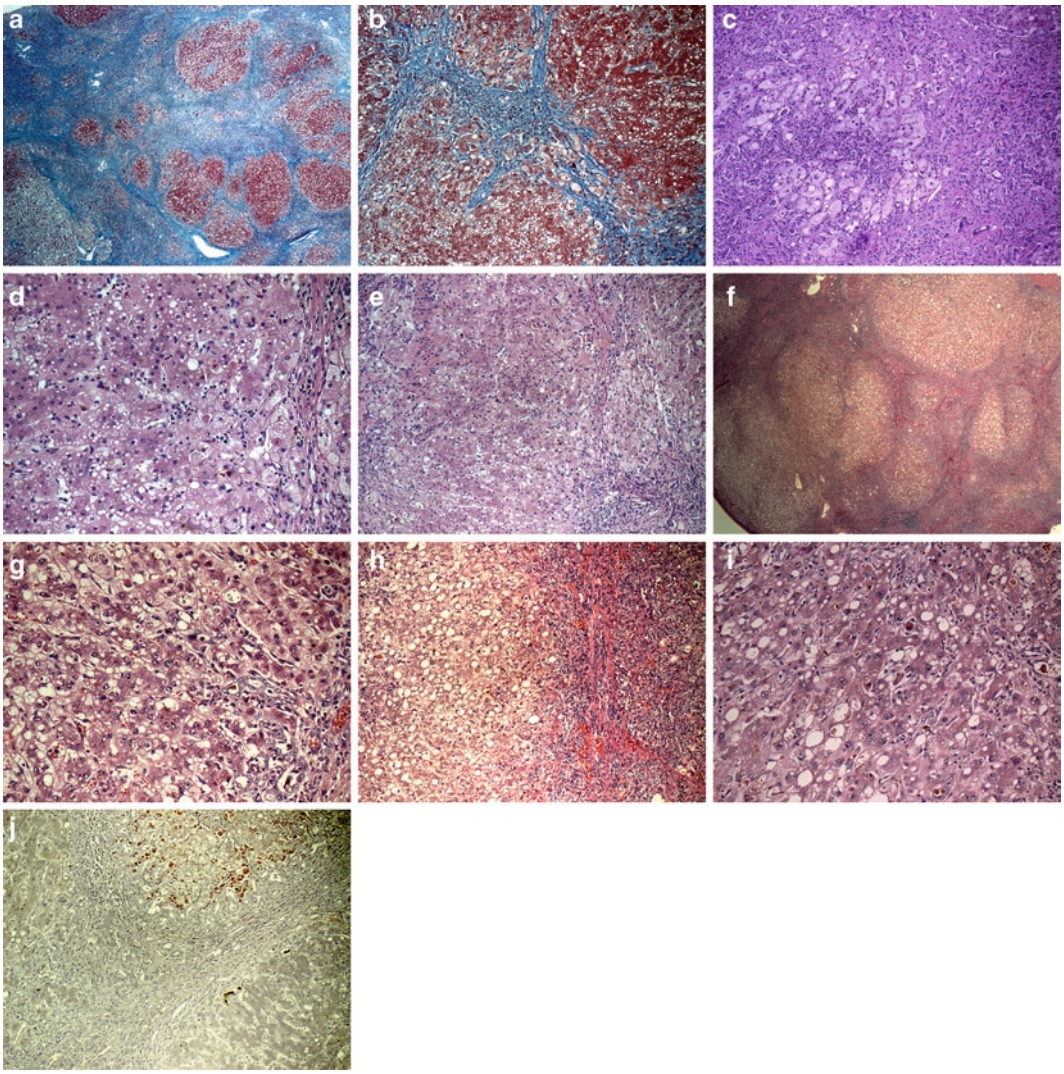

Fig. 43.5 (**a**) (H&E), (**b**, **c**) (Trichrome stains): well developed cirrhosis with associated pericellular/sinusoidal fibrosis (**c**). (**d**–**i**) (H&E images): lobular collapse with prominent hepatocellular balloon degeneration, feathery degeneration, abundant Mallory hyaline and prominent hepatocellular apoptosis/necrosis with prominent cholangiolar reaction (**f**) and variable, sometimes marked cholestasis (**h** and **i**). (**j**) (Rhodanine stain): prominent but patchy accumulation of copper

Table 43.1 Common ATP7B mutations [52, 53]

Mutation – nucleotide (amino acid)	Exon	Location	Frequency of affected allele (%)
– 441/– 427 del	5' UTR	Sardinia[a]	>50
1934T_G (M645R)	6	Spain	>50
R778L	8	Asia	14–49
p.R778Q (c.2333 G-_A)	8	China	35
2299insC (G710S)	8	Central, Eastern and Northern Europe	<10
3400delC	15	Central, Eastern and Northern Europe	<10
R969Q	13	Central, Eastern and Northern Europe	<10
c.3034G_C p.G1012R	13	Greece	25
c.3207C>A (H1069Q)	14	Central, Eastern and Northern Europe	30–90

[a]The Wilson disease gene encodes a copper transporting P-type ATPase (ATP7B). Molecular genetic analysis reveals distinct mutations in the ATP7B gene among specific population groups with varying allele frequencies. A few dominant mutations may be present in populations, for instance _441/_427del, 213–214delAT, 1512–1513insT, R778W, 2463delC, and V1146M combined account for ~85% of affected chromosomes in the Sardinian population

advanced liver disease unresponsive to medical therapy and ALF due to Wilson disease. Patients with ALF should also be evaluated for liver transplant as early as possible, as there is virtually no recovery with medical therapy alone [59]. While awaiting transplantation, specific treatment that acutely lowers copper in the circulation can be initiated. Treatments such as plasmapheresis and hemofiltration, or with other devices such as MARS or SEPAD that provides albumin dialysis exchange may be utilized [60, 61]. Removing excess copper can help reduce hemolysis and other secondary copper induced renal tubular damage. Liver transplant for neurological disease in the absence of liver failure is controversial.

Acknowledgement We would like to acknowledge the Wilson's Disease Association and the Yale New Haven Transplantation Center. There are no financial disclosures, no conflicts of interest to report.

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Chapter 44
Hemochromatosis

James E. Nelson, Debbie Trinder, and Kris V. Kowdley

Introduction

Hereditary hemochromatosis (HH) is one of the most common genetic disorders among Caucasians. The first clinical description of hemochromatosis was over a century ago by Tousseau [1]. The disease is characterized by increased iron deposition in multiple organs due to increased iron absorption from a normal diet [2]. The consequences of iron overload in this setting are end-organ damage in multiple tissues such as the liver, heart, anterior pituitary gland, pancreas, skin, and joints. The main clinical complications in patients with HH include cirrhosis and hepatocellular carcinoma (HCC), hypogonadotropic hypogonadism, arrhythmias, insulin-dependent diabetes, and arthropathy, especially involving the metacarpophalangeal (MCP) joints [2, 3]. The mainstay of therapy is iron depletion by phlebotomy. Early diagnosis is important because treatment prior to the development of cirrhosis is associated with normal life expectancy and drastically reduces the risk of cirrhosis and HCC [2, 3]. By contrast, patients who have cirrhosis at the time of diagnosis have an increased mortality risk due to end-stage liver disease and liver cancer. In this chapter, we summarize the molecular aspects of iron metabolism, the cellular and molecular abnormalities associated with HH, and other forms of iron overload.

HH is currently classified into five types based on mutations in different genes responsible for iron metabolism [4]. Type 1 HH, or the *HFE*-associated HH, is the most common form of HH, and is inherited as an autosomal recessive trait. This mutation is very common in Caucasians especially in Northern Europe. The prevalence of the homozygous C282Y mutation is as high as 1:70 in Ireland [5], and approximately 1:250 in the United States [6]. The penetrance of this mutation is fairly low and clinical expression is generally mild in the majority of patients. However, many patients may have clinically silent disease for many years and the classic phenotypic features may not be present in all patients, leading to under-diagnosis. Furthermore, the symptoms associated with clinical disease may be nonspecific (such as fatigue, abdominal pain, impotence, and joint symptoms) leading to lack of clinical suspicion of HH.

Type 2 HH is also called "juvenile hemochromatosis" because of the presence of massive iron overload early in life, leading to severe end-organ damage, in particular cardiomyopathy, endocrine dysfunction, and cirrhosis. Type 2 HH is associated with mutations in genes encoding for hepcidin or hemojuvelin, which are major iron regulatory proteins, as discussed below.

Type 3 HH is characterized by mutations in the transferrin receptor 2 gene (*TFR2*); this gene has a high degree of homology to transferrin receptor 1 (*TFR1*) and is expressed in the liver. The phenotype of Type 3 HH is similar to Type 1 HH. Iron overload develops gradually and patients usually present in middle or middle-to-late age.

Type 4 HH is associated with mutations in the gene encoding ferroportin, the major iron export protein in cells; this form of HH has a distinct phenotypic pattern compared to other forms of HH. Patients with Type 4 HH generally have normal serum transferrin-iron saturation (TS) and elevated serum ferritin levels. In addition, these patients may not tolerate phlebotomy due to prompt development of anemia. The histologic patterns of hepatic iron deposition in the various forms of HH are described below.

The diagnosis of HH generally begins with measurement of serum TS and ferritin, followed by *HFE* gene testing. An overwhelming majority of Caucasian patients with the phenotype of HH are homozygous for the C282Y mutation in the *HFE* gene or compound heterozygotes for the *HFE* C282Y and H63D mutations. Clinical testing is widely available via commercial laboratories for these *HFE* mutations. Among patients with suspected HH who lack these mutations, the diagnosis is generally made on phenotypic grounds (i.e., liver biopsy and measurement of quantitative hepatic iron) since genetic testing is not yet available on a clinical basis for Type 2–4 HH [3].

K.V. Kowdley (✉)
Director, Liver Center of Excellence, Benaroya Research Institute at Virginia Mason, 1201 Ninth Avenue Seattle, WA 98101-2795
e-mail: jnelson@benaroyaresearch.org

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_44, © Springer Science+Business Media, LLC 2011

Iron Deposition Patterns in Hemochromatosis

In HH types 1–3, iron preferentially accumulates in periportal hepatocytes with a decreasing gradient in acinar zones two and three; referred to as the classic HH iron deposition pattern [7–10]. However, in later stages of HH characterized by heavy iron loading, iron deposition can spread into reticuloendothelial cells (REC) [7–10]. In contrast to the autosomal recessive HH types 1–3, HH Type 4 (also known as ferroportin disease) is an autosomal dominant disease manifested by either REC iron accumulation or the typical HH pattern depending on the location of the mutation in the ferroportin coding region [11]. Two types of ferroportin mutations have been identified; those that are defective in cell surface localization resulting in failure of iron export and REC iron accumulation, and those that are not responsive to hepcidin regulation due to a decreased ability of ferroportin to be internalized and degraded following hepcidin binding leading to the typical HH pattern of iron deposition [11]. Thus, ferroportin disease provides an example of how different genotypes can alter the hepatic iron deposition phenotype.

Overview of Iron Metabolism

The liver plays an important role in iron metabolism. It is the primary site of iron storage in the body and is central in the regulation of iron homeostasis. In healthy individuals, approximately 1–2 mg of iron is absorbed from the diet each day to maintain iron balance. Once absorbed, iron is bound by the plasma protein transferrin and is transported to the tissues where it is used for the synthesis of heme and non-heme proteins or stored as ferritin. Most of the iron is taken up by the bone marrow for incorporation into hemoglobin for erythropoiesis, and to a lesser degree by the muscle for the synthesis of myoglobin and respiratory enzymes. Excess iron is stored primarily in the hepatocytes. In addition, macrophages phagocytose senescent erythrocytes, heme is degraded and iron is recycled back to the plasma to be re-utilized by the bone marrow and other tissues [12].

Iron metabolism is tightly regulated by the hepatic iron regulatory hormone hepcidin. It is synthesized predominately by hepatocytes and secreted into the circulation where it binds to the iron exporter ferroportin, which is expressed on the surface of enterocytes, macrophages, and hepatocytes. Hepcidin induces ferroportin degradation, thus limiting the absorption of dietary iron by the intestine and the release of iron from macrophages and iron stores in the liver. Excess iron increases hepcidin, which in turn limits iron availability for erythropoiesis and other iron-dependent processes, while low iron down-regulates hepcidin increasing iron bioavailability (discussed in detail below) [13].

Over recent years, our understanding of iron metabolism and the molecular pathogenesis of HH has increased greatly due to the identification of a number of iron-regulatory molecules and cellular iron transport proteins that mediate intestinal iron absorption and hepatic iron transport. As described above, in HH In HH, mutations have been identified in a number of genes (*HFE*, *HJV, HAMP*, and *TFR2*) that encode key regulatory proteins. These mutations result in impaired hepcidin synthesis and dysregulated iron homeostasis by enhancing iron absorption and recycling, with deposition of the excess iron, particularly in the liver, causing iron overload. Too much iron is detrimental; as excess iron can generate free radicals causing oxidative stress and liver damage [14].

Liver Iron Transport

Hepatocytes and Kupffer cells (KCs) both have the capacity to bi-directionally exchange iron with the blood. Under normal physiological conditions, they acquire transferrin-bound iron (TBI) from the plasma by several pathways including transferrin receptor (TFR) mediated processes and release iron back to the plasma via the iron exporter ferroportin. KCs, like other macrophages, obtain most of their iron by erythrophagocytosis.

Transferrin-Bound Iron Uptake

Uptake of TBI can occur through different mechanisms.

High-Affinity Uptake: Transferrin Receptor 1

Hepatocytes and KCs, like other cells, express TFR1, which is a transmembrane protein that binds holotransferrin (iron-loaded transferrin) with high-affinity at the cell surface. The receptor-transferrin complex is then internalized into endocytic vesicles where the iron is released from transferrin by the low pH of the endosome. The iron is reduced from a ferric to a ferrous form by a ferrireductase called six-transmembrane epithelial antigen of prostate 3 (STEAP3) and transported across the endosomal membrane to the cytosol by divalent metal transporter 1 (DMT1). Iron is then utilized for iron-dependent processes or stored as ferritin. The receptor-apotransferrin (iron-deficient transferrin) complex is recycled to the cell surface where, at the higher extracellular

pH, the affinity of TFR1 for apotransferrin is reduced and the apotransferrin is released from the receptor into the circulation to bind more iron [15, 16].

Uptake of iron by the TFR1-mediated process is dependent on the expression of TFR1, which is regulated by cellular iron levels. Depletion of cellular iron upregulates TFR1 expression and TBI uptake, while increased iron reduces TFR1 expression and iron uptake. This regulation is achieved via an iron responsive element-iron regulatory protein (IRE-IRP) post-transcriptional mechanism. TFR1 is also regulated by cellular proliferation, hypoxia, and inflammation [15, 16].

Low-Affinity Uptake: Transferrin Receptor 2

For many years it has been recognized that hepatocytes take up the majority of their iron by a low-affinity, high-capacity, TFR1-independent pathway. With the identification of a second type of TFR expressed in hepatocytes, it was suggested that TFR2 may be responsible for the low-affinity uptake of iron. TFR2, has a high degree of homology to TFR1, but binds holotransferrin with a 25–30-fold lower affinity [17]. TFR2 has been shown to increase cellular iron uptake by the internalization and recycling of transferrin, suggesting that TFR2 mediates iron uptake by an endocytic process that is similar to TFR1. However, recent studies suggest that TFR2 plays a relatively limited role in hepatic iron transport. In mice that lack functional TFR2, it was found in vivo that hepatic iron uptake from plasma transferrin was reduced by only 20% when compared with wild-type mice with similar iron status [18]. Additionally, in mouse hepatocytes, increased TFR2 expression was not associated with enhanced TBI uptake while, in hepatoma cells, over or under-expressing TFR2 altered TBI uptake modestly despite large variations in TFR2 expression [19]. Together these results suggest that TFR2 can mediate TBI uptake, but it is unlikely to make a major contribution to hepatocyte iron transport. Therefore, it is likely that another high-capacity process, which is responsible for significant iron uptake, is operating in hepatocytes. The mechanism responsible for this process remains unclear.

There is evidence of another iron uptake pathway in hepatocytes that does not involve endocytosis; rather, iron is released from transferrin at the cell surface and enters the cell by a carrier-mediated process. Furthermore, in hepatic cells, it has been shown that the uptake of TBI is inhibited by non-transferrin bound iron (NTBI) and vice-versa suggesting that both forms of iron may be taken up by a common transporter. In non-hepatic cells, over-expression of TFR2 is involved in the uptake of NTBI, but whether this mechanism operates in hepatocytes is unknown [15].

TFR2, unlike TFR1, is not regulated by cellular iron levels, but is post-translationally regulated by transferrin saturation. The binding of holotransferrin to TFR2 upregulates TFR2 by increasing protein stability and directing the receptor to be recycled to the cell surface whereas, in the absence of holotransferrin, TFR2 is directed to lysosomes for degradation via a multivesicular body pathway. TFR2 expression is also altered by cellular proliferation and differentiation [20].

Non-Transferrin Bound Iron Uptake

Iron is also present in the circulation in the form of NTBI. This is a pool of plasma iron which binds with low-affinity to molecules other than transferrin. Under normal conditions, plasma NTBI concentration is relatively low, but increases with iron loading. NTBI is toxic and can generate reactive oxygen species (ROS) that may cause lipid peroxidation, DNA, and protein damage. It is cleared rapidly from the circulation, mainly by hepatocytes, with most of the iron removed during the first pass through the liver. NTBI uptake involves reduction of iron and transport across the cell membrane by a carrier-mediated process. Two transmembrane proteins have been identified that transport NTBI in hepatocytes. The first is DMT1, which is also involved in endosomal iron transport and iron absorption, and has the capacity to transport a number of divalent metals including iron, manganese, cobalt, and zinc. The second is Zrt-like, Irt-like protein 14 (ZIP14), which was first described as a zinc transporter, is highly expressed in hepatocytes and can also transport NTBI. Whether there are additional hepatic NTBI transporters remains unclear [15, 16].

Iron Release

Ferroportin, the iron export protein is highly expressed on the basolateral surface of KCs and to a lesser degree in hepatocytes. Ferroportin mediates the release of iron across the cell membrane in its ferrous state. Iron is then converted to the ferric ion by the multi-copper oxidase, ceruloplasmin, and bound by circulating transferrin. Iron export is dependent on ferroportin protein levels, which are regulated by both hepatic iron levels and hepcidin expression. Hepatic iron regulates ferroportin protein levels by an IRE-IRP post-transcriptional mechanism, where increased iron stimulates protein translation and iron depletion decreases translation. Hepcidin regulates ferroportin protein levels by a post-translational mechanism where hepcidin binds to ferroportin on the cell surface and induces its internalization, phosphorylation, and degradation in proteasomes [15, 16, 21]

HFE and Liver Iron Transport

HFE is a major histocompatibility complex class I-like protein that interacts with beta$_2$-microglobulin and is expressed by both hepatocytes and KCs. *HFE* mutations inhibit HFE protein interaction with beta$_2$-microglobulin abrogating cell surface expression and function. HFE binds to TFR1 at a site that overlaps with transferrin binding, lowering the apparent affinity of TFR1 for transferrin and reducing TBI uptake by cells. HFE also interacts with TFR2, but at a site that is distinct from the transferrin binding site; thus it does not interfere with transferrin binding [22, 23]. In addition, HFE has been reported to reduce ZIP14-mediated uptake of NTBI in hepatoma cells by decreasing ZIP14 protein stability [24]. Furthermore, HFE is involved in the iron-dependent regulation of hepcidin, as outlined in detail below, which influences ferroportin-mediated cellular iron export [25]. Thus, HFE plays an important role in hepatic iron transport, regulating both TBI and NTBI uptake, and iron release by the liver.

Liver Iron Transport in Hereditary Hemochromatosis

Numerous studies have been undertaken in HH patients and animal of models HH providing valuable insights into the molecular pathogenesis of iron overload [14, 26]. In HH, plasma iron levels and transferrin saturation are elevated leading to an increase in hepatocyte iron storage levels with a relative absence of iron in KCs. In an *Hfe* knockout mouse model of HH, hepatic TBI uptake in vivo is increased indicating that TBI uptake contributes to hepatic iron loading. Expression of TFR1 is downregulated with hepatic iron loading in HH. However, TFR1-mediated iron uptake by hepatocytes isolated from *Hfe* knockout mice is increased compared to wild-type hepatocytes with a similar iron status. This is likely because the absence of *Hfe* allows more holotransferrin to bind to TFR1. The low-affinity uptake of TBI by hepatocytes also increases with rising plasma iron levels but it is not affected by a lack of *Hfe* [19]. TFR2 protein expression is increased in HH because an elevation in transferrin saturation increases TFR2 protein stability, as described above, but its contribution to liver iron uptake is limited. Therefore, it is likely that another low-affinity pathway, independent of TFR1 and TFR2 also contributes to iron loading in HH.

Plasma NTBI levels are elevated in HH patients and *Hfe* knockout mice. NTBI uptake is increased in *Hfe* knockout hepatocytes and is associated with an upregulation in the NTBI transporter, DMT1 [27]. Furthermore, the absence of HFE may also contribute to promoting ZIP14 expression and NTBI uptake [24]. Hence, increased NTBI uptake likely makes a major contribution to hepatic iron loading in HH.

KCs express low levels of TFR1 but not TFR2, and take up low levels of TBI. Uptake of TBI by KCs from *Hfe* knockout mice is an enhanced possibly due to a lack of Hfe competing with holotransferrin to bind to the receptor. The amount of NTBI taken up by KCs is relatively low compared with hepatocytes and is not affected by the absence of Hfe.

In HH patients and *Hfe* knockout mice, the hepatic iron concentration is increased and hepcidin expression impaired, leading to an upregulation of ferroportin and cellular iron release [14, 15, 21]. Ferroportin expression is high in KCs and an increase in iron export is likely to contribute to the absence of iron loading seen in KCs in HH. However, in the hepatocytes where ferroportin expression is low, any increase in iron release is likely to be relatively minor and has a limited effect on reducing hepatic iron loading.

In summary, hepatic iron loading in HH is due to an increase in the uptake of both NTBI and TBI with limited release of iron by hepatocytes. The mechanisms responsible for increased TBI uptake involve both TFR1 and TFR2-mediated pathways, as well as an additional, less well-defined, low-affinity pathway (Fig. 44.1). In contrast, KCs are relatively spared of iron in HH as a result of significantly enhanced iron export with a limited increase in TBI uptake.

Hepcidin is a Central Regulator of Iron Homeostasis

Hepcidin expression is influenced by the balance of a number of positive and negative regulators with excess iron and inflammation upregulating hepcidin expression and iron deficiency, anemia, and hypoxia downregulating hepcidin expression (Fig. 44.1). The iron-dependent regulation of hepcidin is controlled by HFE/TFR2 and bone morphogenetic protein/ hemojuvelin/mothers against decapentaplegic homologue (BMP/HJV/SMAD) cell-signaling pathways [13, 26].

The mechanism by which HFE and TFR2 regulate hepcidin is not yet fully understood. HFE and TFR2 are hypothesized to act as sensors of plasma transferrin saturation directing hepatocytes to modulate hepcidin synthesis. A current model proposes that both HFE and transferrin compete for binding to TFR1 and as transferrin saturation increases, holotransferrin displaces HFE from TFR1 leaving HFE free to bind to TFR2. Then the HFE-TFR2 complex or HFE and TFR2 separately may induce hepcidin synthesis [28, 29]. The nature of this mechanism is uncertain, but there is evidence to suggest that it involves SMAD and extracellular signal regulated kinase (ERK) signaling pathways [13, 26].

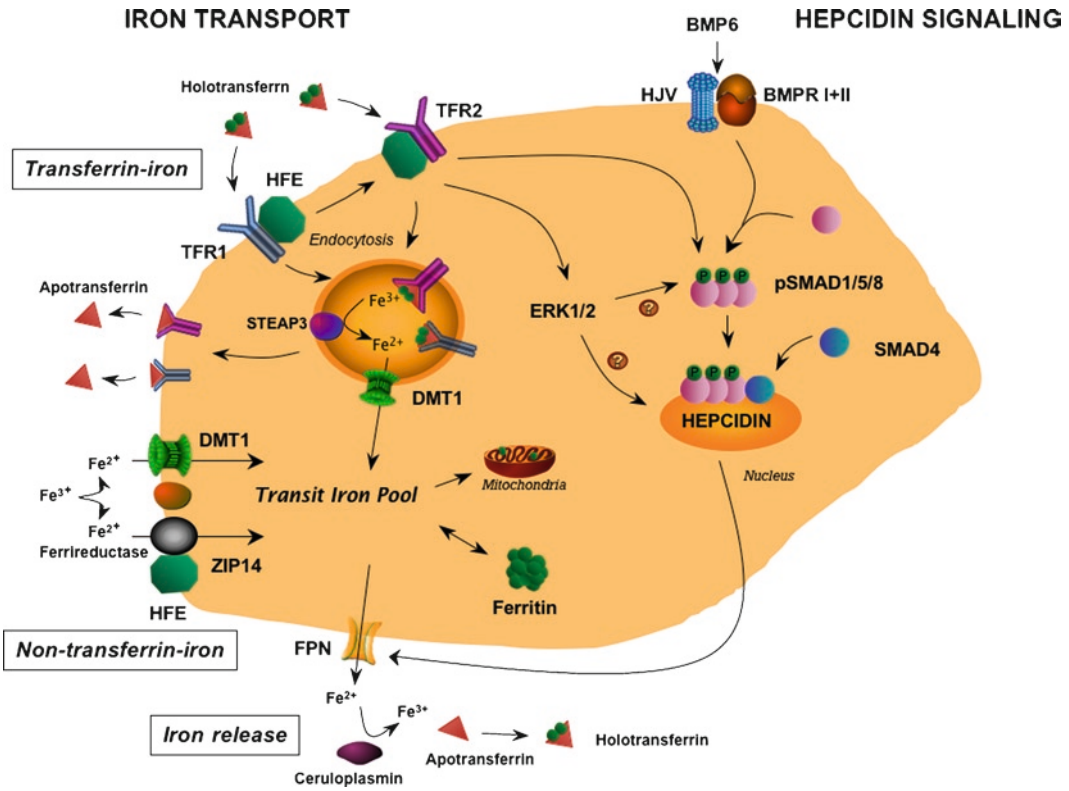

Fig. 44.1 Hepatocyte iron transport and hepcidin signaling. Transferrin-iron uptake: holotransferrin binds to TFR1 and is endocytosed into an endosome where the iron is released from transferrin and reduced by STEAP3. Iron is transported out of the endosome via DMT1 and apotransferrin is recycled. TFR2 mediates uptake of holotransferrin by a similar mechanism to TFR1. Non-transferrin-iron uptake: Iron is reduced by a ferrireductase and transported into the cell by DMT1 and ZIP14. Iron release: iron is exported by FPN, oxidized by ceruloplasmin, and binds to apotransferrin. Hepcidin signaling: BMP6 binds to HJV/BMPRI+II and phosphorylates SMAD1/5/8, which binds to SMAD4 and activates hepcidin transcription. HFE and TFR2 act together, or separately, to induce SMAD1/5/8 phosphorylation and ERK1/2 signaling to activate hepcidin. Hepcidin is secreted by the hepatocyte and binds to FPN inducing its internalisation and degradation. *TFR1* transferrin receptor 1; *TFR2* transferrin receptor 2; *STEAP3* six-transmembrane epithelial antigen of prostate 3; *DMT1* divalent metal transporter 1; *ZIP14* Zrt-like, Irt-like protein 14; *FPN* ferroportin; *BMP6* bone morphogenetic protein; *HJV* hemojuvelin; *BMPRI+II* bone morphogenetic protein receptor I and II; *pSMAD1/5/8* phospho-mothers against decapentaplegic homologue 1,5,8; *SMAD4* mothers against decapentaplegic homologue 4; *HFE* hemochromatosis protein; *ERK1/2* extracellular signal regulated kinase

BMP/SMAD signaling plays a key role in iron-dependent regulation of hepcidin. Liver BMP6 levels are increased with iron loading and, in the absence of BMP6, SMAD signaling and hepcidin synthesis are attenuated causing severe iron overload [30]. BMPs bind to liver BMP receptors and, together with HJV, a BMP co-receptor, augment SMAD signaling [31]. This involves phosphorylation of SMAD 1/5/8, interaction with SMAD4, translocation to the nucleus and activation of hepcidin transcription. There is cross-talk between HFE/TFR2 and BMP/SMAD pathways, as the absence of functional HFE or TFR2 attenuates iron-induced phosphorylation of SMAD 1/5/8. Furthermore, TFR2 activates ERK 1/2, which may act via SMAD 1/5/8 or directly with the hepcidin gene, to regulate transcription [13, 26].

The iron-dependent regulation of the hepcidin-ferroportin pathway that controls iron homeostasis is impaired in HH. Mutations in *HFE, HJV, HAMP,* and *TFR2* genes that cause HH type 1, 2A, 2B and 3, respectively, reduce HFE/TFR2 or BMP/HJV/SMAD signaling and hepcidin synthesis. HH patients with mutations in the *HFE, HJV,* and *TFR2* genes have been shown to have inappropriately low urinary levels of hepcidin allowing unregulated iron absorption and hepatic iron overload [32–34]. HH type 4 is caused by "loss of function" mutations in *FPN* that impair ferroportin-mediated iron export (type 4A) or "gain of function" mutations that prevent hepcidin-induced degradation of ferroportin (type 4B). In all types of HH except for type 4A, ferroportin is upregulated leading to increased dietary iron absorption and macrophage iron recycling, raising plasma TBI and NTBI levels, and promoting iron uptake and deposition in hepatocytes and other parenchymal cells. However, in type 4A, attenuation of ferroportin-mediated cellular iron export results in the accumulation of iron primarily in macrophages [13, 26].

In contrast to iron-mediated upregulation of hepcidin, hepcidin expression is downregulated in response to hypoxia and erythropoiesis via the transcription factors HIF-1 [35]

and CEBP/α [36], respectively. In addition, hepcidin expression is induced during inflammation by activation of the transcription factor STAT3 by the inflammatory cytokines IL-6 and IL-1 [37, 38]. Upregulation of hepcidin via IL-6 is the mechanism responsible for "anemia of inflammation" a condition often observed associated with chronic disease [39]. Two hallmarks of anemia of inflammation are the direct result of IL-6 mediated increased hepcidin; inhibition of duodenal iron absorption and iron sequestration in macrophages [39]. Hepcidin expression has also recently been shown to be upregulated in response to ER stress via transcription factors CREBH [40] and CHOP [41]. Other proposed regulators of hepcidin include leptin [42], p53 [43], estradiol [44], and circadian rhythms [44]. The importance of these alternate pathways of hepcidin regulation in HH is poorly understood.

Iron and Oxidative Stress

The histopathologic consequences of iron overload in the liver is directly related to the role of iron in catalyzing the production of free radicals, which in turn damage organic membranes, resulting in mitochondrial and other organelle dysfunction, cell injury, and death [45]. The molecular mechanisms leading to oxidative stress and its relevance in liver diseases are discussed in Chap. 28. The high redox potential of iron (particularly labile iron) facilitates its function as the catalyst for the generation of hydroxyl radicals (•OH) from the reactive oxygen species (ROS) H_2O_2 (hydrogen peroxide) and superoxide ($\bullet O_2^-$) in a series of chemical reactions named for their discoverers Henry John Horstman Fenton (Fenton reaction) [46], and Fritz Haber and his student Joseph Weiss (Haber-Weiss reaction) [47]. ROS are by-products of normal cellular aerobic respiration resulting from incomplete reduction of dioxygen in mitochondria and other organelles. Iron also catalyzes the production of the highly reactive nitronium ion (NO_2^+) from peroxynitrite ($ONOO^-$), formed through a reaction of $\bullet O_2^-$ and nitric oxide (NO). It should also be noted that NO can also react with Fe^{2+} and Fe^{3+} thus limiting the availability of the iron cations to participate in Fenton and Haber-Weiss reaction [48]. Thus the capacity of iron to generate cytotoxic free radicals is to a large extent dependant on the subcellular milieu of ROS and reactive nitrogen species (RNS). There also exist a number of cytoprotective mechanisms within the cell to control the

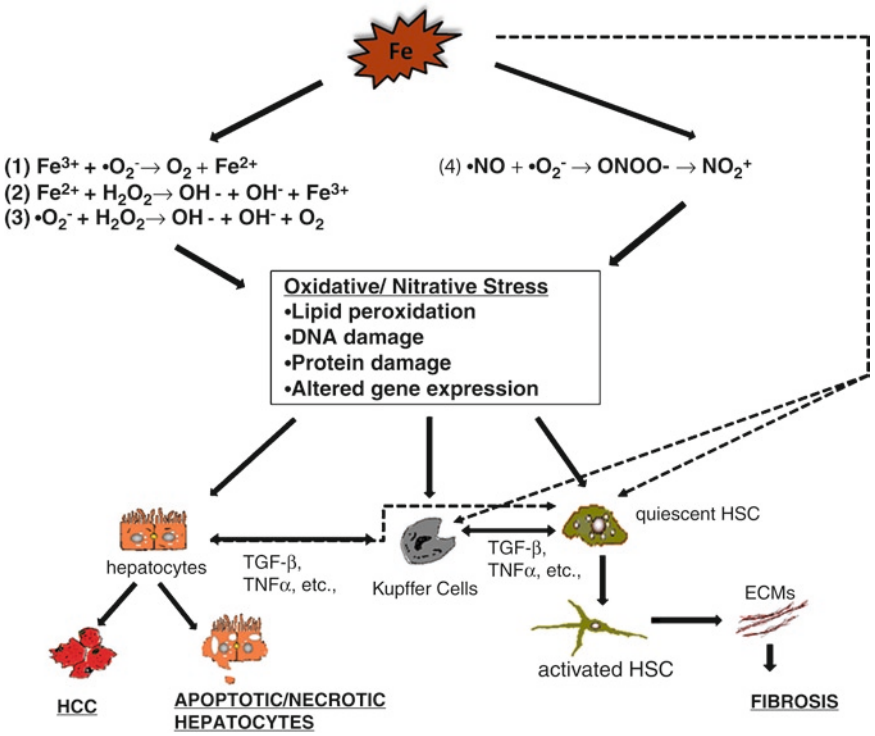

Fig. 44.2 The role of iron in oxidative stress and the pathogenesis of hemochromatosis. Reduction of ferric iron (Fe^{3+}) by superoxide($\bullet O_2^-$) (*1*) is the first step in the Fenton reaction (*2*) where ferrous iron interacts with hydrogen peroxide (H_2O_2) to catalyze the production of hydroxyl (•OH) radicals. The net result is called the Haber-Weiss reaction (*3*). Iron also catalyzes the production of the highly reactive nitronium ion (NO_2^+) from peroxynitrite ($ONOO^-$), formed through a reaction of $\bullet O_2^-$ and nitric oxide (NO) (*4*). Collectively, •OH and NO_2^+ radicals cause lipid peroxidation of organic membranes, resulting in mitochondrial and other organelle dysfunction, damage DNA and proteins, and activate proinflammatory, profibrogenic, and cytotoxic pathways, which can result in activation of HSC, hepatocellular apoptosis and necrosis and ultimately fibrosis and HCC. Iron may also directly potentiate these effects in Kupffer cells and HSC.

toxic effects of free radicals including enzymes like superoxide dismutase, glutathione peroxidase, and glutathione reductase or antioxidants like glutathione and vitamins A, C, and E [49]. When the level of ROS overwhelms the cellular antioxidant capacity, then a state of oxidative (or nitrative) stress occurs (Fig. 44.2). Thus excess tissue iron, through its role in the Fenton and Haber-Weiss reactions, is a potent inducer of oxidative stress, and is the basis of iron toxicity.

Lipid Peroxidation

The highly oxidative •OH and NO_2^+ radicals damage lipid membranes by attacking the double bonds of polyunsaturated fatty acids forming a number of lipid radicals which self-perpetuate by reacting with adjacent fatty acids in a process termed lipid peroxidation [49]. By-products of this process including 4-hydroxynonenal (4HNE) and malondialdehyde (MDA) are often used as markers of lipid peroxidation and both have been shown to be elevated in hemochromatotic livers [50, 51]. Any of the lipid membranes of the cell may be damaged through lipid peroxidation including the plasma membrane and organelles like mitochondria, ER, and lysosomes. In iron overload, iron is sequestered into lysosomes presumably as a protective mechanism, but lipid peroxidation of lysosomes can induce lysosomal fragility and both ferritin and hemosiderin have been shown to induce lysosomal lipid peroxidation in vitro [52]. It has been proposed that release of iron from lysosomes is an important step in mitochondrial dysfunction and cytotoxicity due to oxidative stress [53, 54]; however, it is not clear if this process actually contributes to hepatocellular damage in HH [55] (Fig. 44.2). Mitochondrial lipid peroxidation may contribute to pore formation in mitochondrial membranes or "mitochondria permeability transition" (MPT) [56]. MPT from iron overload can lead to mitochondrial dysfunction and result in uncoupled oxidative phosphorylation [57, 58], mitochondrial DNA damage [59], release of mitochondrial ROS [60], and cell death via apoptosis or necrosis [61]. The alternate mechanisms of cell death either by necrosis through ATP depletion or caspase-dependant apoptosis induced by cytochrome c release both result from MPT, which may explain the coexistence of these pathologic processes in many liver diseases [62]. For more information on modes of hepatic cell death, please see Chaps. 24 and 25.

DNA and Protein Damage

Hydroxyl radicals can damage DNA through production of DNA adducts at many sites such as the modified guanosine base 8-hydroxydeoxyguanosine (8-OHdG) [63], single and double strand breaks, oxidation of the deoxyribose backbone, apurination, and DNA protein cross linking [64, 65]. The potential for iron to catalyze DNA damage and the protective effect of iron chelation have been known for many years [66–69]. The close proximity of chromatin bound iron in the presence of ROS such as H_2O_2, facilitate repeated attacks of *de novo* OH radicals ensuring DNA damage such as double strand breaks [69] (Fig. 44.2). Etheno-DNA adducts, formed from DNA interaction with lipid peroxidation by-products like 4HNE, have been observed in hepatic DNA of HH patients [70]. Thus, iron-induced oxidative DNA damage may contribute to the 200-fold increased risk of developing HCC in HH patients [71, 72]. Multiple studies have found a variety of mutations in the tumor suppressor gene p53 in 64–71% of HCC tumors from HH patients, suggesting a potential connection between iron-induced DNA damage and loss of function p53 mutations in HCC [73, 74].

ROS and RNS can also cause protein damage through oxidation or nitration of specific amino acids, fragmentation or structural changes resulting in altered functionality or inactivation [75, 76] (Fig. 44.2). A common marker for free radical induced protein damage is 3-nitrotyrosine, formed by nitration of tyrosine residues by $ONOO^-$ and other RNS. Cumulatively, the consequences of prolonged protein modification by oxidative and nitrative stress can have profound effects on cell function and viability and has been implicated in the pathogenesis of many human diseases and aging [75, 76].

Iron, ROS, and Cell Signaling

In addition to their direct cytotoxic effect, ROS are thought to directly stimulate a variety of proinflammatory, profibrogenic, and cytotoxic pathways. Activation of these signaling cascades in the liver is mediated by the redox sensitive transcription factor nuclear factor-κB (NF-κB). In cultured KCs NF-κB activation is dependent on Fe^{2+} and precedes the release of tumor necrosis factor-α (TNF-α) [77]. In vivo studies in rats revealed TNF-α and IL-6 expression coincides with deposition of lipid peroxidation markers in the periportal region, all of which was abrogated by iron chelation treatment [78]. TNF-α together with IL-6 and transforming growth factor-β (TGF-β) are the primary mediators of inflammatory, fibrogenic, and apoptotic responses within the liver [79, 80]. TGF-β, a major effector of hepatic fibrogenesis and apoptosis, co-localizes with MDA and iron in hepatic and sinusoidal cells in acinar zone 1 in HH patients, which was normalized following phlebotomy [51]. TNF-α mediated induction of the caspase cascade via the death receptor TNF-R1 is a major pathway of programmed cell death. An interesting paradox is the fact that TNF-α also induces the NF-κB anti-apoptotic survival pathway [81]. In addition to activation of the NF-κB pathway, increased ROS and

oxidative stress also promote apoptosis by activation of the JNK/AP1 serine kinase signaling cascade, but depending on the duration and type of ROS may also promote resistance to apoptosis via the ERK1/2 pathway [82]. For additional details on this signaling cascade please see Chap. 20.

Alcohol consumption is known to exacerbate the pathogenesis of hemochromatosis. This process may be in part mediated by oxidative stress; ethanol can synergize with lipid peroxidation to create MDA-acetaldehyde adducts. These adducts have been shown to directly stimulate the secretion of cytokines and chemokines such as TNF-α, MCP-1, and MIP-2 in liver endothelial cells and hepatic stellate cells (HSC) [83].

Immune Responses in Hemochromatosis

The generation of hepatic inflammation is a complex process involving the interplay between pro-inflammatory and anti-inflammatory cytokines produced by multiple signaling pathways within immune cells and other liver cells including hepatocytes, HSC, and cholangiocytes. Role of inflammation in liver pathology is included in Chap. 27. Despite the induction of a number of cytokines by iron induced oxidative stress, the role of inflammation in the pathogenesis of hemochromatosis is poorly understood. Only mild, scattered inflammatory infiltrate is usually observed in HH [84, 85]. A few studies have characterized the inflammatory infiltrate cell type in hemochromatosis using immunohistochemistry. Multiple studies have found differences in the immunophenotype of tissue macrophages in subjects with HH, including increased CD14 [86] and HAM-56 [85] positive cells and decreased MAC-387 positive cells [85], suggesting mobilization of tissue macrophages may be important in HH. Differences in $CD68^+$ KCs were not observed between HH patients and non liver disease control subjects [85, 86]. $CD3^+$ T cells have been characterized in hemochromatotic livers in a periportal and lobular distribution [85, 87] and were positively correlated to hepatic iron concentration, suggesting a potential role for iron in T cell responses in the liver [85]. A number of studies have shown an increase in the $CD4^+/CD8^+$ ratio in peripheral blood or liver of HH patients [88–91]. Moreover, the decrease in $CD8^+$ T cells was significantly associated with disease severity [90, 91]. The implications of these observations are unclear.

Perhaps due to the lack of overt inflammation in hemochromatosis, few studies have investigated the extent or importance of cytokines and chemokines in the pathogenesis of HH. However, it has been proposed that despite a lack of significant inflammation in HH, a number of proinflammatory and profibrogenic cytokines, such as TNF-α, TGF-β, IL-6, and IL-13, among others, may still have a pivotal role in HH disease progression [45, 85]. Bridle et al. have found that the hepatic expression profile of cytokines interferon-gamma (IFNγ) and IL-10 to be similar to subjects with the inflammatory liver disease HCV; IFNγ expression was increased, while IL-10 mRNA was decreased in HH patients compared to non-disease controls [85]. The authors speculate that since IFNγ inhibits activation of HSC, an initial step in fibrogenesis [92] and downregulates IL-10 which is thought to be a profibrogenic cytokine [93], the expression profile of these cytokines may contribute to the slow progression of fibrosis usually observed in HH. In contrast, this study failed to show any difference in expression of the proinflammatory and profibrogenic cytokines TNF-α or TGF-β between HH patients and controls [85]. A number of studies have looked at correlation between various TNF-α polymorphisms and clinical and histologic features of HH. Most studies did not find any such association [94–97]; however, one study found that carriage of the 308G > A allele seemed to be protective and was associated with lower siderosis and ALT levels [98], whereas another study found this allele to be associated with increased body iron stores (assessed by duration of phlebotomy treatment) or hepatic iron index, but not worsened clinical or histologic features of HH, including fibrosis [99]. Similarly, the role of TGF-β in HH is also controversial with two studies failing to find increased mRNA levels in either HH patients [85] or an iron overload rat model [100]. In contrast, increased levels of TGF-β protein co-localized with iron deposition in hepatocytes and sinusoidal cells in hepatic acinar zone 1 in both rats with iron overload [101] and HH patients [51], which subsequently normalized following phlebotomy treatment [51]. Together, these studies suggest that post-transcriptional regulation of TGF-β may occur with hepatic iron overload. Much work remains to fully elucidate the role of cytokines in HH pathogenesis; however, as in other inflammatory liver diseases, it seems clear that there is interplay between pro and anti-inflammatory mediators occurring in the iron overloaded liver.

The Role of Iron in Fibrogenesis

The process of hepatic fibrogenesis in HH has not been as extensively studied as in other liver diseases, but many similarities do exist although the initial precipitating insult (i.e., iron) may be unique. For additional information on hepatic fibrosis, please see Chap. 30. For example, as described earlier, in contrast to many other liver diseases, massive morphologic inflammation preceding fibrosis does not occur in HH. Moreover, in the hemochromatotic liver, fibrosis is usually observed only subsequent to significant iron loading

of hepatocytes and KCs [7, 8] (Fig. 44.2). In hemochromatosis, like other liver diseases, it is believed that KCs, the major resident hepatic macrophage, are thought to be an important mediator of fibrogenesis. In addition to typical macrophage functions including phagocytosis, antigen processing, and presentation, they are key effectors of the phenotype of surrounding cells including other KCs, hepatocytes, HSC, and innate immune cells via the production of proinflammatory/profibrogenic cytokines, ROS, NO, and prostaglandins [102, 103]. In addition, KCs are a source of tissue ferritin via phagocytosis of senescent red cells and damaged hepatocytes. Interestingly, a recent study has demonstrated that ferritin can act as a proinflammatory cytokine in an iron independent fashion, resulting in induction of the NFκB pathway and expression of a number of NFκB responsive proinflammatory/profibrogenic cytokines in activated HSC [104]. KCs also thought to be a major source of TGF-β, the primary mediator of HSC activation and induction of fibrogenesis [105]. A key step in this paracrine fibrogenesis cascade initiated by KC and other cells is the activation of HSC. During this process, morphologic changes occur that transform this normally quiescent storage cell into a proliferative myofibroblast-like cell producing key components of the extra cellular matrix such as collagen type I and III [101, 105]. This process continues with the remodeling of the extracellular matrix, which is facilitated by matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) which are also secreted by KC, HSC, and hepatocytes. Production of MMPs and TIMPs are regulated by a variety of mediators produced by KC and other liver cells including TNF-α, TGF-β, IL-6, and H_2O_2 [106].

Early in hemochromatosis during mild iron deposition, activated HSC can be seen in the centrilobular zone 3 region, distal to the site of initial iron deposition and fibrosis in the zone 1 periportal region [107]. However, with progressive iron deposition, activated HSC are observed in zone 2 and eventually in zone 1 [107]. Ramm et al. also showed in HH livers there is a strong correlation between HIC and detection of alpha smooth muscle actin (αSMA), a marker of activated HSC, which was detectable at only 60 μmol/g HIC [107]. Furthermore, the number of αSMA positive HSC significantly decreased following phlebotomy therapy [107]. It is tempting to speculate that the location of initial HSC activation removed from the site of early iron deposition suggests that humoral factors produced by iron containing hepatocytes and macrophages may be important in the iron-mediated activation of HSC. However, it is possible that there may be functionally different HSC subtypes with different susceptibilities to the effects of iron within the liver, a hypothesis that is supported by the observation that HSC have differing morphology and marker expression depending on their acinar location [107].

Early in vitro work and studies in iron loaded rodents suggest iron accumulation in KCs and/or hepatocytes may potentiate the process of HSC activation and fibrogenesis indirectly via oxidative stress as described earlier [108–113], or possibly through more direct signaling mechanisms [114–117]. Histologically, detectable iron is not usually observed in HSC; however HSC do express high levels of the iron transport proteins DMT1 and ferroportin, as well as TFR1, and in activated HSC a receptor for ferritin [118–120]. Thus it is possible that NTBI, transferrin, and ferritin-bound iron could potentiate the effects of iron on HSC activation and fibrogenesis via receptor signaling pathways in HSC. Future gene expression profiling studies in HH or iron loaded animal livers will be useful to determine the molecular networks involved in iron-mediated fibrosis.

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Chapter 45
Glycogen Storage Diseases

Mingyi Chen

Introduction

Glycogen is a branched polymer of glucose, which serves as a reservoir of glucose units. The two largest deposits in mammals are in the liver and skeletal muscle but many cells are capable of synthesizing glycogen. Its accumulation and utilization are under elaborate control by a variety of enzymes.

Glycogen in the liver (and to a lesser degree in the kidneys) serves as a form of stored and rapidly accessible glucose, so that the blood glucose level can be maintained between meals. Different hormones, including insulin, glucagon, and cortisol regulate the relationship of glycolysis, gluconeogenesis, and glycogen synthesis. For about 3 h after a carbohydrate-containing meal, high insulin levels direct liver cells to take glucose from the blood, to convert it to glucose-6-phosphate (G6P), and to add the G6P molecules to the ends of chains of glycogen (glycogen synthesis). Excess G6P is also shunted into production of triglycerides and exported for storage in adipose tissue as fat [1]. The glucose and glycogen metabolic pathways in liver are discussed in greater detail in chapter 8 of this textbook and also summarized in Fig. 45.1.

Etiology

Glycogen storage diseases (GSDs) are a heterogeneous group of disorders differing in clinical, biochemical, and molecular features. Defects in basic metabolizing enzymes lead to severe consequences, whereas, with some exceptions, mutations in the regulatory proteins appear to cause a more subtle phenotypic change. GSDs can be genetic or acquired, and are characterized by abnormal inherited glycogen metabolism in the liver, muscle, and brain. Genetic GSDs are caused by inborn errors of metabolism and involve genetically defective enzymes. They are mostly inherited as autosomal recessive disorders and result in defects of glycogen synthesis or catabolism. The acquired GSDs are often caused by intoxication with alkaloids.

M. Chen (✉)
Department of Pathology and Laboratory Medicine, Loma Linda University Medical Center, Loma Linda, CA, USA
e-mail: mchen@llu.edu

Types of Glycogen Storage Diseases

The overall incidence of GSDs is estimated at 1 case per 20,000–43,000 live births. Disorders of glycogen degradation may affect primarily the liver, the muscle, or both. There are over 12 types (divided into types 0–XI), and they are classified based on the enzyme deficiency and the affected tissue (Table 45.1). Glucose-6-Phosphatase deficiency (Type I), Pompe's disease (Type II), debrancher deficiency (Type III), and liver glycogen phosphorylase deficiency (Type VI) are the most common forms in children and myophosphorylase deficiency (Type V) is common in adults. GSD types VIa, VIII, IX, and X are caused by tissue-specific phosphorylase deficiency. Type XI is characterized by hepatic glycogenosis and renal Fanconi syndrome. Although glycogen synthase deficiency does not result in storage of extra glycogen in the liver, it is often classified with the GSDs (as Type 0), because it is also a type of glycogen storage defect and can cause similar problems.

Clinical Features of GSDS

Type 1 GSD

Type 1 GSD (Von Gierke's disease) is due to absence or deficiency of glucose-6-phosphatase activity in liver, kidney and intestinal mucosa with excessive accumulation of glycogen in these organs. It is an autosomal recessive disorder. Patients with type 1 GSD present in the neonatal period with hepatomegaly, hypoglycemic seizures, and lactic acidosis. They may present at 3–4 months with doll like facies due to accumulation of fat on cheeks and growth retardation. Laboratory parameters show hypoglycemia and lactic acidosis on short fast, hyperuricemia, and normal or slightly elevated liver

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5 ,
DOI 10.1007/978-1-4419-7107-4_45, © Springer Science+Business Media, LLC 2011

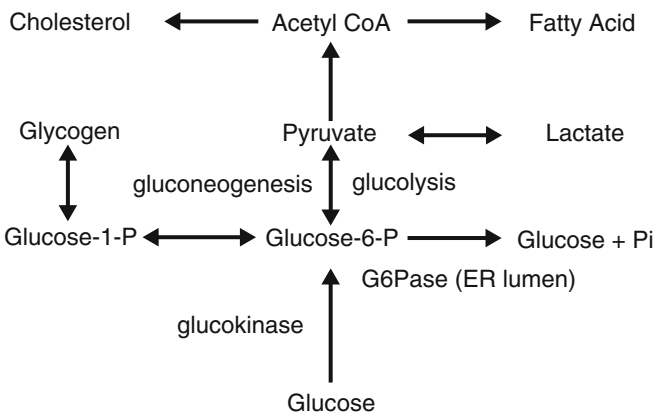

Fig. 45.1 Glucose metabolism in hepatocytes

Table 45.1 Summary of the types of glycogen storage diseases

GSD type	Enzyme deficiency	Eponym
GSD type I	Glucose-6-phosphatase	von Gierke's disease
GSD type II	Acid maltase	Pompe's disease
GSD type III	Glycogen debrancher	Cori's disease or Forbes disease
GSD type IV	Glycogen branching enzyme	Andersen's disease
GSD type V	Muscle glycogen phosphorylase	McArdle's disease
GSD type VI	Liver glycogen phosphorylase	Hers' disease
GSD type VII	Muscle phosphofructokinase	Tarui's disease
GSD type IX	Phosphorylase kinase	–
GSD type XI	Glucose transporter	Fanconi-Bickel disease
GSD type 0	Glycogen synthase	–

enzymes with hyperlipidemia. Liver histology shows not only glycogen, but also presence of fat in the hepatocytes with little associated fibrosis (Fig. 45.2). Long-term complications include gout, hepatic adenomas, osteoporosis, renal disease, and short stature with most patients surviving to mid adulthood [2]. The diagnosis is suspected on clinical presentation and abnormal lactate & lipid levels. Administration of glucagons or epinephrine results in little or no rise in blood glucose. A definite diagnosis is made by determination of enzyme activity on liver biopsy or identification of mutations for G-6-P or translocase gene.

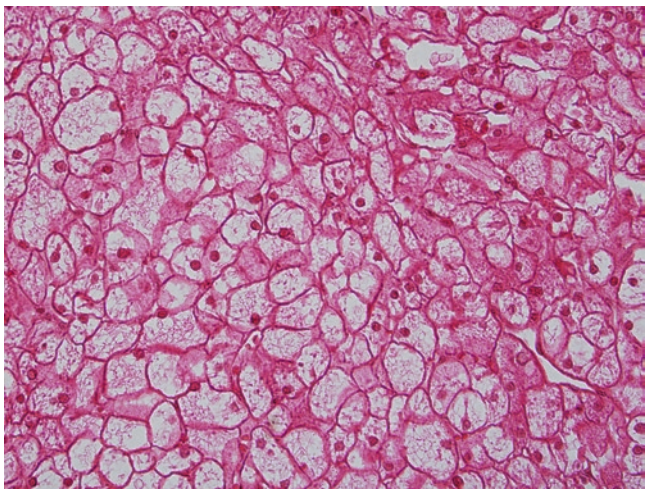

Fig. 45.2 Liver biopsy from an 18-month-old-male who had hepatomegaly for 6 months and was diagnosed with von Gierke's disease. Deficiency of glucose-6-phosphatase results in accumulation of glycogen in hepatocytes. The hepatocytes are swollen and a mosaic histological pattern with compression of the sinusoids is seen. Microvesicular steatosis is also present

Type II GSD

Type II GSD (Pompe's disease) is a prototype of inborn lysosomal storage diseases and involves many organs, but primarily the muscle. It is due to acid maltase deficiency and is an autosomal recessive disorder. The c.–32–13T>G is the most frequent mutation in Caucasian populations [3]. The infantile variety presents at 0–6 months with cardiomegaly, hypotonia and hepatomegaly with death by 2 years. The juvenile form presents as myopathy and cardiomyopathy in childhood, and death by the second decade due to respiratory failure. The adult form presents between the second and seventh decade as slow progressive myopathy without cardiac involvement, but with progressive respiratory failure.

Type III GSD

Type III GSD (debrancher deficiency, limit dextrinosis) is caused by deficiency of glycogen debranching enzyme activity as a result of which glycogen breakdown is incomplete and an abnormal glycogen with short outer branch chains accumulates. It is an autosomal recessive disorder with genetic defect on chromosome 1p21 [4]. Type IIIa involves both the liver and muscle, and IIIb solely the liver. The exon three mutations (17delAG and Q6X) are specifically associated with GSD-IIIb. The splice mutation IVS32-12A>G is found in GSD-III patients having mild clinical symptoms, while the 3965delT and 4529insA mutations are associated with a severe phenotype and early onset of clinical manifestations [5]. The disorder usually affects liver and muscle, although, in 15% of patients only the liver is involved. Patients present in childhood with hepatomegaly, hypoglycemia, hyperlipidemia and growth retardation and may be indistinguishable from type I disease. However, in Type III, blood lactate and uric acid levels are normal and liver enzymes are elevated. Liver symptoms improve with age and disappear after puberty. In patients with muscle involvement, the muscle weakness becomes predominant in adulthood leading to distal muscle wasting and ventricular hypertrophy. Liver histology is characterized by distension of hepatocytes by glycogen and presence of fibrous septa with paucity of fat. Glucagon administered 2 h after a carbohydrate meal provokes a normal rise of blood glucose, but no change after overnight fast. Definitive diagnosis requires enzyme assay in liver or muscle or both. Mutation analysis can also be done. Treatment is symptomatic with frequent feeds and uncooked cornstarch supplementation.

Type IV GSD

Type IV GSD (branching enzyme deficiency/Andersen's disease) usually presents in the first year of life with hepatomegaly and growth retardation. L224P and Y329S are the two most common mutant alleles, and PCR-based mutation analysis is used for prenatal diagnosis of GSD type IV [6]. Patients present with failure to thrive, hypotonia, hepatosplenomegaly, progressive cirrhosis, and death by the fifth year. Liver transplant is an effective treatment modality.

Type V and VII GSD

Types V and VII involve only the muscle.

In Type V GSD (McArdle's disease), muscle glycogenoses is caused by deficiency of muscle phosphorylase and presents in adulthood with exercise intolerance, muscle cramps, and attacks of myoglobinuria.

Type VII GSD is caused by deficiency of muscle phosphofructokinase with clinical features similar to GSD V, but is also associated with hemolytic anemia.

Type VI and IX GSD

Types VI and IX are a heterogeneous group of diseases caused by deficiency of the liver phosphorylase and phosphorylase kinase system. There is no hyperuricemia or hyperlactatemia.

Type VI GSD is caused by deficiency of liver phosphorylase and is a benign condition causing hepatomegaly, mild hypoglycemia, hyperlipidemia, and ketosis. There is no hyperlactic acidemia or hyperuricemia. The mainstay of treatment is high carbohydrate diet and frequent feedings.

Type IX GSD is due to phosphorylase kinase deficiency and the clinical picture depends on the organs involved. X-linked liver phosphorylase deficiency results in growth retardation and incidentally detected hepatomegaly. Hypoglycemia is mild, if present. Symptoms improve with age.

Type VIII and X GSD

Types VIII and X were considered distinct conditions in the past but are now reclassified with Type VI.

Molecular Mechanisms

Type 1 GSD, first described by Von Gierke in 1929, is the most common of the GSDs. It is a group of autosomal recessive disorders with an incidence of 1 in 100,000 [1]. In 1952, Cori and Cori showed that GSD-1 is caused by the absence of glucose-6-phosphatase (G6Pase) activity, establishing for the first time that metabolic disorders could arise from enzyme deficiencies [7]. Deficiency of G6Pase impairs the ability of the liver to produce free glucose from both glycogen and gluconeogenesis. Since these are the two principal metabolic mechanisms by which the liver supplies glucose to the rest of the body during periods of fasting, it causes severe hypoglycemia. Reduced glycogen breakdown results in increased glycogen storage in the liver and kidneys, causing their enlargement. Both organs function normally in childhood, but are susceptible to a variety of problems in the adult years. Other metabolic derangements include lactic acidosis and hyperlipidemia.

G6Pase is an enzyme located on the inner membrane of the endoplasmic reticulum (ER). It has been shown to comprise of at least five different polypeptides: a catalytic subunit, a regulatory Ca2+ binding protein, and three transport

proteins (glucose-6-phosphate, phosphate/pyrophosphate, and glucose) [1]. The catalytic unit is associated with a calcium binding protein and three transport proteins (T1, T2, and T3) that facilitate movement of glucose-6-phosphate (G6P), phosphate, and glucose (respectively) into and out of the enzyme [8, 9]. A defect of these proteins could cause type I glycogenosis. The major subtypes of GSD I are designated as GSD Ia and GSD Ib, the former accounting for over 80% of diagnosed cases and the latter for less than 20%. Molecular and genetic evidence have unequivocally demonstrated that the two major GSD-1 subgroups, GSD-1a and GSD-1b, have different etiologies [8]. GSD-Ia is caused by complete absence of or markedly decreased microsomal G6Pase enzymatic activity in the liver and the kidneys. GSD-Ib has normal G6Pase activity, and is caused by a deficiency in the glucose-6-phosphate transporter (G6PT) systems that abolish or greatly reduce microsomal G6P uptake activity. Both G6Pase and G6PT are associated with the ER membrane. G6PT translocates G6P from the cytoplasm into the lumen of the ER, whereas, G6Pase hydrolyses the G6P into glucose and phosphate. Together, G6Pase and G6PT thus maintain glucose homeostasis. G6Pase is expressed primarily in gluconeogenic tissues, namely, the liver, kidney, and intestine. However G6PT, which transports G6P efficiently only in the presence of G6Pase, is expressed ubiquitously. This suggests that G6PT may play other roles in tissues that lack G6Pase [9]. Both GSD-Ia and GSD-Ib patients manifest phenotypic G6Pase deficiency, characterized by growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia. The current treatment is mainly a dietary therapy that includes frequent or continuous feedings of cornstarch or other carbohydrates [10]. Allopurinol may be given to lower uric acid levels. Other therapeutic measures may be needed for associated problems. GSD-Ib patients also suffer from chronic neutropenia, and functional deficiencies of neutrophils and monocytes, resulting in recurrent bacterial infections as well as ulceration of the oral and intestinal mucosa, which is treated with granulocyte colony stimulating factor to restore myeloid function. The other two possible GSD type I variants; GSD-Ic and GSD-Id were observed in some literatures to be caused by deficiencies in pyrophosphate translocase, and glucose translocase activities, respectively. So far, the molecular basis of these disorders is not fully established [11].

GSD-I has an incidence in the American population of approximately 1 in 100,000–200,000 births [9]. In the past, GSD-1 was diagnosed primarily by clinical symptoms, supported by measurements of G6Pase activity in liver biopsy samples. Reliable carrier testing was not available. Recent advances in molecular analysis of hepatic GSDs have made rapid progress in our understanding of glycogen metabolism and we are able to reevaluate these disorders at the molecular level. With cloning of the GSD-Ia and GSDIb genes, DNA-based diagnostic tests for this disorder have been developed in many laboratories and a database of G6Pase and G6PT mutations has been established [8, 12]. The database provides the foundation for a gene-based diagnosis of carriers in at-risk families and a non-invasive prenatal screening test [8, 13, 14].

The human glucose-6-phosphatase cDNA was first cloned and characterized in 1993 [8, 9]. The G6Pase gene contains 5 exons, spans approximately 12.5 kb, and maps to chromosome 17q21. It encodes a 36-kDa glycoprotein that is anchored to the ER by nine transmembrane helices with its active site facing the lumen. To date, more than 80 separate mutations have been identified in the G6Pase gene [8]. These include missense (D38V, W77R, R83C, R83H, E110K, E110Q, A124T, V166G, P178S, G184E, G188S, G188R, L211P, G222R, W236R, P257L, G270V, R295C, and L345R), insertion/deletion (813insG822delC), nonsense (R170X and Q347X), and codon deletion (DF327) splicing mutations, which can abolish or greatly reduce G6Pase activity. Moreover, a splicing G6Pase mutation (727GÆT) was shown to cause exon-skipping. R83C and Q347X are the most prevalent mutations found in Caucasians [11]; R83C is the only prevalent mutation in the Ashkenazi Jewish population [15]; 130X and R83C are most prevalent in Hispanics; and R83H is most prevalent in Chinese [11, 16].

The G6PT gene maps to chromosome 11q23 and encodes a 37-kDa protein that is anchored to the ER by ten transmembrane helices [8]. DNA-based diagnostic tests for G6PT genes mutation seen in GSDIb have been developed. To date, more than 70 separate mutations have been identified in the G6PT gene. These include missense, nonsense, insertion/deletion, splicing, and codon-deletion mutations, which appear to be scattered throughout the coding region. Of the mutations identified to date, more than 80% are found in Caucasian patients, presumably reflecting their greater ethnic diversity [8]. Within this group, 1211delCT and G339C are the prevalent mutations, accounting for over 40% of all cases [8]. The prevalent mutation of G6PT gene in Japanese patients is W118R [17].

Once the diagnosis is suspected, the multiplicity of clinical and laboratory features usually makes for strong circumstantial evidence. If hepatomegaly, fasting hypoglycemia, and poor growth are accompanied by lactic acidosis, hyperuricemia, hypertriglyceridemia, and enlarged kidneys by ultrasound, GSD-I is the most likely diagnosis. The differential diagnoses include glycogenoses types III and VI, fructose 1,6-bisphosphatase deficiencies, and a few other conditions, but none are likely to produce all of the features of GSD-I. The diagnosis can be supported by liver biopsy with electron microscopy, and assay of glucose-6-phosphatase activity in the tissue. It is further confirmed by specific gene testing, establishing the molecular basis of this disorder. The database of the residual enzymatic activity

retained by the G6Pase and G6PT mutations is facilitating the correlation of the disease phenotype with the patients' genotype [8, 13].

The recent developments of genome-based studies have increased our understanding of the molecular mechanism of GSD disorders, and the progress will facilitate the development of novel therapeutic approaches for these disorders [18].

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Chapter 46
α_1-Antitrypsin Deficiency

David H. Perlmutter

Introduction

The classical form of α_1-antitrypsin (AT) deficiency, homozygous for the Z allele, is the most common genetic cause of liver disease in children and the most frequent genetic disease for which people undergo liver transplantation. As a cause of chronic hepatitis, cryptogenic cirrhosis, and hepatocellular carcinoma with new onset in the adult, this diagnosis has been under-appreciated. Among the liver diseases it has a unique and fascinating pathobiology, related to the hepatotoxic consequences of an aggregation-prone mutant protein.

In the most extensively studied population, the Swedish population, the incidence of the deficiency is approximately 1 in 1,639 live births [1]. Data from eight separate studies suggest that the prevalence of the deficiency in the United States is 1 in approximately 3,000 individuals [2]. It especially affects Caucasians of northern European ancestry [3, 4].

AT is an ~55 kDa serum glycoprotein that inhibits destructive neutrophil proteases, including elastase, cathepsin G, and proteinase 3. Circulating AT is predominantly derived from the liver and serum levels increase three to fivefold during the host response to inflammation/tissue injury. It is the archetype of a family of proteins called serpins because most of the members are serine protease inhibitors that circulate in the blood and body fluids.

Serum concentrations of AT are reduced by approximately 85–90% in homozygotes. A single amino acid substitution results in an abnormally folded protein that also has a tendency to polymerize and aggregate. It is inefficiently translocated through the secretory pathway so that there is a marked decrease in secretion together with accumulation of the polymerization-prone protein in the endoplasmic reticulum (ER) of liver cells.

D.H. Perlmutter (✉)
Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA
e-mail: david.perlmutter@chp.edu

AT deficiency was first recognized as a cause of chronic obstructive lung disease/emphysema (COPD), sometimes with premature onset in the third or fourth decade of life. Cigarette smoking markedly accelerates the onset and increases the severity of COPD in AT-deficient individuals. Most of the evidence in the literature indicates that this is caused by a loss-of-function mechanism in which the decrease in number of AT molecules within the lower respiratory tract allows unregulated proteolytic activity to destroy the connective tissue matrix of the lung [5, 6]. Cigarette smoking is thought to accelerate the lung injury by provoking increased release of active oxygen intermediates by phagocytes in the airways which, in turn, results in functional inactivation of any residual AT [7]. Indeed, the elastase-antielastase theory for the pathogenesis of COPD is based on the concept that oxidative inactivation of AT as a result of cigarette smoking accounts for connective tissue matrix destruction in AT-sufficient individuals, the vast majority of COPD patients [5]. Several observations about polymers of the mutant AT protein in the respiratory epithelium and interstitium have raised the possibility that gain-of-toxic function mechanisms may contribute to the lung injury that is principally due to the loss-of-function effects in most patients [7].

In contrast, liver disease in AT deficiency is entirely caused by a gain-of-toxic function mechanism. This is most powerfully demonstrated by transgenic mouse models in which liver disease is a sequelae of expressing the mutant human ATZ gene in a mouse that is otherwise replete with its own endogenous antielastases [8, 9]. The mechanism by which accumulation of the mutant ATZ molecule in the ER causes liver-cell injury is currently being investigated. A genetic epidemiology study carried out in Sweden has shown that only 8% of homozygotes that are identified in an unbiased newborn screening protocol develop clinically significant liver disease through the first three decades of life [1, 10]. This study has led to the concept that genetic and environmental modifiers play a major role in predisposing a subgroup of homozygotes to liver disease.

The diagnosis of AT deficiency is based on altered migration of the abnormal ATZ molecule in serum specimens subjected to isoelectric focusing. The hepatic histopathologic

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DOI 10.1007/978-1-4419-7107-4_46, © Springer Science+Business Media, LLC 2011

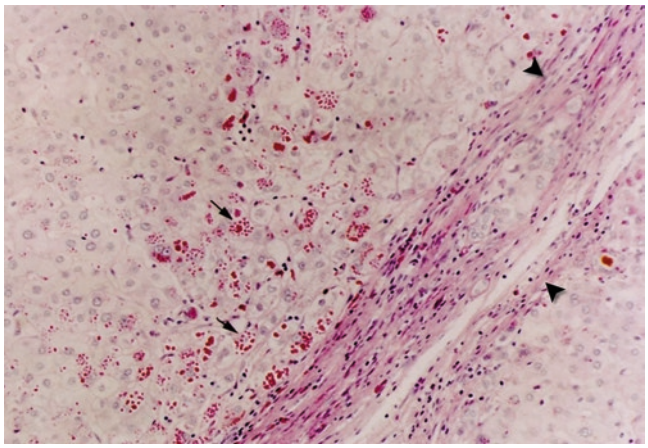

Fig. 46.1 Liver biopsy from a PIZZ individual. *Arrows* point to hepatocytes with typical PAS-positive, diastase-resistant globules and *arrowheads* point to a band of fibrosis. Reprinted from Perlmutter [180], with permission

hallmark of the disease is intracellular globules in hepatocytes that stain with Periodic acid–Schiff after diastase treatment (Fig. 46.1). These globules represent the mutant ATZ that has accumulated in the ER of liver cells.

Treatment of severe liver disease associated with AT deficiency is currently limited to liver transplantation. Milder hepatic phenotypes are managed by supportive measures. Novel pharmacological and cell-based strategies for prevention/treatment of the liver disease are being actively investigated.

AT Structure

AT is encoded by a 12.2-kb gene on chromosome 14q31–32.3 [11]. There is a sequence-related gene approximately 12 kb downstream. Because there is no evidence that it is expressed, the downstream gene is considered a pseudogene.

The gene is composed of five exons and four introns [12]. Exon Ic, the 5′ portion of exon II, and the 3′ portion of exon V are noncoding regions. The first intron is 5.3 kb long, contains a short open reading frame, an Alu family sequence, and a pseudotranscription initiation codon. Apparently, the short open reading frame does not code for protein. The AT mRNA expressed in the liver is 1.4 kb long [13]. In macrophages, the AT mRNA is slightly longer [13]. There are three forms of AT mRNA in macrophages, depending on transcription initiation sites in two upstream exonic structures (exons IA and IB) [13, 14]. Novel transcription initiation sites have also been identified in exon IA for expression in corneal epithelial cells and in a lung-derived epithelial cell line [15].

The AT protein is a single-chain, approximately 55-kDa polypeptide with 394 amino acids and three asparagine-linked complex carbohydrate side chains [16]. There are two major serum isoforms depending on the presence of a biantennary or triantennary configuration of the carbohydrate side chains. AT is the archetype of a family of structurally related proteins called serpins (serine protease inhibitors), which includes antithrombin III, α_1-antichymotrypsin, C1 inhibitor, α2-antiplasmin, protein C inhibitor, heparin cofactor II, plasminogen activator inhibitors I and II, and protease nexin I among others [17]. A serpin-like structure is also found in several cellular proteins, trophic factors, and circulating carrier proteins, such as corticosteroid- and thyroid hormone-binding globulin.

From a higher order structural perspective, AT is composed of two central β sheets surrounded by a small β sheet and nine α helices [18]. The dominant structure is the five-stranded, β-pleated sheet called the A sheet. A mobile reactive center loop rises above a gap in the center of the A sheet [19–21].

The Protease Inhibitor System for Classification of Structural Variants of AT

Variants of AT in humans are classified according to the PI phenotype system, as defined by agarose electrophoresis or isoelectric focusing of plasma in polyacrylamide at acid pH [4]. The PI classification system assigns a letter to variants according to migration of the major isoform. For example, the most common normal variant migrates to an intermediate isoelectric point, designated M. Persons with the most common severe deficiency have an AT allelic variant that migrates to a high isoelectric point, designated Z. With modern DNA sequencing techniques in addition to isoelectric focusing, investigators have identified more than 100 allelic variants of AT [22].

The most common normal variant of AT is termed M1 and is found in 65–70% of white persons in the United States [23]. There are many rare normal allelic variants with allelic frequencies of less than 0.1%. For each of these variants, serum concentration and functional activity of AT are within the normal range.

AT variants in which AT is not detectable in serum are called null-allelic variants. Inheritance of a null-allelic variant with another null variant or a deficiency variant is associated with premature development of COPD [24, 25]. There has been no evidence of liver injury in persons with null variants who have been examined in detail [26]. Potential molecular mechanisms for the null phenotype have been identified with DNA sequence analysis of a number of null variants [22, 24–29]. They include deletion of all AT coding exons, substitutions that result in stop codons, frameshift mutations, and single-base substitutions. In at least three cases, the

frameshift mutation results in an abnormal truncated protein that is retained in the ER (nullHong Kong, nullClayton, and nullSaarbrucken) [26–28, 30]. Detailed study of some of these variants has raised questions about classification. Although AT was not detected in the serum of a patient with what has been called AT nullLudwigshafen [29], this mutant molecule is synthesized and secreted in transfected heterologous cells [31]. Its rate of secretion is slightly decreased and the secreted mutant protein lacks functional activity. It is not yet known whether instability or accelerated catabolism in vivo is the explanation for the inability to detect this mutant AT molecule in serum specimens.

ATPittsburgh is the a well-characterized dysfunctional variant [32]. This variant was identified in a 14-year-old boy who died of an episodic bleeding disorder. A single amino acid substitution, Met to Arg at residue 358, converted AT from an elastase inhibitor to a thrombin inhibitor. The episodic nature of the illness was attributed to changes in the synthesis of the mutant protein during the host response to acute inflammation and tissue injury, the acute-phase response. The AT MMineral Springs [31, 33] and nullLudwigshafen [29, 31] probably are other examples of dysfunctional variants.

Several variants of AT that are associated with a reduction in serum concentrations are called deficiency variants. Some of these variants are not associated with clinical disease, such as the S variant [12, 34]. Other deficiency variants are associated only with emphysema, such as MHeerlen [35], MProcida [36], and PLowell [37]. In two persons with MMalton and one with MDuarte, hepatocyte AT inclusions and liver disease have been found with emphysema [38–40]. In one person with the AT SIiyama variant, emphysema and hepatocyte inclusions were reported, but this patient did not have liver disease [41]. The most common deficiency variant, the Z variant, is associated with both COPD and liver disease, as mentioned above and further discussed later.

Function of AT

AT is an inhibitor of serine proteases in general, but it's most important targets are neutrophil elastase, cathepsin G, and proteinase 3, proteases released by activated neutrophils. Several lines of evidence suggest that inhibition of neutrophil elastase is the major physiologic role of AT. First, persons with AT deficiency are susceptible to premature development of emphysema, a lesion that can be induced in experimental animals by means of instillation of excessive amounts of neutrophil elastase [42]. This has led to the concept that destructive lung disease may result from perturbations of the net balance of elastase and AT within the local environment of the lung [5]. Second, the kinetics of association for AT and neutrophil elastase are more favorable, by several orders of magnitude, than those for AT and any other serine protease [43]. Third, AT constitutes more than 90% of the neutrophil elastase inhibitory activity in pulmonary alveolar lavage fluid [5].

AT acts competitively by allowing its target enzymes to bind directly to a substrate-like region within its reactive center loop. The reaction between enzyme and inhibitor is essentially second order, and the resulting complex contains one molecule of each of the reactants. A reactive-site peptide bond within the inhibitor is hydrolyzed during formation of the enzyme-inhibitor complex. The complex of AT and serine protease is a covalently stabilized structure that is resistant to dissociation by denaturing compounds, including sodium dodecyl sulfate and urea. The interaction between AT and serine protease is suicidal in that the modified inhibitor is no longer able to bind with or inactivate enzyme. There is also a profound alteration in the structure of the enzyme, including disruption of the catalytic site, such that the enzyme becomes inactive and subject to proteolytic destruction [44]. Studies have shown that the irreversible trapping of target enzyme is mediated by a profound conformational change in AT, such that the cleaved reactive-loop binding enzyme inserts into the gap in the A sheet [45]. Carrell and Lomas [45] likened the inhibitory mechanism to a mousetrap: the active inhibitor circulates in the metastable, stressed form and then springs into the stable, relaxed form to lock the complex with its target protease.

The net functional activity of AT in complex biologic fluids may be modified by several factors. First, the reactive-site methionine may be oxidized and thereby rendered inactive as an elastase inhibitor [46]. In vitro, AT is oxidatively inactivated by activated neutrophils [47] and by oxidants released by alveolar macrophages of cigarette smokers [48]. Second, the functional activity of AT may be modified by proteolytic inactivation. Several members of the metalloproteinase family, including collagenase, matrix metalloproteinase 9 and Pseudomonas elastase, and of the thiol protease family can cleave and inactivate AT [49]. Third, DNA, which often is released from neutrophils at sites of inflammatory activation and phagocytosis, can impair the cathepsin G inhibitory activity of AT [50].

Although AT from the plasma or liver of persons with PIZZ AT deficiency is functionally active [51], there may be a decrease in its specific elastase inhibitory capacity. Ogushi et al. [52] showed that the kinetics of association with neutrophil elastase and the stability of complexes with neutrophil elastase were significantly decreased for AT isolated from PIZZ plasma. There was no decrease in the functional activity of PISS individuals.

Results of several studies have indicated that AT protects experimental animals from the lethal effects of tumor necrosis factor [53, 54]. Most of the evidence from these studies

indicates that this protective effect is due to inhibition of the synthesis and release of platelet-activating factor from neutrophils [54, 55], presumably through the inhibition of neutrophil-derived proteases.

Results of several studies indicate that AT has functional activities other than inhibition of serine proteases. The carboxyl-terminal fragment of AT, which can be generated during formation of a complex with serine protease or during proteolytic inactivation by thiolproteinases or metalloproteinases, is a potent neutrophil chemoattractant [56]. The chemotactic response is equivalent to that elicited by formyl-methionyl-leucyl-phenylalanine. The carboxyl-terminal fragment of AT also is responsible for an increase in synthesis of α_1-AT in human monocytes and macrophages when these cells are incubated with exogenous neutrophil elastase [57]. In each case, the biologic effect is mediated by the interaction of a pentapeptide neodomain within the carboxyl-terminal fragment of AT and a novel cell-surface receptor, the serpin-enzyme complex (SEC) receptor [58–60].

Results of a provocative series of experiments suggested that α_1-AT inhibits human immunodeficiency virus 1 [61]. Separate mechanisms for inhibition of infectivity and production of intact virus were implicated, but the results have not been independently corroborated.

There were reports years ago that α_1-AT alters immune function through effects on lymphocytes [23, 62]. However, there are inherent conflicts in these reports, and the data have not been replicated. A recent study reported increased IL-8, IL-6, and IL1-receptor antagonist levels in the serum of AT-deficient patients [63]. Furthermore, purified AT suppressed levels of these cytokines in an in vitro system. The authors concluded that AT is an endogenous inhibitor of proinflammatory cytokine production. Another possibility is that the elevated cytokine levels observed in AT-deficient persons are a reflection of mononuclear phagocyte activation. We know that AT is synthesized in mononuclear phagocytes and that mutant ATZ accumulates in the ER of monocytes and macrophages [64, 65]. In fact another recent study has reported increased serum levels of soluble CD14 in patients with AT deficiency [66]. Soluble CD14 is a fragment of the endotoxin receptor that is thought to reflect monocyte activation. However, it is important to point out that there is no evidence that PIZZ individuals are susceptible to infections or fail to respond appropriately to immunization. Although a variety of vasculitis syndromes that might be considered autoimmune in nature have been described in patients with AT deficiency, many of these reports are anecdotal, include patients that are not rigorously proven to be homozygotes for the Z allele and a definitive epidemiological association study has not been done. Vasculitis has not been described in any of the PIZZ cohort identified by the Swedish newborn screening study.

Biosynthesis of AT

The predominant site of synthesis of plasma AT is the liver. This is most clearly shown by conversion of plasma AT to the donor phenotype after orthotopic liver transplantation [67]. AT is synthesized in human hepatoma cells as a 52-kDa percursor; undergoes posttranslational, dolichol phosphate–linked glycosylation at three asparagine residues [68]; and undergoes tyrosine sulfation [69]. It is secreted as a 55-kDa native single-chain glycoprotein with a half-time for secretion of 35–40 min.

Plasma concentrations of AT increase threefold to fivefold during the host response to inflammation or tissue injury [70]. The source of this additional α_1-AT has always been considered the liver; thus AT is known as a positive hepatic acute-phase reactant. Synthesis of α_1-AT in human hepatoma cells (HepG2 and Hep3B) is upregulated by interleukin 6 (IL-6) but not by IL-1 or tumor necrosis factor, and so IL-6 is thought to be the predominant mediator of the acute phase increase in AT levels [71]. Plasma concentrations of AT also increase during oral-contraceptive therapy and pregnancy [72].

AT is also synthesized by human blood monocytes and tissue macrophages [64]. Expression of AT in monocytes and macrophages is influenced by products generated during inflammation, such as bacterial lipopolysaccharide [73] and IL-6 [71].

Finally, AT is synthesized in a variety of epithelial cells in humans [74]. In the intestine, it is synthesized by Paneth cells and enterocytes, increasing with differentiation from crypt to villus tip [75]. In pulmonary epithelial cells, AT synthesis is regulated by cell type-specific mechanisms and responds to the effect of the cytokine oncostatin M [76–78].

Clearance and Tissue Distribution of AT

The half-life of AT in plasma is approximately 5 days [79]. It is estimated that the daily production rate of AT is 34 mg/kg body weight, 33% of the intravascular pool of AT being degraded daily. There is a slight increase in the rate of clearance of radiolabeled ATZ compared with that of ATM when infused into PIMM individuals, but this difference does not account for the decrease in serum levels of AT in persons with the deficiency [80]. The low-density lipoprotein receptor–related protein (LRP) family probably plays a major role in clearance and catabolism of AT when it is in complex with neutrophil elastase [81, 82]. The SEC receptor may be involved in clearance and catabolism of both complex and modified forms of AT [83, 84], but this has not yet been tested in vivo. The mechanism of clearance of native AT is unknown [85].

AT diffuses into most tissues and is found in most body fluids [5]. Its concentration in lavage fluid from the lower respiratory tract is approximately equivalent to that in serum. AT also is found in feces and increased fecal concentrations of AT correlate with the presence of inflammatory lesions of the bowel [86]. In each case, it has been assumed that AT was derived from serum. However, local sites of synthesis, such as macrophages and epithelial cells, may make important contributions to the AT pool in these tissues and body fluids.

Mechanism for Decreased Serum levels of AT and Fate of Mutant AT in PIZZ Individuals

The mutant ATZ molecule is characterized by a single-nucleotide substitution that results in a single-amino-acid substitution, Lys for Glu 342 [87–89]. There is a selective decrease in secretion of AT, the abnormal protein accumulating in the ER [65, 90–92]. The defect is not specific for liver cells, because it also affects AT synthesis in xenopus oocytes, monocytes [65], and transfected-cell lines [91–94]. Site-directed mutagenesis studies have shown that this single-amino-acid substitution is sufficient to produce the cellular defect [92–94]. Once translocated into the lumen of the ER, the mutant AT protein is unable to traverse the remainder of the secretory pathway because it is abnormally folded. Several studies have provided evidence that the substitution of Glu 342 by Lys in the ATZ variant decreases the stability of the molecule in its monomeric form and increases the likelihood that it will form polymers by means of a so-called loop-sheet insertion mechanism [95]. In this mechanism, the reactive center loop of one AT molecule inserts into a gap in the β-pleated A sheet of another AT molecule. Lomas et al. [95] were the first to detect polymers of ATZ in the ER of hepatocytes by electron microscopic examination of a liver biopsy specimen from a PIZZ individual and then to notice that the site of the amino-acid-substitution in the ATZ variant was at the base of the reactive center loop, immediately atop the gap in the A sheet. These investigators predicted that a change in the charge at this residue, as occurs with the substitution of Lys for Glu, would prevent insertion of the reactive-site loop into the gap in the A sheet during interaction with enzyme; therefore the mutant ATZ would be susceptible to the insertion of the reactive center loop of adjacent molecules into the gap in its A sheet. This would cause the mutant ATZ to be more susceptible to polymerization than the wild-type AT. The results of their experiments [95] showed that ATZ undergoes this form of polymerization to a certain extent spontaneously and to a greater extent during relatively minor perturbations, such as an increase in temperature. Although they proposed that elevated temperatures from febrile illnesses might constitute one of the perturbations, An et al. did not detect an increase in polymerization in the liver of the PiZ mouse model of AT deficiency at fever-range temperature elevations [96]. Similar polymers have been found in the plasma of patients with the AT SIiyama variant and the AT MMalton variant [97, 98]. The mutations in AT SIiyama (Ser 53 to Phe) (41) and in ATMMalton (Phe 52 deletion) [39] affect residues that provide a ridge for the sliding movement that opens the A sheet. Thus, these mutations would be expected to interfere with the insertion of the reactive center loop into the gap in the A sheet and therefore leave the gap in the A sheet available for spontaneous polymerization. It is interesting that the hepatocytic AT globules have been found in a few patients with these two variants. Recent observations suggest that the relatively common S variant of AT also undergoes polymerization [99], and that this may account for its retention in the ER, albeit to a milder extent than that for the Z variant [34].

A relatively recent study of the crystallographic structure of a stable serpin dimer has suggested the possibility that polymerization is a result of altered folding kinetics and involves a domain swapping mechanism rather than loop-sheet insertion [100]. In this model, a substantially larger domain of the AT molecule, including both strand 5a and the reactive-site loop of one AT molecule inserts into the gap in the A sheet of another AT molecule. This new structural information provides an explanation for why reactive site loops of other serpins do not polymerize with ATZ in the ER of liver cells and for what has been called the hyperstability of serpin polymers and may lead to novel strategies for reversing polymerization.

Polymerization of other serpins has been identified in other clinical deficiency states, including antithrombin deficiency [101] and C1 inhibitor deficiency [102]. A striking example of this phenomenon is the familial dementia associated with Collins bodies. Studies by Davis et al. [103] have shown that these neuronal inclusion bodies contain a polymerized mutant neuroserpin.

Yu et al. [104] investigated the folding kinetics of ATZ in transverse urea gradient gels. The results showed for the first time that ATZ folds at an extremely slow rate, unlike the wild-type AT, which folds in minutes. This folding defect leads to the accumulation of an intermediate that has a high tendency to polymerize.

One important issue is whether polymerization is the cause of retention of ATZ in the ER of liver cells. The most powerful evidence for this concept comes from studies showing partial correction of the secretory defect by insertion of a second mutation into the ATZ protein that suppresses polymerization [105–107]. However, those studies do not exclude the possibility that there is an abnormality in folding that is distinct from the tendency to polymerize and that is also partially corrected by the second, experimentally introduced

mutation. More recent studies cast some doubt on the concept that polymerization is the cause of ER retention. First, naturally occurring variants of AT with truncated carboxyl terminal tails, including a double mutant with the substitution that characterizes the Z allele together with the substitution that results in carboxyl terminal truncation, are retained in the ER of liver cells even though they do not form polymers [30]. Second, only a minor proportion (~15%) of the intracellular pool of ATZ at steady state in model cell lines is in the form of polymers [30, 108]. Moreover, because the remainder of ATZ in the ER in vivo is in heterogeneous soluble complexes with multiple ER chaperones [108], the principles by which purified ATZ polymerizes in vitro are probably not applicable to what happens in live cells in vivo. Third, the new structural information from Yamasaki et al. [100] implies that an alteration in folding precedes polymerization. Thus, most of what we know suggests that polymerization is not the cause by which ATZ is retained in the ER but rather is the result of its retention. As it accumulates in the ER of liver cells, polymers of ATZ form and become insoluble. Even though it might not cause the retention and accumulation of ATZ in the ER, the formation of polymers in that intracellular compartment appears to have a specific impact on how the liver cell responds and, moreover, to dictate the pathobiology of the liver inflammation, fibrosis, and carcinogenesis (see below).

Pathogenesis of Liver Injury in PIZZ Individuals

There have been several theories for the pathogenesis of liver injury in AT deficiency. According to the immune theory, liver damage results from an abnormal immune response to liver antigens [109]. This theory is based on the observation that peripheral blood lymphocytes from PIZZ infants are cytotoxic for isolated hepatocytes; however, this is probably a nonspecific effect of liver injury, in that peripheral blood lymphocytes from PIMM infants with a similar degree of liver injury due to idiopathic neonatal hepatitis syndrome also are cytotoxic for isolated hepatocytes. More recent studies have indicated an increase in the HLA DR3-DW25 haplotype in AT-deficient persons with liver disease [110]. However, there is no difference between the expression of major histocompatibility complex (MHC) class II antigen in the livers of these persons and expression in healthy controls [111]. Moreover, an increase in the prevalence of a particular HLA DR haplotype in the affected population does not by itself imply altered immune function. Because of the linkage disequilibrium displayed by genes within the MHC, it is possible that increased susceptibility is caused by the products of unrelated, but linked genes. For example, the MHC contains genes for several heat-shock/stress proteins [112], proteins that play an important role in the biogenesis and transport of other proteins through the secretory pathway.

The accumulation theory, in which liver damage is thought to be caused by accumulation of mutant AT molecules in the ER of liver cells, is the most widely accepted. Experimental results with transgenic mice are most consistent with this theory and completely exclude the possibility that liver damage is caused by proteolytic attack as a consequence of diminished serum AT concentrations. Transgenic mice carrying the mutant Z allele of the human AT gene develop periodic acid–Schiff positive, diastase-resistant intrahepatic globules and liver injury early in life [8, 9]. Because there are normal levels of AT and presumably other antielastases in these animals, as directed by endogenous murine genes, the liver injury cannot be attributed to proteolytic attack. Thus, the mechanism of liver damage in this deficiency is classified as a gain-of-toxic function mechanism.

It has been difficult to reconcile the accumulation theory with the observations of Sveger and Eriksson [1, 10], showing that only a subset of PIZZ persons sustains marked liver damage. Because accumulation of mutant ATZ in the ER causes liver disease by a gain-of-toxic function mechanism, we have theorized that putative genetic and/or environmental modifiers would affect the pathway, or pathways, for disposal of mutant ATZ in the ER or affect the cellular protective responses that are activated by ATZ accumulation in the ER [113]. The effect of such modifiers would be silent in other people because the cells of these other people are not exposed to a chronic burden of mutant proteins. One study has substantiated this theoretical paradigm by demonstrating a lag in ER degradation of ATZ after gene transfer into cell lines derived from homozygotes with liver disease when compared to cell lines from homozygotes completely free of liver disease [113].

To further address this paradigm, we and others have sought to determine the pathways responsible for disposal of ATZ and the signaling pathways that are involved in the cellular protective response. In terms of the disposal pathways, early studies in multiple-cell systems and by multiple laboratories showed that the proteasome played a major role [114–118]. The proteasome is a large cytoplasmic multiprotein complex that degrades many cellular proteins, particularly denatured proteins. Degradation by the proteasome depends on ATP; involves trypsin-like, chymotrypsin-like, and peptide glutamyl peptide hydrolyzing activity; and, for the most part, targets proteins that have been conjugated with ubiquitin. Extensive investigation over the last decade has shown that mutant or misfolded proteins in the lumen of the ER may be transported to the proteasome in the cytoplasm by a retrograde translocation mechanism. The most recent work suggests that this involves a kind of transmembrane extraction mechanism by multiprotein complexes composed

of chaperones, transporters, and ubiquitin E3 ligases that impart specificity [119]. The entire process of transport from the ER to the proteasome in the cytoplasm has come to be termed ERAD, for ER-associated degradation.

It was also evident in early studies of the pathways responsible for intracellular disposal of ATZ that the proteasomal system could not completely account for all of the degradative activity. The first evidence that autophagy may play a role in ATZ degradation came from studies of the ultrastructure of human fibroblast cell lines engineered for expression of mutant ATZ [120]. Autophagy is a general mechanism by which intracellular organelles, or parts of organelles, are degraded and is discussed independently in Chap. 25. Autophagy is a highly evolutionarily conserved process that occurs in many cell types, especially during stress states such as nutrient deprivation and during the cellular remodeling that accompanies morphogenesis, differentiation, and senescence. In the well-characterized form of autophagy, macroautophagy, intracellular proteins and organelles are sequestered in double-membrane vesicles (autophagosomes) and these vesicles are delivered to lysosomes for degradation of the constituents, presumably as a source of energy for the starved or "stressed" cell. In the human fibroblast cell lines expressing mutant ATZ, we found intense accumulation of vesicles that resembled autophagosomes. Several criteria were used to determine that the structures were autophagosomes: intravital staining with monodansylcadaverine and immune electron microscopic labeling with DAMP. Furthermore, there was increased accumulation of autophagosomes in liver cells of the PiZ mouse model of AT deficiency and in the liver cells of patients with AT deficiency [120, 121]. In these initial studies, the degradation of ATZ in cell line-model systems was shown to be partially abrogated by chemical inhibitors of autophagy including 3-methyladenine, LY-294002, and wortmannin. When taken together with the observation that ATZ was present in autophagosomes by immune electron microscopy, these results suggested that autophagy participated in the disposal of ATZ. However, because the actions of 3-methyladenine, LY-294002, and wortmannin are not specific for autophagy and their influence on degradation of ATZ was partial, a definitive conclusion about the role of autophagy in disposal of ATZ was not reached at that time.

To definitively determine whether autophagy contributes to degradation of ATZ, it was expressed in an autophagy-deficient mammalian cell line, murine embryonic fibroblasts from the atg5-null mouse [122]. The results demonstrated a significant delay in disposal of ATZ in the atg5-null background compared with the wild-type background. There was progressive accumulation of insoluble ATZ with very large inclusions extending from the ER into the cytoplasm in many of the atg5-null cells. Furthermore, ATZ was immunolocalized to autophagosomes in wild type murine embryonic fibroblasts when autophagosome–lysosome fusion was inhibited by co-expression of dominant-negative Rab 7T22N. Thus, these studies provide definitive genetic evidence that autophagy contributes to degradation of mutant ATZ that accumulates in the ER. Moreover, the results suggest that autophagy plays a homeostatic role in AT deficiency by preventing toxic cytoplasmic accumulation of ATZ through piecemeal digestion of the insoluble aggregates.

To determine whether accumulation of ATZ in the ER activates autophagy, we mated our Z mouse with hepatocyte-specific inducible expression of ATZ to the GFP-LC3 mouse that renders autophagosomes fluorescent [122]. Although green fluorescent autophagosomes are seen only in the liver of the GFP-LC3 mouse after starvation, they were observed in the liver of the Z xGFP-LC3 simply by inducing the expression of the ATZ gene – accumulation of ATZ in the ER was sufficient to activate autophagy; starvation was not required. This effect was found to be specific for the aggregation-prone properties of ATZ because it was not seen when the GFP-LC3 mouse was mated to our Saar mouse, the latter being a mouse with hepatocyte-specific inducible expression of the AT Saar variant that accumulates in the ER but does not polymerize/aggregate. Together, these studies provide powerful and definitive evidence that autophagy is specialized for the pathobiology of AT deficiency in which an aggregation-prone protein accumulates in the ER, activates the autophagic response, and autophagy plays a critical role in mediating the disposal of the ATZ aggregates.

A study by Kruse et al. [123] using a completely different approach and system also demonstrated the critical importance of autophagy in the cellular response to the mutant protein in AT deficiency. In this case, a yeast model system was used. A library of yeast mutants was screened for defects in degradation of human ATZ. One of the mutants corresponded to the yeast homology of autophagy gene atg6. In the absence of atg6 or another autophagy gene atg14, there was a marked delay in degradation of ATZ. The delay in degradation in atg6-deficient or atg14-deficient yeast was particularly apparent at high levels of expression of ATZ as though the proteasomal pathway was sufficient at low levels of expression, but autophagy was required at higher levels of expression.

Kruse et al. [124] also recently discovered that a mutant subunit of fibrinogen that aggregates in the ER of liver cells in an inherited form of fibrinogen deficiency depends on autophagy for disposal. Interestingly, this type of fibrinogen deficiency is associated with a chronic liver disease, providing evidence for the notion that the accumulation of aggregation-prone protein in the ER is hepatotoxic and for the notion that autophagy is particularly specialized as a cellular response to retention of aggregated protein in the ER.

We now envision that when ATZ accumulates in the ER, it can be degraded by two major mechanisms, the proteasomal

and autophagic pathways. The autophagic pathway is specialized for the polymerized/aggregated forms of ATZ that are accumulating constitutively, but become particularly abundant at high levels of expression that might occur during an acute phase response. The proteasome is probably specialized for the soluble forms of ATZ that accumulate in the ER, presumably bound to multiple chaperones [106]. Indeed, one could imagine a series of events that occur in the ER: an initial response in which soluble ATZ is bound by the ER chaperones designed to prevent its aggregation; chaperone-bound soluble ATZ is directed to the ERAD pathway for degradation by the proteasome; when biogenesis and/or accumulation of ATZ exceeds the capacity of chaperones/ERAD pathway it begins to aggregate, activating autophagy for disposal.

It is important to note that several recent studies on other aggregation-prone proteins have shown that proteasomal function can be impaired by these mutant proteins when they accumulate in the cytoplasm as aggregates [125, 126]. Thus, we could imagine that if autophagy cannot keep up with the formation of aggregated ATZ, such as might occur at high levels of expression, the aggregated ATZ impairs the proteasome and theoretically lead to a vicious cycle that favors massive intracellular accumulation of ATZ.

The studies of Kruse et al. [123] have suggested that there is a third pathway for disposal of ATZ in yeast that involves transit to the trans-Golgi and then targeting to the vacuole. A comparable pathway in mammalian cells has not yet been described. Cabral et al. have also described a pathway for degradation of ATZ in a human hepatoma cell line that is not clearly linked to the proteasome or autophagy [127]. This pathway is characterized by sensitivity to tyrosine phosphatase inhibitors.

Together, the results of the foregoing studies indicate degradation of ATZ as a complex process that involves two or more pathways, each with at least several sequential steps (Fig. 46.2). Theoretically, each of these pathways or its individual steps may be affected in an AT–deficient patient who is "susceptible" to liver disease; that is, there may be heterogeneity among susceptible hosts in the mechanism by which ER degradation is delayed (Fig. 46.3).

According to our paradigm, susceptibility to liver disease could also be determined by genetic and/or environmental modifiers that alter putative cellular protective cellular response pathways. To address this possibility, we characterized the pathways that are specifically activated when ATZ accumulates in the ER. We already knew from the Z x GFP-LC3 mouse described above that autophagy is one of the pathways that are activated when ATZ accumulates in the ER. Using cell lines-models and the Z mouse model with hepatocyte-specific inducible expression of ATZ, we have also found that the NFκB signaling pathway is activated, but the unfolded protein response (UPR) is not [128]. Genomic

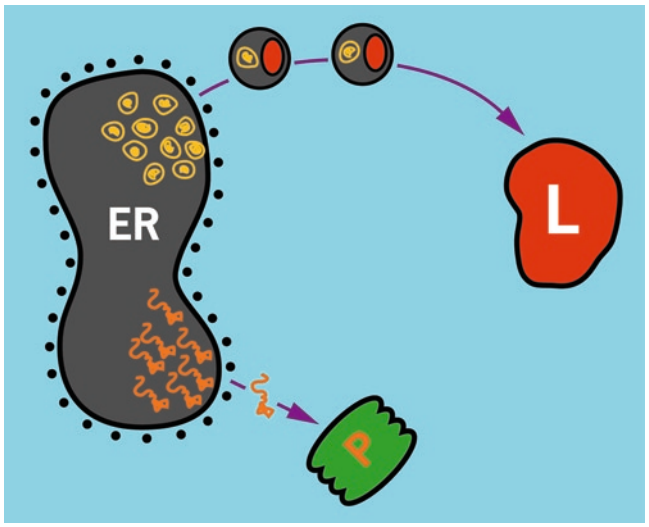

Fig. 46.2 Schematic model for degradation of mutant ATZ that accumulates in the ER. In the *lower* part of the ER, soluble monomeric and oligomeric forms of ATZ (*orange*) are extracted through the ER membrane and degraded by the proteasome (P). In the upper part of the ER, polymerized ATZ (*yellow*) induce areas of ER membrane to transform into autophagosomes that are destined for delivery to lysosomes (L). Reprinted from Perlmutter [181]

analysis of the liver from the Z mouse provided further evidence for the importance of NFκB signaling and the autophagic response by demonstrating changes in expression of selected NFκB targets and marked upregulation of RGS16, a protein which is capable of activating autophagy by antagonizing G protein Gαi3 [129]. Activation of NFκB and autophagy, but not the UPR is a repertoire that appears to be specific for ATZ and probably reflects the formation of insoluble polymers. When the AT Saar variant, which cannot polymerize, accumulates in the ER the repertoire is different with activation of the UPR, NFκB to a lesser extent, but not autophagy [128].

Two recent studies have implicated single nucleotide polymorphisms (SNP) as potential genetic modifiers of liver disease in AT deficiency [130, 131]. In one study, an SNP in the upstream flanking region of the AT gene has been implicated [130]. However, it is not clear that this SNP is significantly associated with liver disease if all of the potential controls are included in the analysis. Furthermore, there is no evidence for a functional difference in the allele bearing the SNP. In the second study, an SNP in the downstream flanking region of ER mannosidase I was implicated in early-onset liver disease [131]. Because ER mannosidase I plays a role in the ERAD pathway, a polymorphism that affected its function would be a prime candidate for a genetic modifier of liver disease in AT deficiency according to the paradigm. However, further epidemiological studies will be necessary to determine if this SNP is truly associated with liver disease and further cellular studies are needed

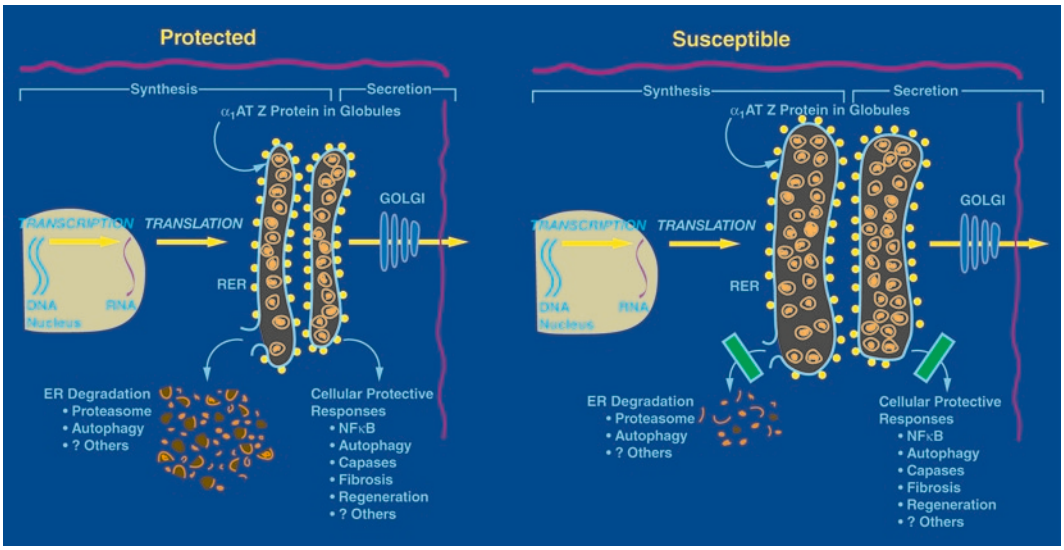

Fig. 46.3 Hypothetical model for how genetic and environmental modifiers might increase susceptibility to liver disease in AT deficiency. For liver cells of the "protected" host on the *left*, ATZ accumulates in the ER, but it can be degraded and it activates cellular responses that play a role in protecting the host from liver inflammation and carcinogenesis. For the liver cells of the "susceptible" host on the *right*, a polymorphism or environmentally-induced change in protein expression or function results in a subtle block in degradation or cellular protective response

to identify how it alters the pathobiology of ATZ accumulation in the ER.

Indomethacin could represent an environmental modifier of liver disease in AT deficiency. In one study, indomethacin administration to the PiZ mouse model resulted in increased hepatic ATZ accumulation and hepatocyte injury [132]. The effect of indomethacin appeared to involve increased expression of AT at the transcriptional level via IL-6 signaling.

Mechanism of Liver-Cell Injury in AT deficiency

There is still relatively limited information about the mechanism by which ER retention of ATZ leads to liver-cell injury. In transgenic mice that express the human ATZ gene, there are focal areas of liver-cell necrosis, microabscesses with an accumulation of neutrophils, regenerative activity in the form of multicellular liver plates, and focal nodule formation during the neonatal period [133]. Nodular clusters of altered hepatocytes that lack AT immunoreactivity also are found during the neonatal period. With aging, there is a decrease in the number of hepatocytes containing ATZ globules; there is also an increase in the number of nodular aggregates of AT–negative hepatocytes and development of perisinusoidal fibrosis [133]. Within 6 weeks, there are dysplastic changes in these aggregates. Adenoma occurs within 1 year, and invasive hepatocellular carcinoma occurs between 1–2 years of age [134]. The histopathology of ATZ transgenic mice is remarkably similar to that of hepatitis B virus surface antigen in transgenic mice and is particularly interesting because hepatitis B virus is retained in the ER or in the ER-Golgi intermediate compartment of hepatocytes, often called ground-glass hepatocytes [135]. Although the liver-cell injury in transgenic mouse models of AT deficiency has been characterized as milder than what is seen in the human disease, our recent studies have shown much more significant fibrosis in these mice than previously appreciated [136]. Two other factors need to be taken into consideration when making this comparison. First, liver biopsies are predominantly done in the AT-deficient population that is more significantly affected by the condition and not in the vast majority of the population that has limited clinical effects of the disease. Second, there are likely to be strain-sepcific factors that condition the hepatic pathological response in transgenic mice just as there are host-specific modifiers in humans.

Mitochondrial dysfunction has been implicated in the liver-cell injury of AT deficiency and is supported by relatively strong evidence [137]. Mitochondrial damage is found in both cell line and transgenic mouse models of AT deficiency including ultrastuctural alterations, functional aterations in mitochondrial polarization, and activation of caspase-3. The implications of these data are that impairment in mitochondrial energy generation and release of oxidants is the final common pathway mediating liver-cell injury. It is not clear how accumulation of ATZ in the ER causes mitochondrial dysfunction, but two possible mechanisms have been proposed. First, mitochondrial dysfunction could be the direct effect of the "stressed" ER. Indeed we know that accumulation of ATZ in the ER activates BAP31

[128], an integral membrane protein of the ER that mediates pro-apoptotic activity signals to mitochondria. There is now extensive evidence for intramembranous connections between ER and mitochondria that play essential roles in calcium signaling and phospholipid metabolism [138]. In this case, mitophagy would then be activated secondarily, to remove damaged mitochondria. A second possible explanation is that mitochondrial dysfunction is an indirect effect of an exuberant autophagic response activated by accumulation of ATZ in the ER; that is, mitochondria are innocent bystanders of the effect of ER ATZ accumulation on the autophagic response. To begin to determine which of these explanations is more likely, we examined the effect of cyclosporine A (CsA) on cell line and transgenic mouse models of AT deficiency [137]. CsA inhibits the mitochondrial permeability transition but its related compound, tacrolimus, does not. The results showed that CsA, but not tacrolimus inhibited mitochondrial depolarization, decreased hepatic caspase 3 activation, and completely prevented mortality in the PiZ mouse in response to the stress of starvation. There was no change in the number of autophagosomes in liver cells. Although definitive testing of this should be done in a system genetically deficient in autophagy, these results are most consistent with the first explanation, that mitochondrial injury is a direct result of changes in the ER. These results also suggest that CsA or other drugs that inhibit mitochondrial dysfunction oxidant release could be effective therapeutic interventions for liver disease due to AT deficiency.

Mechanism of Hepatic Carcinogenesis in AT Deficiency

There is also relatively little known about the pathogenesis of hepatocellular carcinoma in AT deficiency. One of the possible clues to carcinogenesis comes from the longstanding observation that only some of the hepatocytes have intracellular globules. The presence of globule-containing and globule-devoid hepatocytes has been noted in the liver of patients and in the PiZ transgenic mouse model of AT deficiency. Studies in the PiZ mouse model have shown that there is increased hepatocellular proliferation in the resting liver and that almost all of the proliferating liver cells are globule-devoid [139]. Consistent with this, almost all of the adenomas and carcinomas that occur in the human disease and in the mouse model arise in the globule-devoid hepatocytic population [140]. These results have led us to hypothesize that the pathogenesis of hepatic carcinoma involves the interplay of globule-containing cells that are "sick but not yet dead" and chronically stimulating the globule-devoid cells "in trans" in the presence of inflammation [140]. We know that the globule-devoid cells have accumulated aggregated mutant ATZ 41 and activated a number of cellular "alarm" pathways, including ER- and mitochondrial caspases, NF-κB and autophagy [128]. Furthermore, we know that the globule-containing cells are relatively impaired in cell proliferation [139]. Perhaps even more importantly, the regenerative activity in the liver of PiZ mice is directly proportional to the number of globule-containing cells [139]. Thus, we believe that these "sick but not yet dead" globule-containing cells are responsible for regenerative signals but the globule-devoid cells are the ones capable of responding to these signals – that is, the globule-devoid cells have the selective proliferative advantage. The mechanism by which the globule-devoid cells acquire the selective proliferative advantage is not known, but one possibility is that they are younger and have had less time to accumulate ATZ and the damage that accrues from it.

A recent study has provided important new information about cell death in the PiZ mouse liver [141]. Markers of apoptosis were found to be more pronounced in hepatocytes with greater levels of insoluble ATZ. Furthermore, stimulation of the extrinsic apoptotic pathway with antibody to fas resulted in increased apoptosis almost exclusively within the globule-containing cells. These results are consistent with the notion that the globule-containing cells are more susceptible to cell death than are the globule-devoid hepatocytes – that is the globule-containing cells are "sicker" than the globule-devoid hepatocytes. However, these results do not address the possibility that the globule-containing hepatocytes in the AT-deficient liver are relatively resistant to apoptosis when compared with normal hepatocytes. Indeed, the data in this recent study shows an increase in one antiapoptotic protein, cFLIP, in cells with higher levels of insoluble ATZ [141]. This would mean that the globule-devoid hepatocytes were even more resistant than normal hepatocytes to apoptosis. This is probably an important distinction because relative resistance to apoptosis in either of these cellular compartments could potentially contribute to the mechanism of carcinogenesis.

It is not yet known whether autophagy plays a role in the carcinogenic susceptibility. We know that autophagic activity confers resistance to tumorigenesis [142, 143]. On the basis of our previous work in model cell lines, one would predict that there is increased autophagic activity in the globule-containing cells. This prediction should be possible to address by detailed studies in the PiZ xGFP-LC3 mouse and by mating the PiZ mouse to autophagy-deficient mouse models. It is not clear what happens when the autophagic response is chronically, or even constitutively, activated and whether under those circumstances it might even contribute to the inflammation and carcinogenesis.

Liver Disease: Clinical Manifestations

Liver involvement often is first noticed at 1–2 months of age because of persistent jaundice. Conjugated bilirubin and serum transaminase levels are mildly to moderately elevated in the blood. The liver may be enlarged. Such infants usually are admitted to the hospital with a diagnosis of neonatal hepatitis syndrome and undergo a detailed diagnostic evaluation [144]. Infants may also be initially evaluated for AT deficiency because of an episode of gastrointestinal bleeding, bleeding from the umbilical stump, or bruising [145]. A small number of affected infants have hepatosplenomegaly, ascites, and liver synthetic dysfunction in early infancy. An even smaller number have severe fulminant hepatic failure in infancy [146]. A few cases are recognized initially because of a cholestatic clinical syndrome characterized by pruritus and hypercholesterolemia. The clinical features among these infants resemble those of extrahepatic biliary atresia, but histologic examination shows a paucity of intrahepatic bile ducts.

Liver disease associated with AT deficiency may be discovered in late childhood or early adolescence, when the patient is seen with abdominal distention due to hepatosplenomegaly or ascites or has upper intestinal bleeding caused by esophageal variceal hemorrhage. In some of these cases, there is a history of unexplained prolonged obstructive jaundice during the neonatal period. In others, there is no evidence of any previous liver injury, even when the neonatal history is carefully reviewed.

AT deficiency should be considered in the differential diagnosis for any adult who has chronic hepatitis, cirrhosis, portal hypertension, or hepatocellular carcinoma of unknown origin. An autopsy study in Sweden showed a higher risk of cirrhosis among adults with AT deficiency than was previously suspected and indicated that AT deficiency has a strong association with primary liver cancer [147]. This study raised the possibility that the risk of clinical liver disease is as high as 25% among men in the fifth and sixth decades of life.

The only prospective data on the course of AT deficiency–associated liver injury are from the Swedish nationwide screening study conducted by Sveger [1]. In that study, 200,000 newborn infants were screened, and 127 PIZZ individuals were identified. Fourteen of the 127 had prolonged obstructive jaundice, and 9 of the 14 had severe liver disease, as indicated by clinical and laboratory criteria. Another 8 of the 127 PIZZ infants had mildly abnormal serum bilirubin or serum transaminase levels or hepatomegaly. Approximately 50% of the rest of the 127 had abnormal transaminase levels only [10]. Published results of follow-up studies of the original cohort of 127 PIZZ children at 26 years of age [10] have shown that 90% had persistently normal serum transaminase levels with no evidence of liver dysfunction. Issues not addressed by the Sveger study are whether these 26-year-olds have persistent subclinical histologic abnormalities, despite a lack of clinical or biochemical evidence of liver injury, and whether liver disease will eventually become clinically evident during adulthood.

It is still not clear what clinical manifestations or abnormal laboratory test results can be used to predict a prognosis for patients with AT deficiency–associated liver disease. Results of one study suggested that persistence of hyperbilirubinemia, hard hepatomegaly, early development of splenomegaly, and progressive prolongation of prothrombin time were indicators of poor prognosis [148]. In another study, elevated transaminase levels, prolonged prothrombin time, and a lower trypsin inhibitor capacity correlated with a worse prognosis [149]. However, my colleagues and I have found that some children with AT deficiency–associated liver disease can lead relatively normal lives for years after the development of hepatosplenomegaly and mild prolongation of prothrombin time. In a review of 44 patients with AT deficiency seen in the specialty practice at St. Louis Children's Hospital, 17 patients had cirrhosis, portal hypertension, or both [150]. Nine of the 17 patients with cirrhosis or portal hypertension had a prolonged, relatively uneventful course for at least 4 years after the diagnosis of cirrhosis or portal hypertension. Two of these patients eventually underwent liver transplantation, but seven were leading relatively healthy lives for as long as 23 years while carrying a diagnosis of severe AT deficiency–associated liver disease. Patients with the prolonged stable course could be differentiated from those with a rapidly progressive course on the basis of overall life functioning, but not on the basis of other more conventional clinical or biochemical criteria. Thus, prediction of poor prognosis for AT deficiency–associated liver disease and for timing of liver transplantation depends more on the overall functioning of the child than on the histologic findings or laboratory data.

There is currently no evidence that the heterozygous MZ phenotype causes liver disease by itself. Early studies of liver biopsy collections suggested that there was a relation between heterozygosity and the development of liver disease [151]. A retrospective study of liver transplant recipients at the Mayo Clinic showed a higher prevalence of heterozygosity for ATZ than in the general population, including a group of patients without another explanation for liver disease [152]. However, both of these studies were biased in ascertainment and did not include concurrent prospective controls. Results of a cross-sectional study of patients with AT deficiency in a referral-based Austrian university hospital, who were reexamined with the most sophisticated and sensitive assays available, suggested that liver disease in heterozygotes can be accounted for, to a great extent, by infections with hepatitis B or C virus or by autoimmune disease [153]. A more recent cross sectional case-control study did not find a significant association with chronic liver disease or cryptogenic

cirrhosis, but did find a higher prevalence of the heterozygote state in decompensated liver disease [154]. Although the foregoing findings taken together give a strong impression that heterozygotes are susceptible to liver disease and particularly if they have a set of putative predisposing genetic and/or environmental modifiers, the clinical research literature has not been sufficiently powerful to provide definitive evidence for this.

Liver disease has been described for several other allelic variants of AT. Children with compound heterozygosity type PISZ are affected by liver injury in a manner similar to that of PIZZ children [1, 155, 156]. There have been several reports of liver disease in AT deficiency type PIM_{Malton} [38, 39]. This is a particularly interesting association, because the abnormal PIM_{Malton} AT molecule has been shown to undergo polymerization and retention within the ER [39]. Liver disease has been detected in single patients with several other AT allelic variants, such as PIM_{Duarte} [40], PI_{W} [157], and PI_{FZ} [158], but it is not clear whether other causes of liver injury for which there are more sophisticated diagnostic assays, such as infection with hepatitis C and autoimmune hepatitis, have been completely excluded in these cases.

There is still very limited information about the incidence of liver disease among persons with COPD due to AT deficiency. In one study of 22 PIZZ patients with COPD, there was an elevated transaminase levels in ten patients, and cholestasis was present in one patient [159]. Liver biopsy was not performed in this study and may be necessary for accurate determination of the extent of liver injury in these patients.

Diagnosis

Diagnosis of AT deficiency is established by means of serum AT phenotype determination in isoelectric focusing or by means of agarose electrophoresis at acid pH. Serum concentrations can be used for screening with follow-up PI typing of any values below normal (85–215 mg/dL). A retrospective study of all pediatric patients who had both serum concentrations and PI typing done at one center indicated that the serum concentration determination had a positive predictive value of 94% and a negative predictive value of 100% for homozygous AT deficiency [160].

The distinctive histologic feature of homozygous PIZZ AT deficiency, periodic acid–Schiff positive, diastase-resistant globules in the ER of hepatocytes, substantiates the diagnosis. The presence of these inclusions should not be interpreted as confirming the diagnosis of AT deficiency. Similar structures are occasionally found in PIMM individuals with other liver diseases [161]. The inclusions are eosinophilic, round to oval, and 1–40 μm in diameter. They are most prominent in periportal hepatocytes, but also may be present in Kupffer cells and cells of biliary ductular lineage [162]. There may be evidence of variable degrees of hepatocellular necrosis, inflammatory cell infiltration, periportal fibrosis, or cirrhosis. There may be evidence of bile-duct epithelial cell destruction, and occasionally there is a paucity of intrahepatic bile ducts.

Treatment

The most important principle in the treatment of patients with AT deficiency is avoidance of cigarette smoking. Cigarette smoking markedly accelerates the destructive lung disease associated with AT deficiency, reduces the quality of life, and significantly shortens the longevity of these patients [6].

There is no specific therapy for AT deficiency–associated liver disease. Therefore, clinical care largely involves supportive management of symptoms caused by liver dysfunction and prevention of complications. Progressive liver dysfunction and failure in children have been managed with orthotopic liver transplantation with survival rates approaching 90% for children and 83% for adults at 5 years [163]. Nevertheless, a number of PIZZ individuals with severe liver disease, even cirrhosis or portal hypertension, may have a relatively low rate of disease progression and lead a relatively normal life for extended periods. With the availability of living-related-donor transplantation techniques, it may be possible to treat these patients expectantly for some time. Children with α_1-AT deficiency, mild liver dysfunction (elevated transaminase levels or hepatomegaly), and without functional impairment may never need liver transplantation.

A class of compounds called *chemical chaperones* can reverse the cellular mislocalization or misfolding of mutant plasma membrane, lysosomal, nuclear, and cytoplasmic proteins, including CFTRΔ F508, prion proteins, and mutant aquaporin molecules associated with nephrogenic diabetes insipidus, and mutant galactosidase A associated with Fabry disease [164–166]. We found that several members of this class of drugs had a positive effect on secretion of ATZ in cell line models and in the PiZ mouse model of AT deficiency [167]. One of these compounds, 4-phenylbutyric acid [PBA], when administered orally to PiZ mice consistently mediated an increase in blood levels of human AT, reaching 20–50% of the levels present in PIM mice and healthy humans. Because PBA had been used safely for years as an ammonia scavenger to treat children with urea cycle disorders, it was considered an excellent candidate for chemoprophylaxis of target-organ injury in AT deficiency. However, in a pilot study in 10 PIZZ patients with liver disease, PBA did not result in an increase in serum AT levels [168].

The explanation for the discrepancy going from the mouse model to humans is not apparent, but one prominent possibility is that the patients are unable to tolerate the doses required for its salutory effect in mice.

There is some evidence in the literature for potential efficacy of several other strategies. As mentioned above, CsA or other drugs that prevent mitochondrial dysfunction could theoretically prevent liver-cell injury in AT deficiency. This could extend to anti-oxidants as well. Although weaker anti-oxidants such as vitamin E have been mentioned in the literature as potential treatment for AT deficiency, there are no controlled trials that demonstrate its efficacy. Similarly, there are no controlled trials that demonstrate efficacy of ursodeoxycholic acid, which has been tried with the presumption that increase bile flow might ameliorate liver injury in AT deficiency. Iminosugar compounds have been considered with the concept that interfering with oligosaccharide side chain trimming may alter the fate of the mutant protein and we found that one of these drugs, castanospermine mediated increased secretion of ATZ in cell-line models [169]. Another strategy that has been discussed in the literature is small molecules that prevent polymerization by inserting into specific conformationally important sites within the structure of ATZ. Indeed, a drug has been identified using this strategy that mediates an increase in degradation of ATZ in a cell-line model [170]. We have recently shown that a drug which enhances autophagy degradation of mutant ATZ reduces the hepatic ATZ load and hepatic fibrosis in a mouse model of AT deficiency [136]. The drug, carbanazepine has been used safely as an anticonvulseat and mood stabilizer, and so it could be tested almost immediately for therapy of severe lever disease due to AT deficiency.

Patients with AT deficiency and COPD have undergone replacement therapy with AT purified from recombinant plasma and administered intravenously or by means of intratracheal aerosol [6]. This therapy is associated with improvement in serum concentrations of AT and in AT and neutrophil elastase inhibitory capacity in bronchoalveolar lavage fluid without significant side effects. Although results of initial studies have suggested that there is a slower decline in forced expiratory volume in patients undergoing replacement therapy, this occurred in only a subgroup of patients, and the study was not randomized [171]. This therapy is designed for persons with established and progressive emphysema. Protein replacement therapy is not being considered for patients with liver disease because there is no information to support the notion that deficient serum levels of AT are mechanistically related to liver injury.

A number of patients with severe emphysema from AT deficiency have undergone lung transplantation in recent times. The actuarial survival rate has been approximately 50% for 5 years with reports of significant improvement in lung function and exercise tolerance [172].

Replacement of AT by means of somatic gene therapy has been discussed in the literature [39]. This strategy is potentially less expensive than replacement therapy with purified protein and would alleviate the need for intravenous or inhalation therapy. Again, this form of therapy would be useful only in ameliorating emphysema, because liver disease associated with AT deficiency is not caused by deficient levels of AT in the serum or tissue. Of course, it would be helpful to know that replacement therapy with purified AT, as it is currently applied, is effective in ameliorating emphysema in this deficiency before embarking on clinical trials involving gene therapy. Several novel types of gene therapy, such as repair of mRNA by means of transsplicing ribozymes [173], chimeric RNA/DNA oligonucleotides [174], triplex-forming oligonucleotides [175], small fragment homologous replacement [176], or RNA silencing [177] are theoretically attractive alternative strategies for the management of liver disease in AT deficiency because they would prevent the synthesis of mutant ATZ protein and its accumulation in the ER.

Studies have shown that transplanted hepatocytes can repopulate the diseased liver in several mouse models, including a mouse model of a childhood metabolic liver disease called *hereditary tyrosinemia* [178, 179]. Replication of the transplanted cells can almost completely replace the liver as long as there is a selective proliferative advantage engendered by the presence of injury and the accompanying regenerative response. These results provide evidence that it may be possible to use hepatocyte transplantation techniques to manage hereditary tyrosinemia and, perhaps, other metabolic liver diseases in which the defect is cell autonomous. For example, AT deficiency involves a cell-autonomous defect and therefore would be an appropriate candidate for this strategy.

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Chapter 47
Hepatic Artery Diseases

Ton Lisman and Robert J. Porte

Introduction

Blood supply to the liver is accomplished via a dual vascular system consisting of the hepatic artery and portal vein. With respect to blood flow, the liver is a unique organ in that the majority of the blood entering the liver is venous blood, which enters the liver from the portal vein. The portal vein is responsible for 65–75% of the blood flow to the liver, whereas the remainder of the blood enters the liver through the hepatic artery. The liver's oxygen demand is met by the arterial blood for approximately 50%, while the other half is derived from the portal blood [1]. The arterial inflow of blood is tightly related to the venous inflow, so that the total blood flow through the liver remains constant. This regulatory mechanism is mediated by the hepatic artery, as the portal vein is unable to regulate blood flow. Arterial flow is regulated by site-specific release of adenosine, a powerful vasodilator. When portal flow is high, adenosine is rapidly washed away, resulting in arterial vasoconstriction. Vice versa, when portal flow is low, adenosine-mediated arterial vasodilation enables the arterial flow to increase [2].

As the hepatic artery supplies only half of the liver's oxygen and only 25–35% of blood, the clinical picture of a diseased hepatic artery can be relatively mild, although there are potentially fatal hepatic artery diseases. Also, permanent ligation of the hepatic artery, which in the past was performed frequently in patients with liver trauma [3], can be without serious clinical consequences. Even though the bile ducts are entirely dependent on the hepatic artery for their blood supply, the hepatic artery can be safely ligated proximal to the takeoff of the gastroduodenal artery, as sufficient collateral flow then remains. Moreover, hepatic artery ligation distal to the gastroduodenal take off is well tolerated by hemodynamically stable patients with normal portal flow [4,5]. Although in the latter case, transient increases in ALT/AST and bilirubin may occur, these patients rapidly develop a collateral circulation. The situation is different in liver transplant recipients, in which a blockade in arterial flow early after transplantation, usually by a thrombus, is associated with a high risk of bile duct necrosis and subsequently increased morbidity and mortality [6]. The transplanted liver differs from native livers in that arterial blood supply from accessory arteries and collateralization is completely interrupted during hepatectomy.

Isolated hepatic artery occlusions outside the setting of liver transplantation are rare, usually asymptomatic, and thus diagnosed primarily at autopsy. Hepatic artery occlusions may be caused by atheroma, thrombosis or emboli, tumor invasion, polycythemia vera or trauma. Thrombosis-induced hepatic artery occlusion has a better prognosis when the thrombus develops slowly as compared to a sudden block, while simultaneous occlusion of the hepatic artery and portal vein is almost always fatal.

This chapter will review the two most common hepatic artery diseases, aneurysms and liver transplantation-associated hepatic artery thrombosis.

Hepatic Artery Aneurysms

Prevalence and Etiology

Aneurysms of the hepatic artery are rare, but they still comprise approximately 20% of all visceral aneurysms [7]. The exact prevalence of hepatic artery aneurysms is not known, but one study reported an incidence of 0.03% of all trauma and surgical admissions during 7 years in a single large referral center [8], while another study reported an incidence of 0.002% in a population of two million patients seen at the Mayo Clinic [9]. The majority of hepatic artery aneurysms are extrahepatic, while a minority is located intrahepatically [10]. Men are more frequently affected than women, and they are usually in their middle ages [9]. Congenital anomalies associated with hepatic artery aneurisms have been described, but are extremely rare [11]. More frequently, acquired aneurysms

T. Lisman (✉) and R.J. Porte
Department of Surgery, University Medical Center Groningen, Groningen, The Netherlands
e-mail: j.a.lisman@chir.umcg.nl

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5 ,
DOI 10.1007/978-1-4419-7107-4_47, © Springer Science+Business Media, LLC 2011

occur, which may be the result of vessel wall damage, injuries, or inflammatory processes including cirrhosis. In patients with abdominal sepsis, septic emboli can become deposited at a hepatic artery bifurcation and result in the formation in an aneurysm [12], but nowadays mycotic hepatic artery aneurysms are rarely seen, because of earlier antibiotic treatment of infections [9]. Older literature describes atherosclerosis as a predisposing factor for aneurysm formation. Although post-mortem examinations indeed show atherosclerotic sites [10], it is now recognized that this is secondary to the aneurysm and not a causative factor. The hepatic artery is in general extraordinarily protected against atherosclerosis, even in the presence of wide spread atherosclerotic disease in other arterial beds [13].

Half of hepatic artery aneurysms are in fact pseudo- or false aneurysms, commonly associated with hepatobiliary (surgical) procedures or trauma [8]. True aneurysms are permanent, localized dilatations of the vessel wall, in which the vessel wall architecture is still intact. False aneurysms on the other hand consist of a disrupted arterial wall with a contained perivascular hematoma. Table 47.1 shows possible causes of hepatic aneurysms.

Dissection of a hepatic artery aneurysm rarely occurs, but the aneurysms, in particular false aneurysms, do rupture, which requires immediate intervention. The prevalence of rupture varies between studies, with the most conservative estimate at 20%, while estimates of up to 70% have also been reported [14,15].

Table 47.1 Possible causes for hepatic artery aneurysm

Congenital anomalies
Vessel-wall damage
Polyarteritis nodosa
Connective tissue disorders (Marfan syndrome, Ehler-Danlos syndrome, Osler-Weber-Rendu disease)
Fibromuscular-hypoplasia
Injuries
Abdominal trauma (motor vehicle accidents)
Bile-duct surgery
Intra-arterial chemotherapy
Liver biopsy
Transhepatic interventions
Hepatectomy
Liver transplantation
Inflammatory processes
Bacterial endocarditis
Syphilis
Tuberculosis
Cholecystitis
Appendicitis
Pancreatitis
Cirrhosis
Other
Multiple pregnancies

Clinical Features

Patients with hepatic artery aneurysms remain asymptomatic for long periods of time. The first presenting symptom is abdominal pain in 75% of cases [16], which can be evident months before rupture occurs, although it is frequently misinterpreted as biliary or pancreatic pain. Smaller aneurysms may not produce any symptoms and are only discovered incidentally during imaging procedures or surgery [9]. Hepatic artery aneurysms are usually not palpable, but in rare occasions in which the aneurysm is extraordinarily large, a pulsatile mass or thrill in the right upper quadrant may be detected.

The majority of patients with an aneurysm of the hepatic artery are discovered coincidentally when imaging studies of the upper abdomen are performed for unrelated symptoms or reasons [9]. Few patients present for the first time when the aneurysm has ruptured, although one study reported that five of eight hepatic artery aneurysms seen in a single hospital presented with hemorrhagic shock [15]. Rupture predominantly occurs with non-atherosclerotic aneurysms [9]. Hepatic aneurysms may rupture into the biliary tree, into the portal vein, or in the peritoneal cavity, each with distinct symptoms. Rupture into the biliary tree presents as hemobilia, epigastric pain, and icterus, rupture into the portal vein is associated with portal hypertension and variceal bleeding, while rupture into the peritoneal cavity results in aggravation of abdominal pain and shock. Approximately half of the hepatic aneurysms rupture into the peritoneal cavity, which frequently is fatal due to rapid exsanguination. Rupture into the biliary tree may result in bile duct obstruction by blood clots, which result in jaundice within days. Classically, the triad of abdominal pain, jaundice, and gastrointestinal bleeding is considered suggestive of a hepatic artery aneurysm, but this combination of symptoms actually occurs in only 30% of patients [17].

Diagnosis

Due to the low prevalence of hepatic artery aneurysms, the diagnosis is frequently missed, as it is simply not considered. Laboratory values are normal, except when there is biliary stasis. Initially, ultrasonography will result in a suspicion of an aneurysm, which may be confirmed by magnetic resonance angiography or contrast-enhanced computed tomography (CT) scan [18]. Asymptomatic aneurysms may also be detected during radiologic investigations including CT, ultrasound, and angiography that are performed for other reasons than a suspicion of an aneurysm [10].

Treatment and Outcome

Literature on the treatment strategy for non-ruptured hepatic artery aneurysms is conflicting. While some argue for conservative treatment when the patient is asymptomatic, other authors prefer interventions in aneurysms larger than 2 cm, especially in atherosclerosed arteries [7,9]. False aneurysms do require treatment, as the proportion of ruptures is much larger compared to true aneurysms. Extrahepatic aneurysms can be treated by ligation or resection [8,19]. The location of the aneurysm will determine whether vascular reconstruction will be required. Aneurysms of the common hepatic artery can usually be safely ligated without reconstruction due to residual perfusion via the gastroduodenal and right gastric branches, provided baseline liver function is acceptable [10]. In patients with cirrhosis, reconstruction is recommended [20]. Arterial reconstruction is recommended for all patients with an aneurysm in the proper hepatic artery [10]. Common hepatic artery aneurysms may also be treated by endovascular embolization of the feeding artery using coils, injectable thrombin, or gelatin sponge [19,21–23]. The choice of open vs. endovascular repair depends on many factors including the size and the location of the aneurysm, the general health of the patient, and the accessibility of the aneurysm to endovascular device placement. Endovascular embolization is also useful for intrahepatic aneurysms [8]. A small proportion of intrahepatic aneurysms have to be treated with liver resection when ligation or embolization fails. The development of new and smaller diameter covered endovascular stents has opened a new therapeutic strategy to exclude a hepatic artery aneurysm and to prevent or treat rupture and bleeding [24]. However, data on long-term patency and safety of this technique are still sparse.

Outcome of non-ruptured aneurysms is good, but complications do occur. After surgical repair, necrosis of the liver due to inadequate perfusion may occur. Endovascular repair may be complicated by endoleaks, aneurysm growth, and failure of complete occlusion after embolization.

Ruptured aneurysms require immediate attention by volume resuscitation followed by either emergency surgery or transcatheter embolization. Mortality following a ruptured hepatic artery aneurysm is high, up to 20–30% [9,25,26].

Hepatic Artery Thrombosis

Prevalence and Etiology

Hepatic artery thrombosis almost exclusively occurs in patients following liver transplantation. Isolated hepatic artery thromboses probably do not occur as the hepatic artery is profoundly protected against atherosclerosis, and thus no trigger for thrombosis develops during life [13]. Hepatic artery thrombosis occurs in approximately 5% of adult liver transplant recipients [27], while the prevalence in children is substantially higher [27,28]. As the transplanted liver (initially) lacks any collateral circulation, the absence of arterial flow results will invariably lead to ischemia and necrosis of the right hepatic lobe if flow is not restored in time. Furthermore, as the biliary system is fully dependent on arterial flow, rapid biliary ischemic damage occurs in case of a hepatic artery thrombosis. Thrombosis of the hepatic artery is thus associated with morbidity and graft loss [6]. Hepatic artery thrombosis can occur early (within 2–3 months) after transplantation, but may also occur years after the procedure. The clinical outcome of late hepatic artery thrombosis is usually more benign compared to early thrombosis, as collaterals may have developed over time, especially when occlusion of the hepatic artery is preceded by a slowly worsening stenosis [29]. As early and late hepatic artery thrombosis may have a different etiology, they will be dealt with separately in the following sections.

Early Hepatic Artery Thrombosis

Early hepatic artery thrombosis is envisioned primarily as a surgical complication. Technical imperfections with the anastomosis related, for example, to aberrant donor or recipient arterial anatomy or complex back-table arterial reconstruction, kinking of the artery, prolonged clamping of the hepatic artery, or the use of an arterial conduit, indeed increase the risk for hepatic artery thrombosis substantially [6,30,31]. The incidence of early hepatic artery thrombosis has substantially decreased since the first decades of liver transplantation, which also suggests that surgical factors contribute substantially to this complication. Furthermore, low volume transplant centers or less experienced surgeons have a higher rate of hepatic artery thrombosis, which again indicates that the complication has a surgical component. However, additional non-surgical factors also contribute to the risk of early hepatic artery thrombosis. These factors include damage to the graft for example by prolonged cold or warm ischemic times, preoperative transarterial chemoembolization for HCC, or an otherwise damaged artery for example due to complications during organ procurement [31–33]. Furthermore, retransplantation is also an important risk factor for early hepatic artery thrombosis, with the risk even increasing further in a second retransplantation [33,34]. Also, insufficient blood flow through the artery, for example in patients with a splenic artery steal syndrome, increases the risk for hepatic artery thrombosis, although in a splenic artery steal syndrome, ligation of the splenic artery is sufficient to restore adequate arterial flow [31]. A low recipient weight is also a

clear risk factor in pediatric patients. Finally, the combination of a cytomegalovirus (CMV) positive donor with a CMV negative recipient predisposes to early hepatic artery thrombosis [35], possibly related to coagulation activation (see below).

A Role for the Hemostatic System?

A perhaps underestimated contributor to development of early hepatic artery thrombosis is the hemostatic system [36,37]. The thrombus occluding the hepatic artery is a true hemostatic thrombus, and thus triggers that initiate hemostasis should be present for such a thrombus to develop. Multiple triggers that can initiate hemostasis are present in the early postoperative period after transplantation. First, the surgical damage inevitably triggers activation of coagulation. Furthermore, stasis as a result of clamping of major vessels and activatory factors of the graft itself may result in coagulation activation. In particular, the graft potentially activates hemostasis through its endothelium, which is activated as a consequence of multiple processes during organ procurement and preservation. Already in the organ donor, inflammatory processes take place, which result in activation of endothelium cells of endothelial cells [38]. Subsequently, during cold and warm ischemia and reperfusion, additional activation of the endothelium takes place. Activated endothelial cells become adhesive to platelets [39], and start to express tissue factor [40], the physiological activator of coagulation. Indeed, biopsy studies show adhesion of platelets to the sinusoidal endothelium in a transplanted liver after reperfusion [41]. Although this attachment of platelets to the activated endothelium does not directly lead to the formation of large, occlusive thrombi, it does result in apoptosis of the underlying sinusoidal endothelial cells [39], which likely aggravates the already present ischemic damage. Furthermore, endothelial-cell apoptosis further facilitates hemostasis activation, for example, by expression of negatively charged phospholipids on the cell surface, which can support coagulation reactions [42]. Another important trigger of coagulation is CMV, which also results in hemostasis activation by endothelial-cell activation [43]. All these hemostatic triggers happen in a patient, in which the coagulation system is profoundly altered as compared to healthy individuals, and undergoing dynamic changes [44]. Most proteins involved in coagulation and fibrinolysis are present in low levels in patients with liver failure, and levels drop even further during liver transplantation. In the early postoperative period, levels of these proteins recover, although not all at the same rate. There is evidence for a hypercoagulable status in the early postoperative period after liver transplantation [45]. Specifically, platelet function is stimulated by substantially elevated levels of von Willebrand factor, combined with decreased levels of the protease that regulates von Willebrand factor reactivity (ADAMTS13) [46]. Furthermore, levels of the natural anticoagulant proteins antithrombin, protein C, and protein S are low, and in addition to this, activation of the anticoagulant protein C system has been shown to be defective in vitro, up to 10 days after transplantation (47). Finally, immediately after surgery, a temporary lack of fibrinolytic capacity, a phenomenon referred to as "postoperative fibrinolytic shutdown" contributes to a hypercoagulable status [48]. This hypercoagulable state combined with multiple activating triggers of coagulation in the vicinity of the anastomosis may contribute to the development of hepatic artery thrombosis. Furthermore, the hypercoagulable status can be aggravated when a graft from a donor carrying one of the inherited thrombophilic polymorphisms in coagulation factors is transplanted into a wild-type recipient [49]. Most coagulation factors are synthesized in the liver, so liver transplantation is a unique situation in which the phenotype of coagulation factors may change. However, the evidence that inherited thrombophilia, e.g., by the factor V Leiden or prothrombin G20210A mutation increases the risk for early hepatic artery thrombosis is based on small studies and case reports only [49,50]. Moreover, these polymorphisms are relatively rare (around 5% for factor V Leiden) so that screening for these polymorphisms is not recommended.

Clinical evidence for a role of the coagulation system in early hepatic artery thrombosis has emerged from a study in which patients transplanted for familial amyloidotic polyneuropathy (FAP) were shown to have a substantially increased risk for hepatic artery thrombosis as compared to patients transplanted for end-stage liver disease [51]. In contrast to patients with end-stage liver disease, patients with FAP have a fully competent hemostatic system, as the synthetic capacity of the liver of FAP patients is not compromised. Furthermore, the surgical procedure, and the arterial reconstruction is generally much less complicated in a patient with FAP compared to the end-stage liver disease patient because of the absence of a disturbed liver architecture, portal hypertension, venous collaterals, or perihepatic inflammatory lesions . Although experimental evidence is lacking, it is likely that patients with FAP have an increased hypercoagulable status posttransplant compared to the end-stage liver disease patients and this notion combined with the substantially reduced technical difficulty of transplantation in the FAP patient suggests that the increased incidence of hepatic artery thrombosis in the FAP patient is a result of their increased hypercoagulable status [37].

Late Hepatic Artery Thrombosis

The prevalence of late hepatic artery thrombosis after transplantation is unclear. While some studies suggest that the incidence of late hepatic artery thrombosis is much lower as compared to the early form, other studies show the opposite

[27,29,52,53]. It has to be noted that "early" and "late" hepatic artery thrombosis are poorly defined in literature, and some studies do not even make this distinction. Furthermore, late hepatic artery thrombosis may be clinically silent, and only detected during protocol ultrasonographic examination.

There is no consensus on risk factors for late hepatic artery thrombosis, but donor age, severe acute rejection, back-table surgery for anatomic variations, blood group-incompatible grafts, active cigarette smoking, usage of a donor iliac artery interposition graft to the aorta, and use of a graft from a donor who died of a cerebrovascular accident have all been suggested as risk factors [29].

A Role for the Hemostatic System?

Late after transplantation, multiple factors may result in hypercoagulation or may trigger hemostasis. It is well known that a substantial proportion of transplant patients will develop multiple classical arterial risk factors including diabetes, dyslipidemia, obesity, and hypertension [54,55]. The cause of these complications is multifactorial, but use of immunosuppressants is a clear contributor. The increased arterial risk profile is associated with an increased risk of (cardio)vascular events as compared to the general population, but the association between these risk factors and the occurrence of late hepatic artery thrombosis has been poorly studied, although one study showed transplantation-related diabetes to be associated with an increased risk for late hepatic artery thrombosis [56]. Furthermore, CMV infection is also a risk factor for late hepatic artery thrombosis, which may be related to endothelial cell activation resulting in activation of hemostasis [43].

Clinical evidence for a role of the hemostatic system in development of late hepatic artery thrombosis is scarce. One study retrospectively analyzed the effect of aspirin administration to patients with risk factors for late hepatic artery thrombosis (in this study employment of a donor iliac artery interposition graft to the aorta, and use of a graft from a donor who died of a cerebrovascular accident) [57]. Patients who received aspirin indefinitely had a substantially decreased risk of late hepatic artery thrombosis (3.6 vs. 0.6%, which is a relative risk reduction of 82%), which suggests that (excessive) platelet activation may be involved in the pathogenesis of this complication [38].

Clinical Features

Early Hepatic Artery Thrombosis

In the recently transplanted patient, blood supply to the bile duct is fully dependent on the hepatic artery as no collateral circulation is present. Bile duct ischemia and necrosis is thus an inevitable consequence of hepatic artery thrombosis, and also massive injury of hepatocytes occurs. The clinical presentation of early hepatic artery thrombosis is thus by elevated transaminases and bilirubin levels, in combination with biliary complications including biliary sepsis with fever and leucocytosis. As early hepatic artery thrombosis usually results in a rapid deterioration of liver function and patient condition, rapid diagnosis and treatment is required. Nevertheless, even in case of timely intervention, morbidity and mortality is substantial with 33% mortality, and 50% graft loss [6].

Late Hepatic Artery Thrombosis

The clinical course of late hepatic artery thrombosis is much more benign than that of early hepatic artery thrombosis. Notably, liver function tests may be normal, and patients may remain asymptomatic or present with cholangitis, bacteremia, and relapsing fever.

Diagnosis

Early Hepatic Artery Thrombosis

Doppler ultrasonography is the gold standard for the detection of early hepatic artery thrombosis [58]. This test can be performed easily and with repeated frequency, with low costs and high accuracy on any intensive care unit. An absence of arterial flow on Doppler ultrasound is an indication for conventional or CT-angiography, which then definitively confirms the diagnosis, although the accuracy of Doppler ultrasonography alone is >90% [59]. Some centers will not perform angiography if no signal by Doppler is detected and clinical and laboratory symptoms are suggestive of an early hepatic artery thrombosis. Routine Doppler ultrasonography screening at regular intervals in the early postoperative period is recommended to facilitate early detection of hepatic artery thrombosis [58]. Also, some centers use an implantable Doppler probe which continuously measures arterial flow, but the value of this method is not yet definitively established [60].

Late Hepatic Artery Thrombosis

Late hepatic artery thrombosis (generally defined as >1 month after transplantation) may be detected coincidentally by routine protocol Doppler ultrasonography [30]. In some patients, a late hepatic artery thrombosis becomes evident by a minimal rise in liver enzymes, although intrahepatic bile duct necrosis and subsequent biliary sepsis may also occur.

Treatment and Possible Prophylactic Options

Early Hepatic Artery Thrombosis

Early hepatic artery thrombosis requires immediate treatment. Traditionally, urgent retransplantation was the treatment of choice, but this approach is restricted by the donor shortage. A second option is urgent revascularization by thrombolysis, thrombectomy/angioplasty, or implantation of an arterial conduit. Successful revascularization will prevent a retransplantation, but revascularization attempts are successful in only about 50% of cases. Consequently, graft survival after revascularization is low, at around 40% in symptomatic patients [61], although much lower salvage rates (as low as 10%) have been reported in single center series [27]. However, when a thrombosis is detected early in the absence of symptoms, graft survival after revascularization is much better, at around 80% [61], which stresses the need for frequent routine ultrasound screening. The outcome of urgent retransplantation for early hepatic artery thrombosis is also poor, with a 1 year graft survival of around 50% [6].

Thus, the occurrence of early hepatic artery thrombosis is clearly associated with a poor outcome, and therefore improvements in detection and consideration of possible prophylactic strategies are important. Given the suspected contribution of coagulation activation in development of early hepatic artery thrombosis, the use of anticoagulants is an attractive therapeutic possibility. Use of anticoagulation in the early postoperative period has long been avoided, since the perceived bleeding risk in patients with liver disease. However, there is accumulating evidence that patients can be in a hypercoagulable state in the early postoperative period, even though routine laboratory tests such as the PT/INR suggest otherwise [47,62,63]. Since platelets are the hallmark of arterial thromboses, prophylaxis with anti-platelet agents including aspirin, P2Y12 blockers (e.g., Plavix), or $\alpha IIb\beta 3$ blockers (e.g., ReoPro) might be an attractive option. Anticoagulants including heparin(oids) and vitamin K antagonists are potentially also effective. A drawback of heparin use is the low levels of antithrombin in some patients; as antithrombin is required for the therapeutic effect of heparin, the efficacy can be unpredictable in patients with low antithrombin levels. A drawback of vitamin K antagonists is the difficulty in monitoring the INR in the early postoperative period, as the INR may already be elevated in the absence of vitamin K antagonists. New classes of anticoagulants, including direct Xa or thrombin inhibitors are potentially of interest, although a major drawback is the absence of proven antidotes for these agents.

Although theoretically the use of thromboprophylaxis is an attractive strategy to lower the risk of hepatic artery thrombosis, the hemostatic balance in this period is probably precarious [63], so a bleeding risk with the use of any anticoagulant is probably real. However, given the poor outcome of patients with early hepatic artery thrombosis, the bleeding risk may outweigh the potential benefit, especially in high-risk patients, including pediatric transplant recipients.

No studies assessing the potential benefit of early thromboprophylaxis with respect to hepatic artery thrombosis have been performed, and each center has its own protocols, which are in general empirically derived, but not evidenced-based.

Late Hepatic Artery Thrombosis

The management of late hepatic artery thrombosis is controversial, and depends on the presence of associated diseases and symptoms. When a patient with late hepatic artery thrombosis has severe liver function impairment with persistent biliary problems, retransplantation without a prior attempt to revascularization is usually preferred. Thrombolysis is usually not successful, probably because the thrombus has developed over time, and matured thrombi are usually resistant to thrombolysis. Also, the outcome of thrombectomy and/or revision of the anastomosis are only successful in a small proportion of patients. One study reported a success rate of 100% in seven patients treated with systemic anticoagulation only [27].

Prevention of late hepatic artery thrombosis would theoretically require management of classical arterial risk factors such as diabetes, hypercholesterolemia, hypertension, and obesity, in addition to thromboprophylaxis. No randomized studies to the use of thromboprophylaxis to prevent late hepatic artery thrombosis have been performed. One retrospective uncontrolled study showed a remarkably reduced incidence of late hepatic artery thrombosis in high risk patients who received indefinite aspirin prophylaxis [57]. The indefinite anticoagulation regime appeared safe, as no bleeding complications were observed in 160 patients who were followed up for more than 4 years.

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Chapter 48
Hepatic Venous Outflow Obstruction

Yusuf Bayraktar

Introduction

The liver is the largest organ in the body and its dual blood supply makes it a unique organ. Although it makes up less than 3% of total body weight (about 1,800 g in men and 1,400 g in women), the liver receives one-quarter of the total cardiac output via the hepatic artery and portal vein [1]. Classically, the liver has been divided in to two lobes, right and left lobe. For more details on liver anatomy, please see Chap. 1. However, today the terms "left" and "right" liver has been used on the basis of the distribution of vessels and ducts by a line extending between the inferior vena cava (IVC) and gallbladder. Furthermore, the liver can be divided into eight segments. The left liver is composed of a classical left lobe and a caudate lobe. The portal vein carries blood from the digestive tract (proximal stomach, upper rectum, and pancreas), spleen and gallbladder. Many portal vein anomalies have been reported, either congenital or as a result of neonatal omphalitis or portal vein thrombosis, in which nodular hyperplasia or left lobe atrophy occur because of remodeling of the liver.

The three main hepatic veins carrying blood from the liver to the IVC are the main essence of this chapter. The middle and left hepatic veins join before entering the IVC in 80% of individuals. The large hepatic veins divide at an acute angle into branches of equal diameter in order to compose an axial tree receiving smaller tributaries at right angles. In obstruction of liver drainage, the development of intrahepatic anastomoses, which are normally found between branches of the hepatic veins, is essential. In addition to the three main hepatic veins, several veins drain caudate lobe blood directly into the IVC. Because caudate veins are usually patent in Budd-Chiari syndrome (BCS), compensatory hypertrophy of this lobe allows for the drainage of the other parts of the liver through intrahepatic collaterals.

It is well known that hepatocytes have a polyhedral form with spherical nuclei. The sinusoidal surface is composed of a layer of endothelial cells which block the extravascular space of Disse. In this space, lymphocytes and stellate cells are located. The latter can function as the principal hepatic fibroblasts when activated by different molecules, particularly by cytokines, which is a very important step in chronic congestion or inflammation. Kupffer cells are the other sinusoidal cells functioning as hepatic macrophages. The main difference between hepatic sinusoids and systemic capillaries is the presence of fenestration and lack of subendothelial stromal material allowing entrance of large macromolecules into Disse space. During the chronic inflammation of congestion, Disse space is widened by accumulation of collagen tissue and endothelial fenestration becomes smaller, leading to a reduced transport across the hepatic sinusoidal wall. The acinus and lobule are two contrasting models of micro-organization of the hepatic parenchyma. The models differ in the shape of the isobars surrounding terminal portal venules. The acinus is bulb-shaped, defined by zone 1 to zone 3 surrounding the terminal portal venules. The lobule is made of several wedge-shaped portions which have cylindrical isobars. In the acinar model, cells more distant from the portal supply (zone 2 and 3) respond differently to hypoxia and toxin exposure, as in veno-occlusive disease (VOD). On the other hand, hepatocytes close to acinar zone 1 have more oxygen and nutrition supply.

Blood is drained from the hepatic acini through central veins into sublobular veins and then into the right, left and middle hepatic veins, the IVC, and finally the right atrium [2]. Any obstruction along the above mentioned route from the central vein to the right atrium can result in a spectrum of clinical abnormalities ranging from acute hepatic failure to passive hepatic congestion depending on the acuity, level of obstruction, and the number of involved vessels. In these clinical conditions, hepatic venous outflow obstruction (HVOO) can be divided into three main groups with respect to the level of obstruction starting from heart (Fig. 48.1):

Y. Bayraktar (✉)
Department of Internal Medicine, Gastroenterology Section, Hacettepe University, Ankara, Turkey
e-mail: bayrak@hacettepe.edu.tr

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5 ,
DOI 10.1007/978-1-4419-7107-4_48, © Springer Science+Business Media, LLC 2011

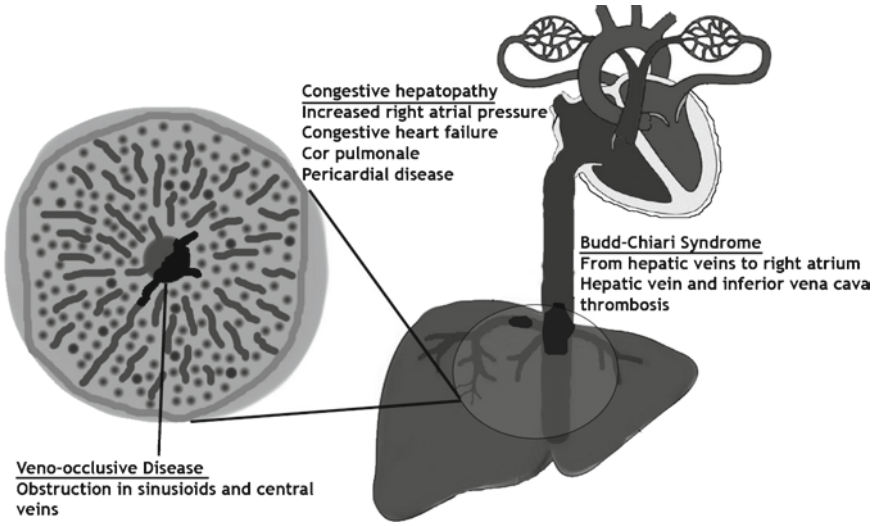

Fig. 48.1 Site of obstruction in three syndromes mimicking each other clinically

- Congestive hepatomegaly (CH): venous stagnation rather than obstruction at the level of the heart.
- Budd-Chiari syndrome (BCS): from sublobular hepatic veins to the superior end of IVC.
- Veno-occlusive disease (VOD): at the level of sinusoids and the terminal venules (central vein).

Although in this group of disorders there is outflow obstruction, the etiological factors are distinct. It is necessary to stress that VOD develops within three weeks of an acute insult to the sinusoidal endothelial cells (SEC), while BCS may develop within a few days or several years of venous thrombosis. On the other hand, CH related with heart disorders may take years to develop. The variation in the "acuteness" of the venous obstruction or occlusion leads to subtle differences in the clinical presentation of these three disorders. Although patients with HVOO usually present with abdominal pain, jaundice, and ascites, chronic BCS patients may first present with cirrhosis or its complications. BCS patients with IVC obstruction may also have leg edema and venous collaterals over the trunk. On the other hand, jugular venous distention, leg edema, and dyspnea may be seen in patients with CH. Nevertheless, the histopathological findings in all three disorders are almost the same and comprise of sinusoidal congestion and hepatocytes necrosis predominantly in the perivenular area of hepatic acini, eventually leading to bridging fibrosis between adjacent central veins [1–3]. More distinctly, in VOD, there are narrowing and obliteration in the central veins diffusely.

The biochemistry of these patients is similar. Hyperbilirubinemia probably due to hepatocellular dysfunction, hemolysis, and biliary canalicular obstruction secondary to distended hepatic vein is seen in almost all patients, except in patients with constructive pericarditis and well drained chronic BCS. Serum alanine amino transferase (ALT) and aspartate amino transferase (AST) levels may be mildly elevated in all HVOO patients, but in fulminant BCS and CH with severe cardiac impairment causing hepatic ischemia these levels may exceed 1,000 IU/L. On the other hand, alkaline phosphatase (ALP) level is high in BCS, particularly with caudate lobe hypertrophied patients. New markers for diagnosis of VOD such as plasminogen activator inhibitors-1 are under investigation.

The diagnosis of BCS is mainly dependent on radiological and histological studies. Furthermore, physical exam and a careful medical history are very important in order not to overlook this protean syndrome. Subsequent Doppler ultrasonography is usually enough to obtain diagnosis. If necessary, hepatic venography may be performed to confirm the diagnosis.

The following provides a detailed review of three main syndromes mimicking each other clinically, CH, BCS, and VOD, listed anatomically, starting from the heart to the liver (Table 48.1).

Congestive Hepatopathy

This entity refers to hepatic manifestations of passive hepatic congestion caused by right side heart failure of any cause, including constrictive pericarditis (CP), tricuspid regurgitation, cor pulmonale and any type of cardiomyopathy. Development of hepatic ischemia and infarction secondary to left-sided heart failure, which often results in congestive hepatopathy, is out of scope of this work. However, it should be kept in mind that the clinical picture in this disorder varies greatly depending on both the degree and acuity of congestion and presence or absence of hepatic ischemia and infarction.

Table 48.1 Definition, classification, etiology, radiologic findings, histology and treatment in CH, BCS, and VOD

	Congestive hepatopathy	Budd-Chiari syndrome	Veno-occlusive disease
Site of blockage	Heart, pericardium	From small hepatic vein to the superior end of IVC	Hepatic sinusoids, hepatic terminal venules
Etiology	Any reason increasing right atrial pressure: CHF (CAD, CMP), cor pulmonale, constrictive pericarditis	Hepatic vein thrombosis, IVC webs, compression of hepatic vein or IVC by tumors, cyst or abscess	Sinusoidal endothelial injury caused by stem cell transplantation, chemotherapy, radiotherapy, and pyrrolizidine alkaloids
Histology	Predominantly in perivenular area sinusoidal congestion and hepatocyte necrosis Bridging fibrosis between central veins leading to cardiac fibrosis[a]	Predominantly in perivenular area (exception presence of concomitant PVT) Sinusoidal congestion followed by ischemic-cell necrosis and bridging fibrosis between central veins[a] Caudate lobe hypertrophy with fibrosis and atrophy in the rest of the liver	Changes in perivenular area Gaps in SEC barrier leading to subendothelial edema. Narrowing of central veins with congestion of sinusoids and hepatocytes necrosis Collagen accumulation in sinusoids and venules leading to bridging fibrosis between central veins[a]
Radiology	Dilatation in all hepatic veins on sonogram ECHO: increased pulmonary artery pressure, dilatation of right side of heart, TR, abnormal diastolic ventricular filling due to pericardial disease	Web or occlusion in IVC, osteal or complete obstruction of hepatic veins, enlargement in caudate lobe, abnormal flow in hepatic veins in Doppler. Intrahepatic collateral vessels	Hepatomegaly Doppler may show reverse blood flow in the portal vein
Treatment	Consists of underlying heart disease Pericardiectomy in CP	Prevention of thrombus extension: anticoagulation with heparin and warfarin Restoration of blood flow: thrombolytic therapy, percutaneous angioplasty, TIPS or shunt surgery Liver transplantation	Prevention: UDCA, heparin, LMWH and defibrotide Symptomatic care, defibrotide, tPA, and AT-III
Prognosis	Liver disease rarely contributes to mortality	5-year survival rate is 42–89% in hepatic vein thrombosis	Mortality rate between 9–98% depending on the severity

CHF congestive heart failure; *CAD* coronary artery disease; *CMP* cardiomyopathy; *IVC* inferior vena cava; *PVT* portal vein thrombosis; *TR* tricuspid regurgitation; *CP* constrictive pericarditis; *UDCA* ursodeoxycholic acid; *LMWH* low molecular weight heparin; *tPA* tissue plasminogen activator; *AT-III* antithrombin III

[a]In all of the above, fibrosis takes place between central veins

Clinical Features

Liver dysfunction resulting from chronic CH, whatever the cause, is usually mild and asymptomatic, and is demonstrated incidentally on routine biochemical investigation. Mild jaundice can be seen in symptomatic patients. It is interesting that jaundice is never seen in CP. In severe heart failure, jaundice may be so deep as to suggest cholestasis [4]. In these cases, right upper quadrant pain and discomfort due to stretching of liver capsule and ascites may also occur. Very rarely, fulminant hepatic failure leading to death has been reported to be caused by congestive heart failure, in which hepatic congestion and ischemia were the main findings [4–6].

Although splenomegaly is rare, tender hepatomegaly, sometimes massive, with a firm and smooth liver edge are the main physical findings. Ascites due to transmitted elevated central venous pressure is an often-encountered dependable finding. Jugular venous distention and hepatojugular reflex may be present and are helpful in differentiating congestive hepatopathy from BCS and primary liver disease characterized by hepatomegaly.

Liver may be pulsatile, particularly in patients with tricuspid regurgitation. In these cases, loss of hepatic pulsatility suggests that cardiac fibrosis has been established.

Laboratory Findings

Hyperbilirubinemia is predominant, particularly the indirect type, in 70% of patients. High hyperbilirubinemia may occur in patients with severe, usually acute right-sided heart failure. The levels correlate well with right atrial pressure, but not with cardiac output. It is necessary to stress that in patients with deep jaundice, serum ALP level is usually only mildly increased, which helps to distinguish congestive

hepatopathy from obstructive jaundice where bilirubin, particularly the direct type, and ALP are equally elevated. Serum ALT and AST levels also show mild elevation unless cardiac output is impaired. As stated above, in patients with severe acute heart failure, aminotransferase levels may be extremely high, secondary to hepatic ischemia and hepatic necrosis, and the degree of elevation of the enzymes correlates well with the extent of necrosis as seen on liver biopsy specimens. Interestingly, prompt treatment of heart failure may cause a decrease of enzyme levels in a relatively short period of time. Moreover, prothrombin time may be mildly abnormal, albumin levels may be decreased in chronic cases, and serum ammonia levels may be elevated [7–9].

Pathology

The characteristic gross appearance of the liver in this condition is referred to as "nutmeg" liver resulting in contrasting areas of red caused by sinusoidal congestion and bleeding into necrotic regions surrounding the enlarged hepatic veins, and yellow due to the normal or fatty liver tissue. In histology, sinusoidal engorgement and hemorrhagic necrosis are seen in perivenular areas of hepatic acini. Additionally, cholestasis with occasional bile thrombi may also be found in variable degrees. As expected, fibrosis develops in perivenular areas, ultimately leading to bridging fibrosis between adjacent central veins in chronic heart failure. This process results in cardiac fibrosis, inappropriately referred to as cardiac cirrhosis, which is distinct from primary liver cirrhosis in which fibrous bands tend to link adjacent portal areas. The regeneration of periportal hepatocytes in cardiac fibrosis may result in nodular regenerative hyperplasia. If heart failure is treated properly, the early histological changes of congestive hepatopathy may resolve, and even cardiac fibrosis may regress histologically and clinically [2].

Diagnosis

The diagnosis of right sided heart failure should be considered in patients with hepatomegaly with or without jaundice. CP is particularly difficult to differentiate from primary liver cirrhosis and BCS because of its relatively nonspecific clinical findings. In the diagnosis, a careful medical history and physical examination are essential. Some symptoms such as exertional dyspnea, orthopnea and angina, and physical findings like jugular venous distention, heart murmurs, and rales may be helpful to differentiate congestive heart failure from primary liver diseases. In addition to liver biochemical testing, viral serology, and abdominal ultrasonography with Doppler studies of the liver, ECG and echocardiography should be performed when CH is suspected. However, a normal echocardiogram does not always rule out this disorder. Upon the finding of dilatation of all three main hepatic veins on abdominal ultrasonogram (Fig. 48.2a), physicians should always look for cardiac causes leading to such hepatic venous dilatation, which is a very important sign of hepatic outflow obstruction, not resulting from hepatic disease but post sinusoidal extrahepatic diseases. Diagnostic paracentesis, if ascites is present, may be very helpful. It reveals a high ascitic fluid protein content and serum to ascites albumin gradient > 1.1 g/dL, reflecting the contribution of "hepatic lymph" and portal hypertension to the ascites.

Treatment and Prognosis

Treatment of the underlying heart and lung disease is fundamental to the management of CH and is outside the scope of this chapter. Jaundice and ascites usually respond significantly to diuretics. In treating these patients, the physician must be careful regarding excess use of diuretics, decreasing cardiac output, and resulting hepatic ischemia. These patients

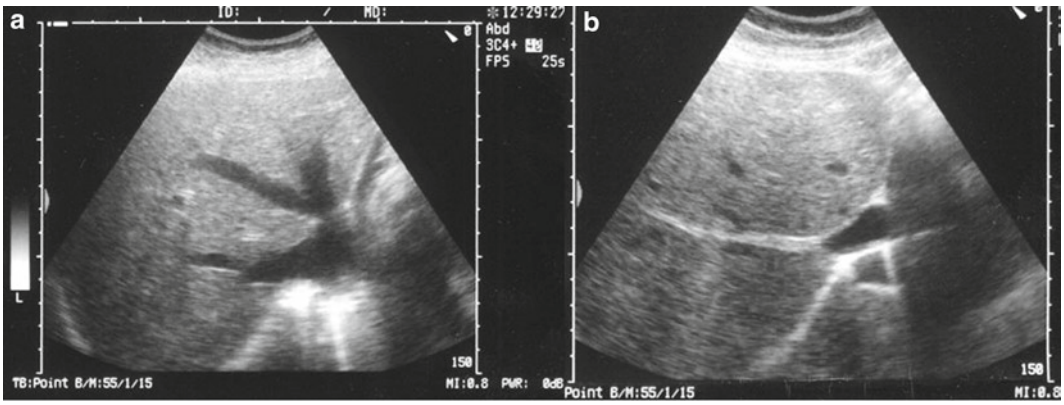

Fig. 48.2 (**a**) Dilatation in all three hepatic veins in a patient with right heart failure. (**b**) Thickness in the hepatic vein wall with near-total occlusion of the lumen in a patient with BCS

usually die of cardiac causes. Liver abnormalities rarely contribute to morbidity and mortality. Patients with cardiac fibrosis rarely develop serious complications of portal hypertension such as variceal bleeding and hepatic encephalopathy as seen in primary liver cirrhosis. HCC is a rarely seen complication of cardiac fibrosis. However, the incidence of HCC and liver failure due to CH is likely to increase, as survival is prolonged, with advances in the management of heart related disorders.

Constrictive Pericarditis

Although CP is an example of CH, it deserves mention separately because its diagnosis can be easily missed and its clinical manifestations are different from those seen in CH due to other causes. CP is the result of scarring and loss of elasticity of the pericardial sac restricting cardiac filling. Tuberculosis, cardiac surgery, radiotherapy, and connective tissue disorders are among the common causes. However, most of the cases are idiopathic or viral. CP patients may present with symptoms of fluid overload such as edema and ascites, or symptoms of diminished cardiac output like exertional dyspnea and fatigue. Hepatomegaly, massive ascites, and peripheral edema are common findings. Interestingly, CP patients do not develop jaundice [10] for reasons that are unclear. As its manifestation is protean, it is easy to mistake CP for BCS and liver disease [11]. Jugular venous distention which was noted in 93% of patients in a large series is a critical finding in the diagnosis of CP [12]. Pericardial knock, pulsus paradoxus, and Kussmaul's sign may rarely offer additional clues to the diagnosis. ECG may show low voltage and nonspecific ST and T wave changes. Chest X-ray taken in the lateral position may demonstrate pericardial calcification and a relatively small heart. Echocardiography is essential in the diagnosis, but left and right heart catheterization with hemodynamic evaluation may be required to confirm the diagnosis [13–15]. Frequently, cardiac fibrosis develops more rapidly in CP than in other causes of right sided heart failure, perhaps due to higher hepatic vein pressure, leading to severe zone 3 congestion and necrosis, and accordingly fibrosis. Pericardiectomy is the well known standard treatment modality.

Budd-Chiari Syndrome

BCS is an uncommon but potentially life-threatening condition caused by obstruction of the hepatic venous outflow tract at any level from the small hepatic veins (sublobular hepatic veins) to the junction of the IVC with the right atrium, in the absence of the right failure, whatever its cause, or CP.

Epidemiology

This disorder is relatively rare. The prevalence and incidence in Japan and France seems to be 0.2 and 2 per million inhabitants [16, 17], respectively. On the other hand, the syndrome appeared to be at least ten times more common in Nepal [17, 18]. It has been known for a long time that there were differences between Asians and Western individuals with BCS. Terminal IVC was the most frequently occluded area in Asians, and usually patent in Western patients. Regarding sex differences, the ratio of male to female is close to 1:1 and mean age of about 45 years [19]. In Turkey, located between Asia and Western countries, this disease is not uncommon [20].

Classification and Etiology

BCS can be classified into two categories: Primary, in which obstruction is located in the luminal area either by thrombus or webs and secondary, in which obstruction leading to blood flow blockage is caused by intraluminal invasion by parasite, or malignant tumor, or by extraluminal compression by an abscess, cyst, or solid tumors [21, 22]. Intravascular thrombosis is the most common mechanism leading to occlusion of the hepatic venous system. At least one hereditary or acquired procoagulative disorder can be identified in 75% of patients with BCS [23].

Myeloproliferative disorders (MPD) including polycythemia vera, idiopathic thrombocytosis, and myelofibrosis are the main cause of this syndrome. Additionally, in paroxysmal nocturnal hemoglobinurea, hepatic-veins thrombosis develops in about 12 % of the cases [24, 25]. As many as 30% of the patients with BCS are found to have factor V Leiden mutation, which is present in the majority of pregnancy- or oral contraceptive-related cases of hepatic vein thrombosis [26, 27]. Protein S, protein C, and antithrombin levels may be decreased nonspecifically because of impaired hepatic function in patients with BCS, but levels below 20% of normal level suggest inherited deficiency of these anticoagulant proteins.

Primary membranous obstruction of the IVC is the most common cause of BCS in South Africa and Asia, and is thought to be a consequence of IVC thrombosis [28]. However, it is less common in western countries. For unknown reasons, nearly half of patients with known membranous obstruction ultimately develop HCC even in the absence of liver cirrhosis [29]. BCS may also be seen as a complication of Behçet's disease [30]. Additionally, HCC has been reported to be a complication of hepatic-vein obstruction in some studies [31, 32]. Table 48.2 shows main etiologic factors for BCS.

Table 48.2 Causes of Budd-Chiari syndrome

Hypercoagulative states	Inherited thrombophilic disorders
	Antithrombin III deficiency
	Protein C deficiency
	Protien S deficiency
	Factor V Leiden mutation
	Prothrombin gene mutation (heterogenous G200210A prothrombin)
	Acquired thrombophilic disorders
	Myeloproliferative disorders (MPD)
	V617F JAK2 positive MPD
	V617F JAK2 negative MPD
	Paroxysmal nocturnal hemoglobinuria
	Antiphospholipid syndrome
	Behçet's disease
Other acquired disorders	Pregnancy
	Use of oral contraceptives
	Tumoral invasion (hepatocellular carcinoma, renal cell carcinoma, and adrenal carcinoma)
	Alveolar cyst hydatid disease
Miscellaneous	Aspergillosis
	Inferior vena caval web
	Trauma
	Inflammatory bowel disease
	Dacarbazine therapy
Idiopathic	

BCS is the hepatic expression of underlying prothrombotic conditions involving particularly the hepatic veins. Thus, let us overview the first blood diseases leading to BCS.

Myeloproliferative Disorders or Prothrombotic Conditions

At least one hereditary or acquired procoagulative disorders can be identified in 75% of patients with BCS [23]. Among the procoagulant disorders, MPD account for approximately 50% of BCS cases. A recent advance in MPD has been the identification of a particular somatic mutation (V617F) in the Janus Tyrosine Kinase-2 (JAK-2) gene in myeloid cells [33]. This gene is coupled to the growth factor receptors on the myeloid cells. Activation by erythropoietin, thrombopoietin, or other growth factors elicits the signals for proliferation and differentiation of the myeloid precursors into mature cells through JAK2 phosphorylation. V617F-JAK2 mutation produces constitutive activation of signal transduction leading to independence from, or hypersensitivity to growth factors. This single somatic V617F mutation can be isolated in leucocytes or other blood cells of the myeloid series. JAK2 mutation has been found in about 80% in PVR and 50% in essential thrombocythemia or idiopathic myelofibrosis [34]. In patients with primary BCS, this V617F mutation has been demonstrated in 35–45% of cases [34–39]. Approximately 80% of BCS patients with a MPD harbor this mutation [34, 35]. In the remaining 20% of this patient group, evidence for underlying MPD is derived from bone marrow biopsy findings. The biopsy and JAK2 mutation test have replaced formerly used tests utilizing cultures of erythroid progenitors to reveal endogenous colonies.

Normally, patients with BCS, in the absence of MPD, are expected to have pancytopenia or bicytopenia, which develop as a result of hypersplenism, hemodilution, and iron deficiency. However, clinicians must be careful with a particular group of BCS patients with underlying MPD, where the peripheral blood count is mostly within normal limits. In this setting, if a patient with BCS has normal or slightly above normal value of peripheral blood count, especially with the presence of portal vein thrombosis, in which splenomegaly is larger than expected, as a possible underlying disorder, MPD should be considered and thoroughly investigated. The reason for this normal blood count, despite hypersplenism, might be MPD, in which normally the cell count should be elevated. Hypersplenism might compensate the elevated blood count. Currently, although uncertainties remain as to which of these patients with an essentially normal blood cell count should receive cytoreductive therapy, I personally think that this treatment might be given to BCS patients resulting from MPD who have normal or mildly elevated blood count.

Pathogenesis and Pathophysiology

In the primary group, at presentation, obstruction may be a result of a fresh thrombus, of mere stenosis or due to a thrombus

superimposed on a previous stenosis. The stenosis can be seen the entire length of one or two hepatic veins or IVC, or may be limited to a short portion of IVC. In all instance of primary BCS, the primary lesion is most probably a thrombus which by time evolves into fibrous tissue [40]. This may be seen as a short length stenosis in distal part of IVC or involved major hepatic veins [41]. To establish this clinical syndrome, at least two major hepatic veins must be involved. Rarely, involvement of all three hepatic veins can be seen simultaneously. After obstruction, the development of collateral circulation starts to bypass the obstacle by connecting blocked territories to patent territories. These collaterals can emerge in intra- or extrahepatic areas.

The wide clinical spectrum of this syndrome is largely due to diversity of the location of the obstruction, the varying acuity of the obstructive process, the combined involvement of IVC and hepatic veins, and the series of underlying procoagulant disorders. It has been shown that intrahepatic portal perfusion is diminished [42]. In these patients there was a strong relationship between loss of portal blood perfusion and liver-cell loss in the related areas. This decreased portal vein perfusion can result either from intrahepatic portal vein thrombosis, or focal (or locally) retrograde portal blood supply, or as a combination of both. It can be advocated that maintenance of portal blood perfusion may be essential for preventing progression of liver pathology. Regenerative macro-nodules and sometimes parenchymal space occupying lesions mimicking focal nodular hyperplasia or occasionally liver tumors are found in the arterialized liver, which are focal areas that are deprived of portal vein supply [20, 42].

In addition to intrahepatic portal vein thrombosis, extrahepatic portal vein thrombosis is also seen [30, 36, 43] either as a superimposed condition or coimposed (coincidental). In these cases, PVR and Behçet's disease particularly should be looked for. In a patient having both venous system involvement, hepatic and portal vein, clinical manifestations are normally expected to be more severe than in those of having only hepatic vein involvement. Superimposed extrahepatic portal vein thrombosis might result from either portal vein stasis or multiple thrombotic factors. Portal venous system is usually affected in patient with BCS because: (1) increased sinusoidal pressure decreases flow through the portal system, (2) hypertrophic caudate lobe may compress the main portal vein branches and the intrahepatic portal venules, and (3) risk of portal vein thrombosis is increased. According to my own view, although portal vein supply is very important in preventing hepatocyte loss, portal vein thrombosis may be beneficial at the beginning of the disorder, particularly in case of three main hepatic vein involvement. Portal thrombosis might be a preventive factor for hepatic over-congestion by diminishing hepatic inflow. Additionally, it is well known that the degree of hepatocellular necrosis depends on the acuteness of the obstruction of the outflow, and increase in pressure leads to centrilobular congestion followed by necrosis. In this situation, a diminished portal vein supply due to portal thrombosis might be preventive by decreasing central vein pressure.

In computed tomography (CT) and ultrasonography, there is usually an obviously dysmorphic liver in BCS because of asynchronous involvement of hepatic vein and resulting atrophy-hypertrophy mechanisms. It is well known that the caudate lobe venous drainage is directly into the IVC by numerous small veins. As the disorder evolves, hypertrophy takes place in the caudate lobe and the central part of the liver because of the adequate drainage of these areas. Secondly, the part of the liver is better perfused than the peripheral areas in most cases. The caudate lobe becomes so hypertrophic that it compresses the IVC. The collateral venous drainage helps to relieve hepatocellular congestion, but does not prevent peripheral atrophy. Obstruction of the hepatic venules induces shunting of flow from the hepatic arteries into the portal veins. Hepatic fibrosis in BCS is different regarding histological location, such as mainly demonstrating centrilobular distribution from alcoholic and virus related-liver fibrosis. Furthermore, the expression of the RNA regarding fibrogenic and angiogenic factors differs from that of other chronic liver diseases such as alcoholic and viral liver diseases [44].

Pathology

The extent of HVOO and acuity of the syndrome are the main factors in determining parenchymal hepatic damage and histological abnormalities. Rapid occlusion of three hepatic veins or two veins, one of which should be the right hepatic vein, carrying large volume of blood leads to diffuse hepatic congestion and enlargement, the consequence of which is ischemic necrosis followed by fibrosis, predominantly in the perivenular area. However, in patients with concomitant portal vein thrombosis, fibrosis prevails in the periportal zone [45, 46]. In chronic disease, direct blood flow from the caudate lobe to the IVC compensates for hepatic outflow obstruction. Over time, the caudate lobe becomes hypertrophic while atrophy and cirrhosis develop in the rest of the liver which means that drainage of hepatic congestion is the main determinant factor in the outcome. Compensatory nodular regenerative hyperplasia is common in areas of hepatic parenchyma that have an adequate blood supply, with progression to fibrosis and cirrhosis at a later stage of the disease [45, 47].

Clinical and Laboratory Findings

There is a wide clinical presentation ranging from a fulminant to chronic feature, rarely an asymptomatic condition, depending

on the location, extent and rapidity of the obstructive process, and on whether portal vein thrombosis coexists or not. The chronic form is the most frequent one. Asymptomatic forms appear to account for 15% of all BCS patients [48]. In these forms, some preserved hepatic veins and IVC, or with large intrahepatic or extrahepatic collaterals exist. Obstruction of a single main hepatic vein is clinically silent [49]. On the other hand, sudden occlusion of all major hepatic veins may lead to fulminant hepatic failure.

The most common signs and symptoms are ascites, hepatomegaly, abdominal pain, and splenomegaly. Portal hypertension is another important feature. Prominent dilatation of subcutaneous veins of the trunk is seen particularly in patients with long-standing IVC obstruction. More interestingly, each or all of these features may be absent. In a series of 237 patients, obstruction was found in hepatic veins, IVC, or both in 62%, 7%, and 31% patients, respectively. For descriptive purposes this syndrome can be divided into an asymptomatic, fulminant, acute, subacute, or chronic [48]. However, one should be stressed that the correlation between clinical history and the duration of the occlusion process is weak and this classification's prognostic significance is not obvious [50].

Patients with the asymptomatic form are usually diagnosed on investigation of abnormal liver function tests. In some studies [48, 51] these patients accounted for 5–20% of BCS cases. Absence of ascites and abdominal pain may be attributed to large intrahepatic portosystemic venous collaterals or patency of one large hepatic vein, particularly the right hepatic vein. Severe, sometimes unbearable right upper quadrant pain, hepatomegaly, ascites, and jaundice develop within a few weeks in patients with acute BCS. This variant accounts for 20% of BCS [48]. ALT and AST levels are always elevated because of ischemic hepatocellular damage. ALP is also elevated usually between 300 and 400 IU/mL. Even if transaminase levels are normal in chronic cases, ALP is always high in concentration. Liver function might deteriorate quickly, resulting in fulminant hepatic failure.

The fulminant form is not common and typically follows rapid and complete occlusion of all main hepatic veins. Patients quickly develop hepatic encephalopathy, renal failure, and coagulopathy. Serum ALT and AST levels are markedly high. Unlike the findings in fulminant viral hepatitis, the liver is enlarged and tender. The subacute form has a more insidious presentation spread over several months. Patients may have minimal ascites, hepatosplenomegaly, and vague discomfort or pain in the right upper quadrant. In chronic variant of BCS, accounting for 60% of cases, signs and symptoms are seen at least for 6 months and patients present with complications of cirrhosis, including bleeding from esophagus and stomach, encephalopathy, coagulopathy, and hepatorenal syndrome. Moreover, hepatopulmonary syndrome has been defined in up to 28% of patients [52] and renal impairment is present in one-half of the cases [23].

There is currently no consensus on the association of disease severity with disease duration. Additionally, the duration of symptoms do not correlate with the degree of histological damage to the liver. It has been observed that 58% of cases of an acute clinical onset may have considerable liver fibrosis, suggesting a long preclinical course or these observations can be explained by the development of recent thrombosis superimposed on previously established lesions [49, 53]. IVC compression of thrombosis generally presents with less severe symptoms compared with hepatic vein thrombosis and is characterized by leg edema or venous collaterals over the trunk in addition to hepatomegaly, ascites, and abdominal pain. The clinical course is chronic with repeated acute episodes eventually leading to congestive cirrhosis [18, 54]. Whatever causes in chronic cases, ALP levels are always high compared to ALT and AST levels.

The underlying thrombophilic conditions such as PVR, idiopathic thrombosis, and Behçet's disease [55] may also contribute to portal vein thrombosis, which leads to depletion of liver perfusion [56]. However, in our own experience, the clinical picture in patients with both hepatic and portal vein thrombosis is not worse than expected. This may be due to decreased blood flow to the liver resulting in reduced liver congestion.

The other issue in BCS patients is the clinical findings of underlying disease such as an antiphospholipid syndrome, Behçet's disease, and PVR. Clinically and by laboratory findings, it is difficult to differentiate the symptoms and signs that how much of them come from BCS and from original disease. For instance, hepatosplenomegaly is present in both BCS and PVR. Additionally, HCC appears to be a significant long term complication as it is in other chronic liver diseases. HCC developed in 11 of 97 patients of a recent cohort followed for a mean of 5 years [57]. In our own study [31], it seems to be that in BCS, due to Behçet's disease, HCC is common and more aggressive than in other causes. Serum AFP appeared to be more specific for a diagnosis of HCC in patients with BCS than with other liver diseases. Patients with long standing IVC obstruction carried a risk of developing HCC what was 70-fold higher than those with pure hepatic vein involvement [58]. To clarify the high incidence of development of HCC in this group, multivariate analysis in a large cohort and molecular studies should be done.

Prognosis and Survival

The spectrum of clinical results ranges from progressive deterioration of the general condition to a few reports of spontaneous regression of the acute manifestation. On the other hand, the natural course of this syndrome is poorly understood, mainly because most patients receive some sort

of treatment. After the administration of anticoagulant treatment and early recognition of asymptomatic cases, mortality rates have decreased over time [48]. In one study [59] consisting of 120 patients, survival rates of 77%, 65%, and 57% were found at 1, 5, and 10 years, respectively.

Murad et al developed a model predicting survival in BCS based on the presence of ascites, encephalopathy, prothrombin time, and serum bilirubin levels, classifying patients into three categories with statistically different 5-year survival rates of 89%, 74%, and 42% [51].

Histological abnormalities of the liver were not found to determine the prognosis [51, 59, 60] except in one study in which advanced fibrosis was associated with increased mortality [61].

IVC obstruction has a good short-term prognosis, but there is limited data regarding long-term prognosis. In a study from Japan, there was a 25% mortality rate over 5 years in patients with obliterative cavopathy. The main causes of death were liver failure, variceal bleeding, and HCC [16, 40]. In a series of 237 patients, portal vein thrombosis with or without splenomesenteric vein thrombosis was identified in 18 (9%) and 15 (7%) of patients, respectively (total 33 patients, 10%). The 5-year survival was 54% in patients with BCS and portal vein thrombosis and 85% in isolated BCS. Death can be related to hepatic failure, bleeding to varices, refractory ascites, or a combination of the above mentioned. The underlying disease, presentation, and level of hepatic outflow obstruction seem to be predictors.

Diagnosis

Diagnosis of BCS requires a high index of suspicion. The diagnosis is more likely when there is no other more common cause of liver disease or when there is a known underlying procoagulative condition. In the diagnosis, an obstructed hepatic venous outflow tract should be demonstrated. BCS should be considered in all patients presenting with ascites, hepatomegaly, and right upper quadrant pain. Although liver biopsy is essential, the diagnosis is largely made by imaging studies.

Real-Time and Doppler Ultrasonography

These procedures are noninvasive, and highly sensitive and specific. The lack of blood flow, thrombus in the hepatic veins, and wall thickness of the hepatic veins are main ultrasonographic findings (Fig. 48.2b). Hepatic veins or even IVC may be partly or completely occluded by thrombus or tumor tissue [21, 62]. Flow discontinuity can be demonstrated between IVC and hepatic veins by Doppler ultrasound. In chronic cases, reversal of blood flow can be seen within hepatic veins. According to the degree of portal hypertension, portal vein blood flow direction can be hepatofugal, away from liver, or hepatopedal. In this case, determinant factor is the site and severity of occluded veins. During ultrasonographic examination, the normal variation of IVC caliber which occurs during respiration is lost. The IVC may detect reduced or no blood flow within the vessel. In nonobese patients, an echogenic membrane can be demonstrated in IVC. The development of collaterals and the demonstration of their presence are very important in determining the treatment modalities. These vessels can develop capsular or/ and intrahepatic areas carrying the blood via the caudate lobe into the IVC. In stable, chronic cases, if these collaterals develop, there is no need for additional shunt procedures. Doppler ultrasonography is very helpful during follow-up period particularly in patients who have undergone TIPS or shunt surgery to evaluate the patency of shunt.

Computed Tomography

In acute BCS, the features on CT imaging vary widely according to the involved vessels and location of the obstruction. An enlarged liver, ascites, and the lack of opacification of the hepatic veins and in homogenous liver with a mottled appearance particularly on contrast figures are the essential CT findings. As portal blood flow direction is reversed, the liver peripheral area can appear hypoattenuated, leading to atrophic liver in peripheral zone. In chronic cases, caudate-lobe hypertrophy (Fig. 48.3a), peripheral atrophy, and large multifocal regenerative nodules are the main findings. The porta hepatis, accordingly the portal vein, may be displaced anteriorly as long as the caudate lobe is hypertrophied (Fig. 48.3b). Although ultrasonography and CT are essential, in some cases, plain X-ray and histology (Fig. 48.3c, d) may be helpful. Regenerative nodules are benign and are usually larger than 5 mm in diameter. Due to their hypervascularity, contrast material enhances their appearance in the arterial phase of the procedure (Fig. 48.4a). It is difficult to visualize them on nonenhanced CT.

Venography

The femoral or jugular vein is the entry to the venous system. At the same session, it is possible to demonstrate the IVC and hepatic veins. Although it seems to be an invasive procedure, venography is the "gold standard" imaging technique in confirming the diagnosis. By this modality, occlusion by thrombus or tumor, a web in the IVC, and compression and lateral displacement of the IVC by an enlarged caudate lobe at the renal and hepatic level can be seen. Venography can demonstrate not only thrombosis, but also its level and dimen-

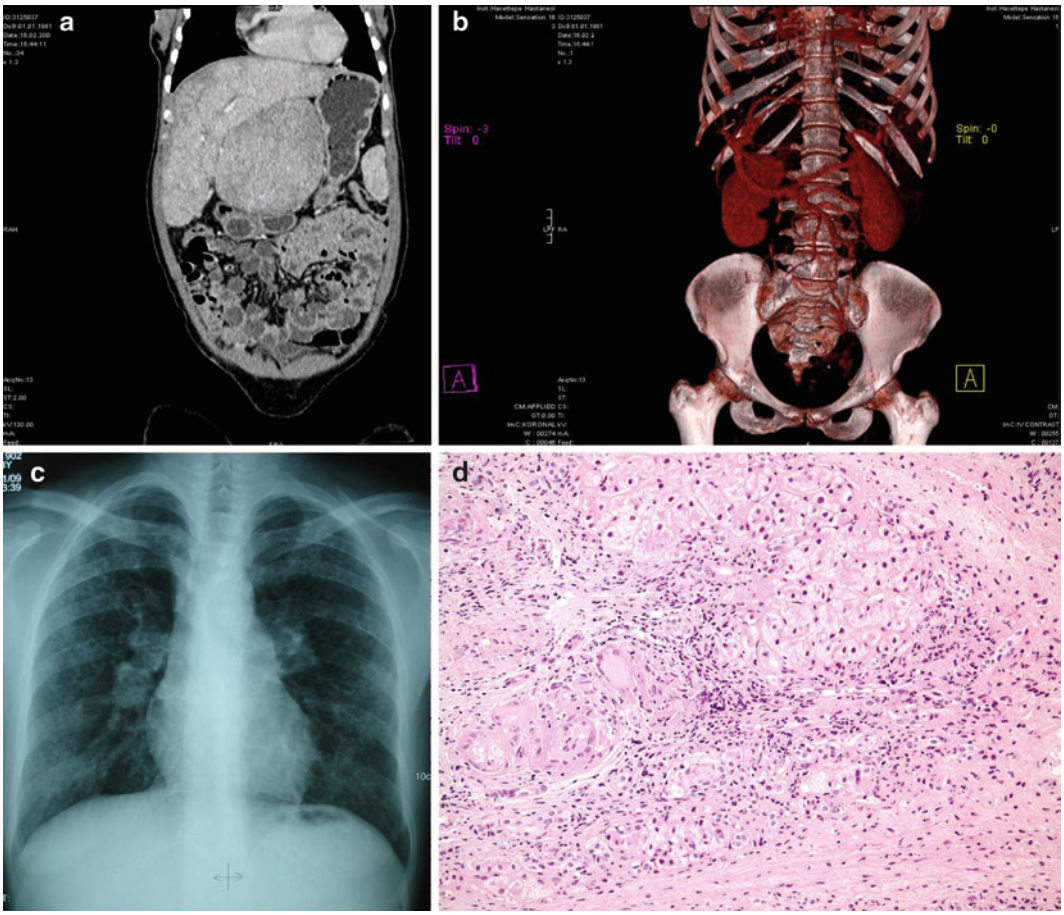

Fig. 48.3 Radiological and histological findings of a systemic sarcoidosis patient complicated with BCS. (**a**) Coronal computed tomography of the abdomen reveals a very large hypertrophied caudate lobe. (**b**) Three-dimensional reconstructed abdominal computerized tomography shows anterior displacement of the main portal vein by the caudate lobe. (**c**) Plain chest X-ray showing bilateral enlargement of hilar lymph nodes and diffuse nodular appearance in lung parenchyma. (**d**) Noncaseating granuloma formation occupying the whole field of the microphotograph of a liver biopsy (H&E ×230)

sion including other abnormalities such as caval aneurysm and developed collaterals (Fig. 48.4b). In selective hepatic venography, a spider-web pattern consisting of the many collaterals that form between hepatic venules and systemic veins and hepatic vein, stenosis often located near the orifice of the major hepatic veins are the main findings. When the roots of the hepatic veins are occluded, percutaneous transhepatic approach can be beneficial. By injection of contrast material into the liver parenchyma, the collateral channels can fill and spider-web appearance can be obtained.

Liver Biopsy

Liver biopsy may demonstrate congestion, hepatocyte necrosis, and fibrosis in the centrilobular area, findings that are similar to those seen in congestive hepatopathy. Liver biopsy is particularly necessary in the rare form of the syndrome where thrombosis is limited to the small intrahepatic vein, i.e., with a normal appearance of the large veins at noninvasive imaging [63]. It is necessary to stress that all features (coagulative necrosis and loss of hepatocytes) are located in the centrilobular area. These findings are also seen in veno-occlusive, cardiac, or pericardial diseases. Thrombosis of the hepatic veins is an uncommon finding in needle-biopsy specimens. Edematous and dilated central veins are the most dependable histopathological findings (Fig. 48.5a). In rare cases, such as sarcoidosis, granulomas can compress along the hepatic veins and its tributaries (Fig. 48.3d), leading to BCS.

Differential Diagnosis

In all congestive hepatopathy, VOD should be ruled out, in which there is thickening in the central venous wall with partial occlusion of the lumen (Fig. 48.5b). Particularly, clinicians must suggest CP, which is the most challenging diagnosis in every patient presented with ascites and hepatomegaly.

Treatment

The aim of management is to alleviate venous obstruction and prevent extension of thrombosis in the hepatic veins and preserve hepatic function by decreasing the centrilobular congestion. Diagnostic workup to identify the underlying cause for hepatic venous thrombosis must be considered at the time of initial diagnosis. Appropriate treatment of underlying disease, if any, should be started early. There are many recent advances in the understanding of myeloproliferative disorder and newly available drugs for PNH will lead to a clinical benefit for BCS patients. On the other hand, for many clinical conditions, such as antiphospholipid syndrome and hereditary thrombophilia, anticoagulant treatment remains the available treatment modality. Recombinant antithrombin is available for different patients, but only for use over a limited period of time [64].

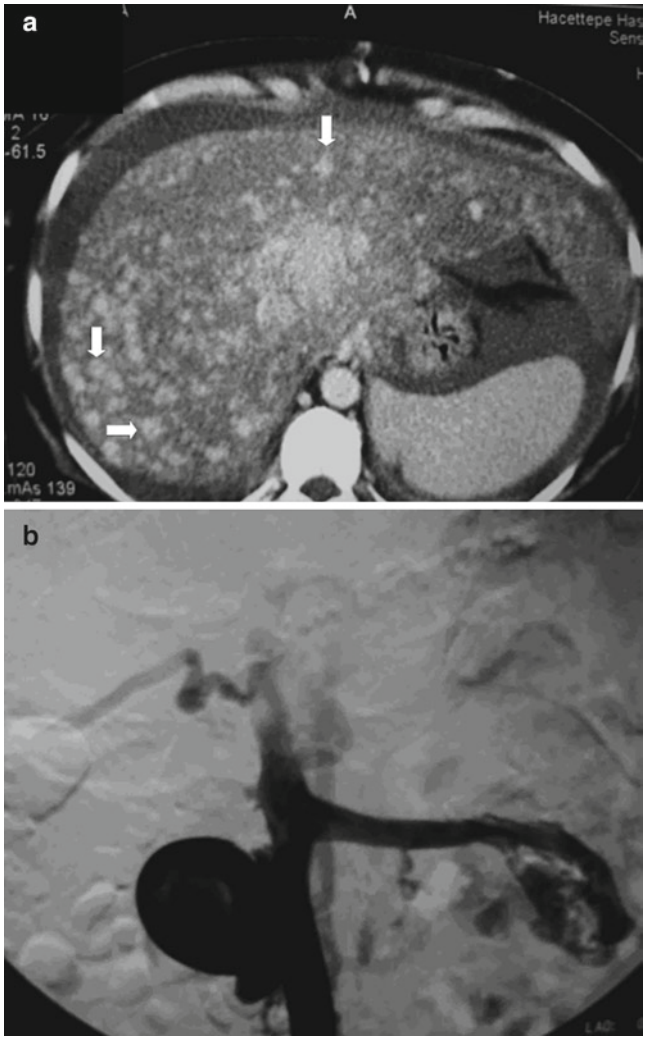

Fig. 48.4 (**a**) Contrast enhanced transverse CT in a patient with Behçet's disease complicated with Budd-Chiari Syndrome shows a background of low attenuation due to fatty infiltration and enhancing regenerative nodules (*white arrows*). (**b**) Inferior vena cavography shows total occlusion of the inferior vena cava, collaterals, and caval aneurysm

Medical Therapy

Medical treatment consists of efforts to control ascites, to prevent extension of thrombosis with anticoagulant therapy, to dissolve the clot and relieve hepatic congestion with thrombolysis, and to treat the complications. To start early, anticoagulation therapy is essential for all patients regardless of whether the underlying procoagulant condition has been clarified or not [63, 65]. Anticoagulation treatment will be sufficient in controlling the liver disease in 10% of patients, mainly those with mild disease [64]. A high incidence of thrombocytopenia induced by heparin has been reported with unfractionated heparin [64], and more recently with low molecular weight heparin [66]. These surprising findings have remained unexplained. However, it is safe to recommend that unfractionated heparin be avoided as it can be replaced by low molecular weight heparin for initiation of anticoagulation. There is no evidence that the efficacy of warfarin (vitamin K antagonist) differs from that of heparin. Although there is no well-accepted consensus on the type and duration of optimal anticoagulation therapy, most centers give lifelong anticoagulation, initially with heparin followed by warfarin, titrated to maintain INR between 2–3 [28, 67]. In choosing anticoagulation therapy, pharmacokinetic consideration and the acuteness of the condition will be the determinant factors.

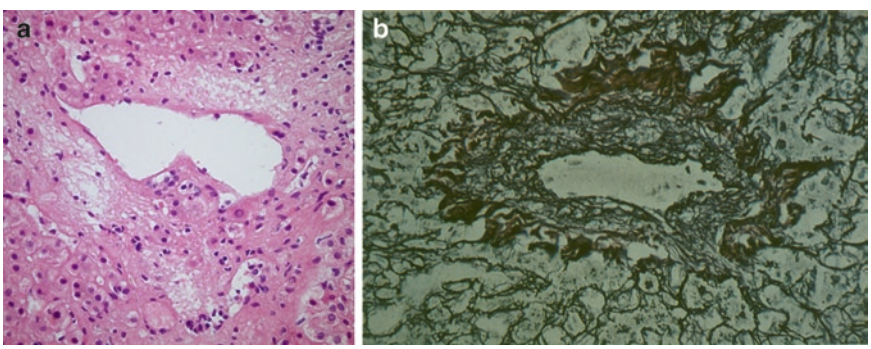

Fig. 48.5 (**a**) Histological examination shows dilated and edematous central vein in a patient with BCS (Magnification 230×; H&E). (**b**) Microphotograph reveals partial central vein occlusion and thickness in the wall in a patient with veno-occlusive disease (Magnification 230×; Silver impregnation)

Ascites is treated by low-sodium diet, diuretics, and therapeutic paracentesis when needed. During active variceal bleeding, balloon tamponade or endoscopic therapy, either ligation or sclerotherapy is preferred to vasoconstrictive agents because the splanchnic blood flow induced by vasoconstrictors can theoretically precipitate thrombosis of the portal vein and its tributaries. Medical treatment alone can be a good option for patients in whom there is no ongoing hepatic necrosis, as indicated by the presence of minimal symptoms, relatively normal liver function results, and ascites that is easily controlled [68]. In chronic cases with well developed collaterals, there is no need to use either vigorous medical treatment or radiologic intervention except anticoagulation treatment.

Restoration of Hepatic Blood Flow

The relief of venous obstruction is the essential part of the treatment of BCS. To reach this goal, thrombotic treatment, percutaneous angioplasty, TIPS, or surgery can be used. In patients with acute presentation, especially when angiography demonstrates presence of a fresh thrombus, thrombolytic therapy can be administered systemically or directly into the vein. Promising data has been reported with thrombolysis carried out 2–3 weeks after the onset of symptoms [69–71]. About a third of patients with BCS harbor short length stenosis located either in hepatic veins or in IVC. Such patients are the cases for percutaneous transluminal angioplasty. Balloon angioplasty has been used to relieve HVOO secondary to caval webs. Although short-term results are excellent, the sustained patency rate is only 50% at 2 years after the procedure [72]. However, the use of intraluminal stents has been shown to increase the long term patency to nearly 90% [73–75]. Once inserted, the stents cannot be removed, and placement of a stent above the intrahepatic IVC may interfere with liver transplantation. Failure of angioplasty or stenting should prompt consideration for a surgical portosystemic shunt or TIPS [74]. TIPS decompressed the liver by creating a second venous outflow route. It is particularly useful either alone or as a bridge to liver transplantation, in patients with an acute presentation such as those with variceal bleeding and patients with fulminant hepatic failure, in whom thrombolysis and angioplasty were unsuccessful [76, 77]. The procedure may be preferred over surgical shunting because it avoids laparotomy, and has less periprocedural morbidity and mortality. Its efficacy is not affected by caudate lobe hypertrophy, and it can be done in patients with PVT [78–80]. Although long-term patency rate is only about 50%, patients in whom stent stenosis occurs do not generally worsen, perhaps because the shunt allows time for collateral circulation to develop [81]. However, extension of the stent into the suprahepatic IVC may preclude liver transplantation and should be avoided. Available data show that in patients where the symptoms of the syndrome are not controlled by the other options, the next step should be TIPS insertion. The advent of covered stents has provided maintained patency for prolonged periods [82]. Improved survival rates in TIPS treated patients are most obvious among those with the most severe disease at baseline. In that subset of patients, 5-year survival rose from 45% in a reference cohort — where TIPS was only marginally used [51], to 71% in a series of TIPS treated patients [83].

To perform surgical treatment, pressure differences between portal vein and intrahepatic IVC must be more than 10 mmHg for portocaval and mesocaval shunt to function. Five years survival ranges between 57–94% and the absence of IVC occlusion is associated with better outcome [84–86]. In addition to shunts, other surgical procedures can also provide adequate decompression in selected patients. Transatrial membranectomy can be effective in patients with IVC membranes [86–89]. Dorsocranial resection of the liver is the only feasible procedure, if there is both portal and superior mesenteric vein obstruction [90].

Patients who have decompensated cirrhosis, fulminant hepatic failure, or biochemical evidence of advanced liver dysfunction are the best candidates for liver transplantation. Two recent retrospective analysis of the outcome in large series of transplanted patients have shown that 5-year survival rates have reached 80% [91, 92]. Development of postoperative portal-vein thrombosis occurred in about 12% of patients. Liver transplantation can cure almost any hereditary thrombophilia; however, thrombosis can still occur and anticoagulation is necessary [78].

The summary of the treatment strategy of Primary BCS cases: Anticoagulant drugs must be started vigorously as low molecular weight heparin and then shift to oral anticoagulation as soon as possible aiming INR 2–3. Also, it is essential to consult with a hematologist to evaluate MPD. If they exist, start cytoreductive treatment immediately.

Veno-Occlusive Disease (Sinusoidal Obstruction Syndrome)

VOD, or sinusoidal obstruction syndrome, is characterized by tender hepatomegaly, fluid retention, weight gain, and jaundice. High dose abdominal radiotherapy, use of certain chemotherapeutic agents, ingestion of pyrrolizidine, and hematopoietic stem cell transplantation (HSCT) can cause this condition. In 1954, Bras et al described VOD in a Jamaican child who developed occlusion of the tributaries of the hepatic veins with subsequent centrilobular, or nonportal fibrosis associated with poisoning with senecio-like alkaloids [93]. The term sinusoidal obstruction syndrome was introduced in 2002 by DeLeve et al to replace VOD based on their studies demonstrating the primary site of injury on the SEC, which results in fibrosis and obstruction of venous outflow [94].

As HSCT is the most common cause of VOD, we will focus on VOD resulting from this transplantation.

Clinical Presentation

In most patients, clinical signs and symptoms of VOD are seen within the first three weeks of HSCT. The most dependable signs are suddenly developed weight gain and hepatomegaly. Hyperbilirubinemia is seen after a few days of increased serum transaminase and ALP levels. Acute congestion of the liver causes right upper quadrant pain. Physical examination usually reveals a tender hepatomegaly and ascites in 83% and 39% of patients with histologically proven VOD, respectively. Patients with VOD may develop renal failure as a result of hepatorenal syndrome. The prevalence of renal, cardiac, and pulmonary failure was noted to be higher in patients with VOD than those without VOD in a report of 355 patients who underwent HSCT [95].

As the overall mortality is reported to be between 3–67% [96, 97], the severity of VOD can be classified as follows: (1) mild (requiring no treatment and with complete resolution); (2) moderate (requiring treatment with diuretics, and pain relievers and with complete healing); (3) severe (requiring treatment and no resolution before death or by transplant). Patients with the severe form differ from those with mild and moderate form in the amount of weight gained, increment speed of total serum bilirubin level, and frequency of edema and ascites.

Risk Factors

The following are the risk factors of VOD: the presence of liver injury prior to HSCT, prior radiotherapy to the abdomen, the presence of hepatic metastases of solid tumors, and hepatic infection. Additionally, advanced age and previous HSCT may be associated with high incidence of VOD. It is believed that the incidence of VOD is higher after allogenic stem-cell transplantation than after autologous stem-cell transplantation. Additionally, patients receiving stem cell from unrelated or mismatched donors have a higher risk of developing VOD.

Histology and Pathophysiology

DeLeve et al reported that the initial histological changes in murine models of VOD were the loss of SEC fenestrae and appearance of gaps in SEC barrier [98]. A more detailed account on SECs is provided in Chap. 7. Other early histological findings were narrowing of the sublobular and central veins due to subendothelial edema, likely secondary to disruption of SEC barrier and congestion of hepatic sinusoids surrounded by pale necrotic hepatocytes [3]. Fragmented red blood cells, factor von Wilebrand, and fibrinogen can be found in the subendothelial space of the central vein, making this lumen more narrowed and perivenular zones of hepatic acini [99]. In the later period, sinusoidal and venous lumen become obliterated by type I, III, and IV collagen accompanied by increase in stellate cells that line the sinusoids [99, 100]. It is believed that there is a depletion of liver glutathione reserve. In this condition, liver's ability to detoxify acrolein, an inactive but hepatotoxic metabolite of cyclophosphomide is diminished. SECs are prone to toxic effects of acrolein because of their lower glutathione levels compared to hepatocytes [101, 102]. It is well known that drugs are metabolized mainly in zone 3 hepatocytes which are rich in p450 enzymes, which is why the initial morphologic changes are seen in SECs in zone 3. Glutathione may also suppress expression of matrix metalloproteinase- 9 in murine models [94]. Additionally, an immunologic mechanism may have a pathogenic role in VOD.

Treatment

The use of diuretics and sodium restriction are necessary in most patients. If ascites causes abdominal discomfort or pulmonary compromise, repeated paracentesis may be necessary. Intravascular volume and renal perfusion should be monitored. Administration of antithrombin III concentrate might decrease the mortality in patients with severe VOD [103, 104]. Clinical improvement was seen in all patients after 5 days of AT-III infusion. Prostaglandin E and glutamine/vitamin E have been used in some cases with success. TIPS in isolated cases seem to be beneficial. Liver transplantation has been attempted successfully in a few patients [105–107].

Conclusion

Clinical manifestations and liver histology are similar in CH, BCS, and VOD because of common underlying mechanisms of hepatic injury and adaptation in response to increased sinusoidal pressure. The severity of the clinical picture depends mostly on the rapidity and extent of the obstructive process. Physicians should consider congestive hepatopathy and BCS in all patients with tender hepatomegaly and ascites. Presence of jugular venous distention is an invaluable clue to differentiate congestive hepatopathy from BCS and other primary liver diseases. VOD should be kept in mind in patients with

rapidly developing ascites and jaundice with hepatomegaly after HSCT. The advent of interventional procedures has improved survival in patients with HVOO, although prevention is still the mainstay in management of VOD.

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Chapter 49
Primary Biliary Cirrhosis

Carlo Selmi and M. Eric Gershwin

Introduction and Clinical Features

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by high-titer serum antimitochondrial autoantibodies (AMA) and an autoimmune-mediated destruction of the small and medium sized intrahepatic bile ducts. PBC is a peculiar, yet representative, autoimmune disease from a clinical standpoint [1] and our knowledge on its pathogenesis fits well into this book. The disease affects women more frequently than men with a female to male ratio of 10:1 and the average age at diagnosis is within the fifth and sixth decades of life, with exceptional cases described in pediatric ages.

The diagnosis of PBC is confirmed when two out of three internationally accepted criteria are fulfilled, that is, presence of serum AMA, increased enzymes indicating cholestasis (i.e., alkaline phosphatase) for longer than 6 months, or a compatible or diagnostic liver histology [2]. Clinical symptoms include fatigue, pruritus, and jaundice; yet the impact, specificity, and etiology of fatigue in PBC remains debated [3]. Indeed, the increasing availability of serological tests for routine AMA has significantly changed the spectrum of disease presentation. In fact, earlier reports were based on a large preponderance of advanced cases with jaundice while patients are now diagnosed most frequently at asymptomatic and early stages [4].

The need for performing a liver biopsy when PBC is diagnosed, remains debated [2] and the procedure is currently indicated only in patients lacking one of the non-invasive criteria, patients requiring accurate staging (although the possibility of sampling errors should be accounted), or in patients enrolled in clinical trials. Discriminating PBC from other autoimmune or inflammatory liver diseases is usually easy, mostly based on serum autoantibody profiles. PBC liver histology can be classified into four stages [5]. At earlier stages, bile duct obliteration and granulomas (possibly found at all stages) are strongly suggestive of PBC. Stage III demonstrates septal or bridging fibrosis, with ductopenia (over half of the visible interlobular bile ducts having vanished), while stage IV corresponds to frank cirrhosis virtually undistinguishable from end-stage liver diseases of different etiologies. Peculiar characteristics of PBC that can be found at any histological stage include epithelioid granulomas with no signs of caseous necrosis, such as in tuberculosis.

PBC results from an environmental insult acting on a genetically susceptible background. Over the past decade, we have witnessed the development of several lines of experimental evidence on the mechanisms ultimately leading to the cell injury observed in PBC, including the role of autoantibodies, autoreactive cells, and more recently, innate immunity. This chapter will discuss what is known about the causes of onset and perpetuation of immune-mediated injury in PBC.

Sex Factors and PBC

Among autoimmune conditions, PBC, Sjögren's syndrome, systemic lupus erythematosus, autoimmune thyroid disease, and scleroderma manifest the highest female predominance, with 80% of the patients being women. Three main hypotheses to explain this observation have been proposed, that is, sex hormones, fetal microchimerism, and sex chromosome haploinsufficiency (Table 49.1) [6–17].

Sex hormones (i.e., estrogens, androgens, and prolactin) have been the first proposed candidates in the sex bias observed in autoimmunity, due to their modulatory functions within the immune response, particularly acting on the development of immune cells. Sex hormones may also directly influence the homing of lymphocytes to a target organ and the process of antigen presentation, thus influencing the organ specificity of AID as well as the breakdown of tolerance. The production of several cytokines including IFN-γ, IL-1, and IL-10 is enhanced

C. Selmi (✉)
Division of Internal Medicine and Hepatobiliary Immunopathology Unit of Internal Medicine,
IRCCS Institute Clinico Humanitas, University of Milan,
Rozzano, Milan, Italy
e-mail: carlo.selmi@unimi.it

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_49, © Springer Science+Business Media, LLC 2011

Table 49.1 Proposed mechanisms influencing female predominance in autoimmune diseases [141]

Autoimmune disease	Female:male ratio	Sex hormones	X chromosome
Systemic lupus erythematosus [10–14]	9:1	Estrogen allows survival of autoreactive B cells and skews their maturation toward marginal zone phenotype Prolactin allows survival of autoreactive B cells and skews their maturation toward marginal zone phenotype, leads to the production of IFN-γ	Triplication of PAR1 region Duplication of TRL7
Primary biliary cirrhosis [6, 7]	10:1	Use of hormone replacement therapies is significantly associated with increased risk of PBC	Increased frequency of X chromosome monosomy in peripheral T and B lymphocytes
Autoimmune thyroid diseases [8, 12, 15]	8:1	Hyperprolactinemia (undefined)	Increased frequency of X chromosome monosomy in peripheral T and B lymphocytes and skewed XCI
Systemic sclerosis [8, 9, 12]	5:1	Estrogens induce fibroblast dysfunction Hyperprolactinemia (undefined)	Increased frequency of X chromosome monosomy in peripheral T and B lymphocytes and skewed XCI
Rheumatoid arthritis [12, 16]	4:1	Estrogen activates synovial-cell proliferation, including macrophages and fibroblasts. TNFα blockers affect estrogen synovial levels	
Multiple sclerosis [12]	3:1	Estrogen determines disease improvement; progesterone seems to have an effect on myelinating and remyelinating the nervous system.	
Myasthenia gravis [17]	2:1	Estrogen promotes AChR-specific Th1 cell expansion	

in vitro by estrogens, while IL-4 and IL-5 levels are reduced in the presence of androgens. The effect of estrogens is different in normal conditions and in autoimmunity with a biphasic effect and lower levels facilitate the immune response, while higher levels suppress it. For example, the effect of estrogens on the secretion of TNF-α is biphasic, with enhancement occurring at low and inhibition at high concentrations. These data indicate that estrogens can modulate both pro- and anti-inflammatory activities of CD4+ T cells and thus have the potential to influence the outcome of CD4+ T cell-mediated immune responsiveness. As a result of these observations, it should be clear that estrogens may be central to the regulation of the balance of Th1/Th2 cytokines within sites of inflammation, and to the appropriate or inappropriate termination of the inflammatory response in infections, tolerance development, or autoimmunity. Several authors have attempted to study sex-hormone changes in women with PBC. These have included epidemiological studies in which a negative association with parity was first denied and ultimately confirmed [6]. Of interest, taking hormonal replacement therapies following menopause was found in this latter study to be significantly associated with PBC, although this may be secondary to the proposed enhanced rate of bone loss in chronic cholestasis [6].

A second hypothesis on female predominance is the persistence of fetal DNA in women following pregnancies (i.e., fetal microchimerism). Microchimeric cells were first found in peripheral blood mononuclear cells from patients with systemic sclerosis and it was suggested that non-autologous cells may be mediating a graft-versus-host disease-like reaction in these patients, but other studies have failed to recapitulate these findings. Several studies found no significant difference in frequency of male microchimerism in female PBC and controls [18]. The available data on the role of fetal microchimerism in autoimmunity in general is still controversial and should be regarded as negative in PBC.

One new fascinating hypothesis on PBC female predominance is based on major defects of sex chromosomes [19]. This theory relies mainly on two observations. First, several genes that are key factors in the maintenance of immune functions and tolerance are located on the X chromosome and, second, diseases associated to constitutive X monosomy or its major structural abnormalities, such as Turner's

syndrome, are frequently associated with autoimmune features and in some cases to chronic cholestasis. X chromosome inheritance displays a peculiar pattern compared to autosomal chromosomes, since women are functional mosaics for X-linked genes. In females, most genes on one X chromosome are silenced as a result of X-chromosome inactivation (XCI). The result of XCI is to achieve equivalent levels of X-linked gene products between males and females. More recent data have mined this long-standing dogma by demonstrating that approximately 15% of X-linked genes are capable of escaping XCI in healthy women [20]. As a result of these observations, a role for X chromosome was first proposed based on experimental evidence that women with autoimmune diseases have a significantly higher frequency of peripheral blood cells with a single X chromosome (i.e., X monosomy) compared to healthy women and this was confirmed in diseases with different organ specificities including PBC [7], scleroderma, and autoimmune thyroid disease [8]. X-chromosome loss is indeed preferential and involves more frequently a parentally inherited one [21], suggesting a possible critical involvement of X-chromosome gene products defects in female preponderance of PBC and other autoimmune diseases, while new factors such as microRNAs [22] or epigenetics [23, 24] should not be overlooked. Other authors have suggested that women affected with specific female-preponderant autoimmune diseases, that is, scleroderma, manifest a skewed XCI pattern in peripheral blood mononuclear cells [9]. Our group failed to demonstrate a preferential inactivation in PBC using four different methylation-prone loci [21]. In a complementary fashion to the X chromosome, its Y counterpart has received significantly less attention in experimental research, possibly due to its limited number of genes. This assumption changed when the genetic bases of the long-known BXSB strain of mice were demonstrated; this model was first found to spontaneously develop a lupus-like autoimmune syndrome affecting male mice with a more aggressive phenotype compared to females [25], secondary to genetic abnormality present in BXSB Y chromosome coined Y-linked autoimmune acceleration (Yaa) [26]. The Yaa mutation is a translocation from the telomeric end of the X chromosome (containing the gene encoding the toll-like receptor [TLR]-7) onto the Y chromosome [10] causing the gene overexpression [27] although its duplication alone is not sufficient to cause Yaa-mediated acceleration of lupus-like disease [28]. Putative mechanistic views are well represented by the higher induction of a type 1 cytokine response by TLR-7 stimulation in female peripheral cells [29]. Suggested candidates include the TLR-8 gene which contributes to the development of a drug-induced humoral autoimmune response mimicking human systemic lupus erythematosus [30]. In the absence of translocation, the Y chromosome may play a protective role in the development of autoimmunity and explain the female predominance as well as the occurrence of autoimmune diseases in men.

PBC Immunobiology

PBC is a model and a paradox for other diseases [31, 32] sharing common features commonly found in autoimmunity, such as female predominance, multifactorial genetic predisposition, presence of autoreactive T cells (CD4 and CD8), and disease-specific autoantibodies, that is, AMA and PBC-specific anti-nuclear antibodies (ANA) (Table 49.2). However, such autoantibodies represent the basis for PBC being a paradox, as their direct pathogenetic role remains poorly defined. Serum autoantibodies are detected in approximately 90% of patients, yet seronegative cases manifest similar histological features and disease progression [33]. Furthermore, most autoimmune diseases are responsive to immunosuppressive therapy, while no such agent has proven effective in PBC [34].

Antimitochondrial Antibodies

Circulating AMA are highly specific for PBC and are detected in approximately 90–95% of patients, when tested using techniques based on recombinant mitochondrial antigens (immunoblotting or ELISA). Conversely, the test specificity and sensitivity is significantly reduced when routine indirect immunofluorescence (Fig. 49.1) is used. The high sensitivity and specificity of AMA make them one of the most specific diagnostic tests of human immune-pathology [35]. AMA specifically recognizes lipoylated domains within components of the 2-oxoacid dehydrogenase (2-OADC) family of enzymes within the mitochondrial respiratory chain.

Table 49.2 Autoantigens for serum AMA and ANA in PBC and the relative specificity and prevalence rates

	Molecular targets (pattern)	Detection systems	PBC-specific	Prevalence in PBC (%)
ANA	Sp100, PML, SUMO (nuclear dots)	IIF	++	30–50
	gp210, NUP62 (rim-like)	IIF	++	30–50
	Anti-centromere	IIF	–	20
AMA	E2 subunits of 2-oxo-acid dehydrogenase complexes, mainly PDC-E2	IIF ELISA, IB	+++	85–95

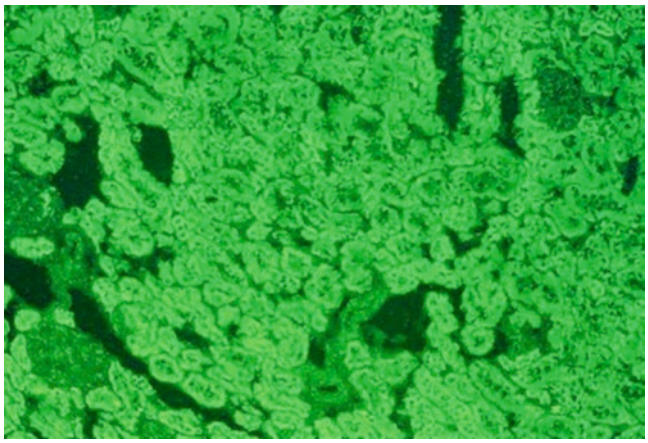

Fig. 49.1 Antimitochondrial antibodies pattern: Typical reactivity to the proximal and distal tubules of the rat kidney (40× original magnification)

All immunodominant epitopes contain DKA motif, with lipoic acid attached to lysine (K), which is necessary and/or sufficient for antigen recognition [36], as explained in further details below. Amongst the 2-OADC constituents, the major autoantigen recognized is the E2 subunit of pyruvate dehydrogenase complex (PDC-E2). Less frequent autoantigens are the E2 components of 2-oxo glutarate dehydrogenase (OADC-E2) and branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) complexes, and the E3 binding protein (E3BP) [37, 38].

Antinuclear Antibodies

PBC-specific serum ANA are detected in as many as 50% of patients and are defined by the immunofluorescence pattern, i.e., multiple nuclear dots (MND) and rim-like (RL) pattern. MND reactivity is based on the recognition of Sp100 and PML (possibly also cross-reacting with small ubiquitin-like modifiers, SUMO) [39]. RL reactivity reacts against proteins of the nuclear pore complexes (NPCs) that include gp210 (a 210-kDa transmembrane glycoprotein involved in the attachment of NPC constituents within the nuclear membrane), nucleoporin p62 (a glycoprotein located on the core of NPC), and the inner nuclear membrane protein lamin B receptor (LBR). Serum anti-gp210 antibodies are detected in about 25% (range 10–40%), while anti-p62 and anti-LBR antibodies are found in about 13 and 1% of patients with PBC, respectively. Unlike AMA, cross-sectional and longitudinal data demonstrated an association between the presence of PBC-specific ANA, especially anti-NPC antibodies and a worse disease prognosis [40, 41]. Whether ANA exert a direct pathogenetic effect remains to be determined.

Autoreactive T Cells

T helper (CD4+), TCR αβ+, and CD8+ T cells are most commonly seen in portal tracts, in particular, around damaged bile ducts in the liver tissue of PBC patients, strongly suggesting the involvement of cellular-immune mechanisms in the biliary damage [42–49]. In the past decades, the nature and the role of cellular adaptive immune response in PBC have been extensively characterized, showing that both CD4 and CD8 T cells participate in liver-tissue damage. Autoreactive CD4 T cells specifically targeting PDC-E2-self-antigen have been reported both in peripheral blood and liver of PBC patients but not in controls. There is a specific 100–150 fold increase in number of PDC-E2-specific CD4 T cells in the hilar lymph nodes and liver, when compared with peripheral blood of patients with PBC, thus further supporting their role the liver damage. Shimoda et al. characterized their antigen specificity, showing that in *HLA DR4*0101* positive PBC patients, a single epitope, that is, 163–176 aa sequence, which encompass the lipoic acid binding residue of the inner lipoyl domain of PDC-E2, is the immunodominant epitope recognized by autoreactive CD4 T cells. Functionally, these cells in PBC patients but not in controls are of pro-inflammatory nature, as demonstrated by their ability to produce pro-inflammatory cytokines such as IFN-γ [44].

Autoreactive CD8 T cells have also been well characterized in PBC, being currently considered the major effectors of the adaptive immunity in the tissue injury encountered in PBC. The HLA class I restricted epitope for CD8 T cells, namely the 159–167 aa sequence, maps in close vicinity to the epitopes recognized by CD4 T cells as well as by AMA. It is of note that the autoepitope for T cells, both CD4 and CD8 T cells, overlaps with the B cell (AMA) counterpart and includes the lipoylated amino acid of the inner lipoylated domain. Similar to CD4 autoreactive T cells, the recent use of tetramer technology has shown a tenfold higher frequency of PDC-E2159-167 specific CD8 T cells within the liver as compared to peripheral blood of patients with PBC. Moreover, the precursor frequency of PDC-E2-specific autoreactive CD8 T cells is shown to be significantly higher in early rather than late stage of the disease. Functionally, it has been shown that autoreactive CD8 T cells in PBC have specific cytotoxicity against PDC-E2 antigen as well as the ability to produce IFN-γ rather than IL-4/IL-10 cytokines [50].

Innate Immune Cells

Adaptive immune system recognizes and responds to antigens via highly specific T-cell receptors, while innate immunity recognizes distinct evolutionarily conserved structures

usually shared by invading pathogens termed pathogen-associated molecular patterns (PAMPs), allowing high efficiency for rapid recognition and elimination of viruses, bacteria, and fungi. PAMPs engages pattern-recognition receptors such as TLRs expressed on cells of the innate immune system, that is, monocytes, dendritic cells, and natural killer (NK), all of which are therefore able to modulate the function of adaptive humoral and cellular immunity. Only recently, study of innate immunity as potential activator of autoimmune response has received a significant impetus and is no longer overlooked by clinical immunologists [51]. Liver has been recognized both structurally and functionally as a major organ of innate immunity. In the liver, resides one of the largest resident populations of cellular components of the innate immune system, including NK and NKT cells. Functionally, liver constantly expresses effective immune responses against a wide range of pathogens from viruses to multicellular parasites. In that sense, the ambience of an inflammatory milieu seems to be critical for effective immunogenic signals to be delivered to intra-hepatic T cells. Mounting evidence shows that innate immunity likely contributes to the initiation and/or progression of liver damage. PBC exhibits specific immunological features, such as presence of epithelioid granulomas, elevated levels of polyclonal IgM, hyper-responsiveness to CpG, increased levels of NK cells and cytokines response that strongly suggests a crucial role of innate immunity in its pathogenesis (Table 49.3).

IgM are commonly elevated in PBC, independently to AMA or ANA status [1], and their reduction is usually observed during treatment [52]. Our group reported that polyclonal hyper-IgM is secondary to a chronic polyclonal innate immune response of memory B cells to specific bacterial PAMPs such as unmethylated CpG motifs [53]. In this study, following stimulation with synthetic oligodeoxynucleotides containing CpG motifs, CD27+ memory B cells in cultured PBMCs from patients with PBC, secreted significantly higher amounts of polyclonal IgM compared to controls. In a subsequent study, our group reported that B cells in PBMCs exposed to CpG motifs express increased amount of TRL9 and CD86 as well as greatly enhance their production of AMAs. This evidence strongly suggests a profound disease-specific dysregulation of B cells, and supports the proposed link between bacteria and PBC pathogenesis. In this scenario, B cells become hyper-responsive to innate stimuli, such as microbial CpG motifs, and therefore contribute to the perpetuation of the autoimmune process, possibly via IgM [54].

Table 49.3 Innate immunity changes observed in PBC

	Peculiarity
Monocytes	Increase in absolute number
	Increase in pro-inflammatory cytokines upon infectious challenge
Elevated IgM	B-cell response to bacterial stimuli
NKT cells	Increased NKT cells in PBC peripheral blood and liver
	Increased NKT cytotoxic activity
Liver histology	Focal duct obliteration with granuloma formation

Monocytes have also been implicated in the pathogenesis of PBC, their pro-inflammatory activity being greatly enhanced in PBC. Functionally, monocytes, once activated by PAMPs through TRLs, are able to release pro-inflammatory cytokines, including IL-1, IL-6, IL-18, IL-12, and TNF-α that are able to amplify adaptive T cell mediated immune response against pathogens. Our data have shown that peripheral monocytes from patients with PBC challenged with different ligands for TLR2, TLR3, TLR4, TLR5, and TLR9 produce a significantly increased level of all pro-inflammatory cytokines compared to healthy controls [55]. From the innate immunity perspective, these findings suggest that peripheral monocytes from patients with PBC are more sensitive to infectious stimuli resulting in secretion of pro-inflammatory cytokines. The mechanisms for such increased sensitivity are currently unknown, but might reflect or be secondary to the higher frequency of recurrent Gram-negative bacterial infections (e.g., urinary tract infections) in PBC.

Therefore, both B cells and monocytes constantly exposed to bacterially derived products from portal blood could participate in the modulation of adaptive cellular immune response and possibly also in its priming.

The role of NKT cells in autoimmunity is also attracting growing attention [56]. NKT cells are innate effector cells, which are regulated by self and non-self glycolipid antigens presented by the antigen-presenting molecule CD1d. This activation allows a rapid NKT cells production of effector cytokines and chemokines, thus modulating both innate and adaptive immune responses. The first evidence of a possible involvement of NKT cells in the pathogenesis of PBC comes from our study reporting a higher frequency of CD1d-restricted NKT cells in PBC patients compared to healthy individuals, and as was the case for autoreactive T cells, CD1d-restricted NKT were more frequent in the liver compared to peripheral blood in PBC patients [49]. Chuang et al. more recently confirmed the increased number of CD1d-restricted NKT cells in the liver of dnTGFβRII mice, our PBC mouse model, compared to controls. He went on investigating the function of such CD1d-restricted NKT, showing an increased IFN-γ production in hepatic CD1d-restricted NKT after exposure to α-galactosylceramide (α GalCer) that represents NKT specific ligand. He also reported that CD1d-deficient dnTGFβRII mice had a decreased hepatic lymphoid-cell infiltrates and milder cholangitis compared to controls [56]. We are well aware that innate immune system hyper-responsiveness is likely not sufficient for immune tolerance breakdown, yet we can hypothesize that these alterations might play a role in the initiation and/or perpetuation of the

autoimmune injury. This is particularly intriguing considering the study by Mattner et al., who demonstrated that in a murine model of PBC (discussed below), *Novosphingobium aromaticivorans* was capable of inducing autoreactive AMAs and chronic T cell-mediated autoimmunity against small bile ducts in an NKT-dependent fashion [57].

T Regulatory Cells

$CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) are known to play a role in the prevention of autoimmune disease as demonstrated in several clinical settings. As an example, in chronic autoimmune hepatitis, therapeutic attempts are ongoing in animal models based on cell expansion [58]. In fact, some studies have demonstrated that the transfer of T cells lacking the $CD4^+$ $CD25^{high}$ Treg subset into athymic nude mice results in the development of various T cell-mediated autoimmune diseases. Experimental data demonstrate that PBC patients displayed significantly lower frequencies of $CD4^+$ $CD25^{high}$ Tregs as percentages of total TCR-$\alpha\beta^+$/$CD4^+$ T cells, which may contribute to the breakdown in tolerance in PBC [59]. The recently defined field of CD8+ FoxP3+ regulatory cells has not been investigated in PBC.

Individual Susceptibility to PBC

Similar to other autoimmune diseases, the pathogenesis of PBC should be regarded as complex or multifactorial, with multiple genetic and environmental factors interplaying to determine disease susceptibility and onset [60].

Genetic Considerations in PBC

It is now clear that PBC genetic components are not related to a single gene mutation nor the disease presents a complete penetrance as indicated by data from monozygotic twin sets. Further, PBC is a complex disease that should be defined as rare, based on its prevalence in the general population (the highest reported being 402 cases per million [4]).

Epidemiology strongly supports the genetic bases of PBC and concordance rates in monozygotic and dizygotic twin pairs estimate the weight of genetic and environmental factors in the susceptibility to this complex disease. The concordance rates in monozygotic twins for autoimmune diseases range between 12% for rheumatoid arthritis [61] and 83% for celiac disease [62] and are on average well below 50%. Until recently, only two case reports addressed the issue for PBC, with one describing a concordant [63] and one a discordant [64] pair defined as monozygotic, although in both cases zygosity was not genetically proven. We have recently reported that concordance rates for PBC are 63% in monozygotic and null in dizygotic twins, with three out of eight monozygotic twin sets being discordant for disease occurrence [65]. Further, in some cases, the natural history of PBC as well as the comorbidities could vary significantly between concordant twins. The phenotypical discordance is particularly interesting as it could be caused by epigenetic factors, differences in exposure to environmental factors, as well as mere serendipity.

In more general terms, family members of patients with PBC have a higher risk of developing the disease and the occurrence of multiple cases within the same family are referred to as "familial PBC" [66] presenting variable frequency rates, possibly following a geoepidemiological pattern. Studies from the UK indicate that such frequency ranges between 1–2.4% [67, 68], possibly increasing over the past decade, likely for the awareness of physicians and novel diagnostic tools. More recently, a population-based study from Minnesota [4] identified 46 cases of PBC and reported prevalence among family members higher than the general population. In the most recent series from the UK, 6.4% of patients with PBC had one familial case, in 1% of cases first-degree relatives were studied leading to a 10.5 sibling relative risk [69, 70]. Finally, we have recently demonstrated that patients with PBC from the genetically peculiar population of Iceland did not present similar anti-mitochondrial patterns while only one affected relative could be identified among 85 first-degree relatives [71].

Until recently, data on the genetics of PBC were derived solely from case-control studies designed to determine associations between specific polymorphic genes and the disease [72]. This approach, albeit meritorious, raised some concerns due to its limits. In fact, association studies present several degrees of potential bias, including those caused by sample size and selection and control matching criteria. For example, patients are often enrolled through tertiary referral centers and possibly over-represent the more advanced spectrum of PBC. As for controls, it is critical that these are matched also by race and geographical background to cases, to minimize false positive results based on population structure differences. The choice of candidate genes warrants further discussion; in most cases it is indicated by immunological features of the disease, as in the case of MHC, or is often derived from data in other autoimmune conditions. Both these methods present several strengths; however, we note how the selection of genes with a solid rationale should in all cases be a priority in association studies. To avoid false associations, all putative association results should be reproduced. It is disappointing to consider that the vast majority of proposed associations in complex diseases would fail this

test [73]. Two more issues need to be addressed. First, there needs to be correction for multiple testing. Recent utilization of statistical approaches such as false discovery rate can obviate some of the problems with the conservative Bonferroni correction that may cause type 1 errors [74]. Lastly, one should also consider the publication bias that favors weakly positive associations over negative, yet rigorous studies thus often causing a duplication of efforts that could be easily avoided. A most recent multi-center study reported the first genome-wide association study and identified interleukins (IL) 12 and its relative receptor as susceptibility genes for PBC and may require additional validation [75]. Among previous association studies, human leukocyte antigens of all three classes were largely predominant and will be discussed first.

Association Studies

In the past, studies performed on small series of subjects (between 21–75) have examined the association between HLA class I molecules and PBC susceptibility [76–82], but the conclusions of such earlier reports were affected by several major flaws. First, the small patient populations evaluated in these studies may account for a limited statistical power of comparisons. Second, technical methods available at the time only allowed the analysis of limited members of HLA class I alleles, therefore raising the possibility of existing associations being underestimated. Nevertheless, our group reported that PBC is associated with various HLA-B alleles in a small proportion of the patients studied [83]. It is possible that positive association might be secondary to *linkage disequilibrium.* Based on the available data, we should therefore regard PBC associations with HLA class I genes as weak and limited to patient subpopulations [84].

HLA class II alleles have been also widely studied in association with PBC in Caucasian and Asian series of patients. In 2001, data from Newcastle, UK, demonstrated that the linkage of DQA1*0401 and DR8-DQB1*0402 is associated with PBC progression and not susceptibility [85]. This association was not confirmed in several other studies from different non-British European populations. The most recent study from the United States demonstrated an association between DRB1*08-DQA1*0401-DQB1*04 haplotype and PBC, albeit in a minority of patients [86]. Finally, studies from Japan failed to provide a consistent picture of HLA class II associations with PBC. Transporters associated with antigen processing (TAP1 and TAP2) also belong to the HLA class II family and have been studied in patients with PBC, since their products determine the transport of antigenic peptides across the endoplasmic reticulum prior to being loaded onto MHC molecules. However, possibly also due to the limited polymorphic nature, no association with PBC onset was found [87]. In summary, we can conclude that the picture of HLA class II involvement in PBC is quite complex. We could somehow assume that, similar to epidemiological data, the genetic background in PBC should also be regarded as following a geographical pattern.

Data from association studies of polymorphisms of tumor necrosis factor alpha (TNF-α) in PBC are conflicting and a cautious interpretation is encouraged [88]. A polymorphism of the gene promoter region produces the more frequent variant TNF1, and the less frequent variant TNF2 [89], with TNF2 associated with increased transcription. The prevalence of the TNF2 allele was reportedly protective against PBC onset [90], while two other studies independently failed to detect any difference in genotype distributions between patients and controls [70, 91]. We note that in the study from Tanaka et al. heterozygous patients had a significantly worse prognosis compared to homozygous TNF1/TNF1 patients [91], as indicated by higher Mayo score value, currently the only validated index for PBC. However, a study from Newcastle, UK, did not confirm this alleged association [70].

Numerous studies of non-HLA SNPs in PBC have been dedicated to molecules involved in regulating the immune response, thus hypothesizing that genomic differences at these levels might confer susceptibility to the loss of tolerance or to an aberrant immune response. Based on the expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) by T cells following activation and the regulatory effect of this molecule on peripheral T cell responses, SNPs of CTLA-4 were suggested as factors facilitating the breakdown of tolerance. Accordingly, the coding A49G SNP was found associated with PBC in a large British study [92] and in 77 Chinese patients with PBC [93], while a smaller study from Brazil failed to confirm the association [94]. Several studied were further dedicated to SNPs of IL, based on their active and critical role in the regulation of the immune response. Prompted by experimental data, such as its dysregulated production by monocytes in PBC [95], SNPs of IL-1 were studied in PBC. First, a study from the UK reported a significantly higher frequency of the IL-1B*1,1 genotype in patients with PBC compared to controls. The difference in the IL-1B*1,1 genotype distribution was even more marked in patients with early stage disease, thus possibly indicating that IL-1 alleles might influence disease progression, albeit in a cross-sectional analysis [85]. Further, based on experimental evidence of cytokine profiles and its involvement in the development of T helper 1 (Th1) cell responses, SNPs of the promoter region of the IL-10 gene were also analyzed in patients with PBC, and controls and data from Italian and Japanese series demonstrated that both groups presented a higher prevalence of the −1082G/G genotype [96].

The 1,25-dihydroxyvitamin D receptor (VDR) gene has been investigated in several studies, based on the dual role of vitamin D in the regulation of bone metabolism and inflammation. We note in fact that accelerated bone loss rates in patients with prolonged cholestasis (as in PBC) have been repeatedly reported, sometimes with conflicting results and in some cases with less than rigorous experimental designs. Differences in the VDR gene might unravel further potential scenarios to help explain the infrequency of PBC in African-American women [97] and could in turn support a possible role for sunlight exposure in PBC onset. This fascinating hypothesis has been most recently strengthened by a multicenter study in Japanese and Italian patient series [98].

Based on different rationales, several other genes have been investigated in the susceptibility to PBC in association studies and their discussion goes beyond the aims of this chapter. Based on the xenobiotic hypothesis in PBC, our group analyzed genetic polymorphisms of cytochrome P450 (CYP) 2E1 and 2D6, multi-drug resistance 1 (MDR1), and pregnane X receptor (PXR). Data demonstrated that no polymorphism was associated with PBC susceptibility, while a weak association of the CYP2E1 c2 allele with disease severity was observed in a small subgroup of patients [99]. Among other studies, the distribution of specific alleles of CD40 ligand [100], CD14 [101], endothelial nitric oxide synthase [102], early T-lymphocyte activation 1/osteopontin [103], T-cell receptor C-beta2 [104], insulin-like growth factor 1, collagen-Ia1 [105], and TLR 9 [53] did not differ between patients and controls. However, in some cases, a significant association with disease severity [102, 104] or bone loss [105] seemed evident.

Environmental Considerations in PBC

Although it is almost certain that PBC susceptibility is conferred by yet unknown genetic factors, genetics are not sufficient to trigger the disease. Exposure to certain environmental factors could result in immune tolerance breakdown, being therefore necessary for PBC onset [106]. Epidemiological, experimental evidence, as well as animal models support the key role of environmental factors and in particular, xenobiotics in the development of PBC. The prevalence of PBC is higher in Northern European countries (particularly the UK and Scandinavian countries) and the Northern United States (Minnesota), but this assumption might be the result of different methodologies for case finding in epidemiological studies, rather than reflect a true difference in the prevalence of cases and, similar to clusters, could be anecdotal. More importantly, no true population-based study has been proposed thus far and all estimates are based on the identification of cases already diagnosed. It has been hypothesized that the incidence of PBC may be increasing, but this assumption is potentially burdened by similar types of bias. Finally, we note that population-based studies on PBC must necessarily rely on AMA detection using indirect immunofluorescence, an assay that fails to determine antibody positivity in up to 15% of PBC cases.

In the UK, Triger et al. reported in 1980 the first epidemiological study on PBC in England [107] and cases were ascertained using physician surveys and follow-up of subjects with positive serum AMA (as tested by indirect immunofluorescence) leading to a prevalence of 54/million and an yearly incidence of 5.8/million. The authors also suggested an association between PBC and environmental factors possibly contained in a residential water reservoir, but chemical analyses failed to suggest a potential candidate component. These observations have recently gained further attention following the report from researchers at Mount Sinai School of Medicine, who reported that PBC cases arrayed by ZIP codes were found more frequently in areas close to Superfund sites, i.e., sites of waste collection for the five New York City boroughs [108]. In the UK, Hamlyn et al. report that PBC has a higher incidence and prevalence than those observed by Triger et al. in Sheffield despite using more strict case-finding criteria [109]. According to this latter study, PBC yearly incidence is 10/million, and prevalence is between 37–144/million [67]. A later study on a larger area in Northern England using the same case-finding criteria as the Sheffield study, reported a yearly incidence of 19/million with prevalence rates ranging between 129–154/million [68]. Stricter case definition criteria were introduced again in 1997 and the incidence rate was estimated to be 22/million/year, with a prevalence of 240/million [110]. In this case and in a study that proposes that PBC is increasing [111], the potential causative effect of increased awareness cannot be ruled out, as suggested by the most recent data from a Canadian health region. In this study, the overall age/sex-adjusted annual incidence of PBC was 30.3 cases per million (48.4 in women). PBC incidence remained stable while the prevalence increased from 100/million in 1996 to 227/million in 2002 [112]. Data on PBC epidemiology are not available in whole continents such as Africa or Asia, with the exception of Japan where serum AMA prevalence in the general population has been inferred [113]. An earlier population-based study identified 84 cases of PBC from the region of Victoria (Australia) using rigorous case-finding methods and first reported a PBC prevalence of 19.1/million, among the lowest rates in the literature. More recently, a study from the same region reported 249 cases with a cumulative, almost tenfold higher than what reported in 1995 [114]. Importantly, prevalence rates were significantly higher for British, Italian, and Greek immigrants, compared to native Victorians. Similarly, data from Canada have provided intriguing observations on subjects from British Columbia of Native Canadian ancestry

for whom PBC has strikingly higher prevalence and incidence rates [115]. In the United States, a large study performed on the population of Olmsted county, MN [4] estimated the age- and sex-adjusted PBC prevalence to be 27/million, among the highest ever reported, and the calculation was based on data from a computerized state index of diagnoses for inpatients and outpatients.

Infectious Agents and PBC

Among proposed mechanisms of environmental impact, molecular mimicry (based on the sharing of epitopes on proteins from unrelated species) has been suggested as a potential mechanism in the immune tolerance breakdown and initiation of autoimmunity [116]. Data supporting the role of bacterial infections in disease development include the significantly higher prevalence of urinary tract infections reported among patients with PBC compared to normal controls [117] and the presence of bacterial products in mononuclear cells surrounding damaged bile ducts in PBC [118]. The molecular mimicry hypothesis has been suggested mostly based on the experimental cross-reactivity of patient AMA and/or autoreactive T cells with prokaryotic antigens for a number of microbes, including *E. coli* [48], which share an ExDK motif within their mimicry epitopes, an amino acid sequence recognized by AMA and autoreactive T cells. These cross-reactivities are not surprising given the highly conserved sequence of PDC in phylogenesis. We have suggested the possible role of *N. aromaticivorans* in PBC based on several observations [119]. The amino acid sequences of two different proteins from *N. aromaticivorans* present one of the highest degrees of homology with the main human PDC-E2 autoepitope (amino acids 208–237) [120]. Importantly, the bacterium is ubiquitous, has not been found to be pathogenic to humans, and shares the capability to modulate estrogen activation. A role for halogenated xenobiotics has been proposed by our group in the induction of PBC [121] and the unique capability of *N. aromaticivorans* to metabolize chemical compounds [122] suggests that this bacterium might link xenobiotics and bacteria in the etiology of PBC. Further, serum reactivity against two lipoylated proteins of 47 and 50 kDa molecular weight was observed in 100% of anti-PDC-E2 positive and in a fraction of AMA-negative sera, regardless of the disease stage. Such reactivity was estimated to be 100–1,000-fold higher than against *E. coli* and was highly specific for PBC sera. The lipoylated bacterial proteins were further identified, cloned, expressed, and phylogenetically defined in our laboratory to confirm their homology to human 2-OADC enzymes [123]. We also demonstrated the presence of the bacterium in the human body in that 25% of the analyzed fecal specimens, regardless of the diagnosis, had detectable specific 16S rRNA. Finally, we confirmed the specificity of our serological findings in independent cohorts of Icelandic patients and controls [71]. For purpose of completeness we note that a human beta-retrovirus has been reported in peri-hepatic lymph nodes and other samples from patients with PBC, thus suggesting its possible role in the pathogenesis of PBC [124]. However, in an independent experimental study conducted with a more comprehensive molecular and immunological approach on a larger series of patients and controls we could not confirm such hypothesis [125]. Most recently, human beta-retrovirus has been found by real-time reverse transcriptase polymerase chain reaction in plasma of patients with different liver diseases, including PBC, AIH and viral hepatitis [126]. Although these data have not been yet confirmed, they would suggest an involvement of human beta-retrovirus in the pathogenesis of etiologically different liver diseases, being therefore not specific for PBC and ultimately discouraging the use of antiretroviral drugs that were proposed for PBC [127].

Chemicals and PBC

Foreign chemicals (coined xenobiotics) may either alter or complex to a defined self or non-self protein, inducing a change in its molecular structure to induce an immune response in the host that may in turn result in the cross-recognition of the self form and perpetuate the immune response. Similar to bacterial agents and molecular mimicry, the process might be self-alimenting and the trigger molecule might be long lost at the time of diagnosis. The main detoxifying organ is the liver, thus potentially exposing hepatocytes and biliary epithelial cells to chemical byproducts. A large number of exogenous compounds, from food preservatives to home detergents to pollutants, potentially lead to these modifications [121]. Lipoic acid is critical to the PDC-E2 epitope recognition [36] and exposed to the exterior part of the complex thus constituting the ideal target for xenobiotic modification. Our group first demonstrated that specific organic structures attached to the mitochondrial antigens were recognized by sera from PBC patients with a higher affinity than native forms of such antigens [121]. Further, a halogenated compound induced AMA production in rabbits without requiring the peptide backbone of PDC-E2 [128], but did not produce liver lesions (possibly in agreement with observations in humans where AMA is present prior to the appearance of liver damage) and disappeared without the stimulus [129]. More recently, Leung et al. reported the induction of PBC-like liver lesions following longer follow-ups in guinea pigs [130]. Utilizing a different approach, our group also demonstrated that 2-nonynoic acid was capable of being recognized by PBC sera with high affinity [131].

This is particularly interesting since this non-naturally occurring compound is known to be found in several cosmetic products and could possibly explain the slight association between PBC and the frequent use of specific cosmetic products [6]. More recently proposed animal models induced by xenobiotics will be discussed in the following paragraph.

PBC Animal Models

Similar to all complex diseases, such as autoimmune disease, the development of an animal model is crucial to elucidate the mechanisms responsible to the initiation and progression of disease. Several models (Table 49.4), mostly murine [132], have been proposed for PBC. Two animal models, that is, dnTGFβRII and IL-2Rα knockout mouse, point out the possible crucial role of Treg deficiency in the loss of immune tolerance with consequent development of autoimmune response against PDC-E2 in PBC. A mouse with dominant negative form of transforming grown factor β (TGFβ) receptor II, (dnTGFβRII) showed PBC-like liver disease, for example, 100% AMA positivity against PDC-E2 [133]. Of note, the depletion of B cells worsens liver disease in this model [134]. On the other hand, a mouse deficient for IL2 receptor alpha (IL-2Rα) that is highly expressed on Tregs developed 100% AMA positivity against PDC-E2, 80% ANA positivity, and lymphocyte infiltration around the portal tracts associated with cholangiocyte injury [135]. Other animal models strongly support the hypothesis that xenobiotics can induce autoimmunity. Among these, the induction of PBC-like lesions was obtained in a non obese diabetic (NOD) background by Wakabayashi et al. and in guinea pigs by Leung et al. exposed to xenobiotic immunization [136, 137] with halogenated compounds. An additional animal model is a variant of the NOD mouse model (NOD.c3c4). It has been described that NOD.c3c4 presents autoimmune cholestasis and PBC-specific serology, showing AMA positivity of 50–60% and ANA positivity of 80–90%. Histologically, it presents lymphocyte infiltration around portal tracts with chronic nonsuppurative destructive cholangitis and epithelioid granuloma formations; nevertheless, the morphological features of bile ducts differ somewhat from those in human PBC [138]. Finally, the induction of a PBC-like phenotype following *N. aromaticivorans* immunization has already been discussed [57].

Table 49.4 Synopsis of comparisons between the innate and acquired immunity compartments in the proposed animal models and the human PBC

Mouse model	Adaptive immunity	Innate immunity
dnTGFbRII	AMA Deficient Treg function	NKT cells worsen liver injury
IL2Ra$^{-/-}$	AMA Portal tract CD4+ and CD8+ cells	–
NOD.c3c4	Lymphocytic infiltrate AMA, ANA	–
N. aromaticivorans on NOD 1101	AMA PBC-like liver lesions Disease transfer by T cells	NKT cells are required
Xenobiotic on C57BL/6	Lymphocytic CD8+ infiltrate AMA PBC-like liver lesions	–

The Resulting Pathogenesis of PBC

Once we have illustrated the available evidence on the immune mechanisms involved in PBC pathogenesis, several hypotheses can be inferred and these should not be regarded as mutually exclusive from any causative factor such as susceptible genes, but rather as terminal mechanisms of the pathway leading to the clinical manifestations.

One major hypothesis for the selective destruction of biliary epithelial cells implies that the immunodominant autoantigen PDC-E2 should be aberrantly exposed on cholangiocytes-cell surface, where it may be recognized by AMA and/or antigen-specific T cells. Studies based on in situ hybridization of PDC-E2 mRNA failed to demonstrate significant differences in its amount in PBC liver compared with other liver diseases [139]. On the other hand, co- or post-translational modifications of PDC-E2 may cause its abnormal turnover leading to its accumulation. Chemicals (i.e., xenobiotics) disposed by the liver may have a role in this scenario by accumulating in the biliary-epithelial cells and modifying PDC-E2 locally. Solid data to support these fascinating mechanisms are lacking or weak and we cannot rule out that the molecules expressed and identified on the ductular surface and recognized by AMA may not be PDC-E2 itself, but possibly unrelated PDC-E2 mimics cross-reacting with human PDC-E2.

As previously discussed, the pathogenetic role of AMA remains unclear. AMA belong mainly to the IgG isotype, in particular IgG3 subclasses, and thus is potentially pathogenic through different mechanisms, e.g., complement activation, antibody-dependent cytotoxicity. However, there is no direct experimental evidence supporting the involvement of these mechanisms in the pathogenesis of PBC.

AMA can be also of IgA isotype. The role of such AMA-IgA has been ignored for long, but may be critical in the pathogenesis of PBC. It is now well established that AMA-IgA, indeed, can be detected not only in sera, but also

in bile, saliva, and urine of patients with PBC, in some cases correlating with disease severity [140]. Moreover, IgA represents the principal Ig isotype in epithelial surfaces, including biliary epithelia. AMA-IgA has been reported to co-localize with PDC-E2 both inside the cell cytoplasm as well as the apical membrane of cholangiocytes in PBC but not in controls. Thus, AMA-IgA and in particular AMA-IgA bound to mitochondrial antigen could be able to disrupt cell metabolism and may also induce cellular dysfunction and damage thus leading to a tissue specific injury. We cannot preclude the possibility that the apical staining obtained with anti–PDC-E2 monoclonal antibodies may be secondary to the presence of immune complexes formed by secreted IgA and AMA antigens, as some line of evidence seems to suggest [139].

Most recently, a solid theory based on the unique apoptosis features in bile duct cells has been proposed [141]. It was first demonstrated that PDC-E2 remains intact and retains its immunogenicity during cholangiocyte apoptosis, secondary to a cell-specific lack of glutathionylation of biliary epithelial cells [142]. The intact PDC-E2 in apoptotic blebs (i.e., apotopes) could be then uptaken by local antigen presenting cells and transferred to regional lymph nodes for priming of cognate T cells thus initiating PBC [143].

In conclusion, the current working hypothesis (Fig. 49.2) could be summarized as follows [144, 145]. Three major events are crucial to the proposed mechanism, that is, bile duct cell apoptosis, female predominance, and genetic susceptibility. A mimicking microorganism (possibly the ubiquitous *N. aromaticivorans*) enters the human system through the digestive mucosa and its PDC-E2-like proteins are modified within the liver by xenobiotics to form immunoreactive adducts. These modifications could be then sufficient to trigger the innate immune system to initiate a cascade of local inflammatory events resulting in local dendritic-cell activation and antigen processing. Mucosal antigen-presenting cells in turn could activate autoreactive T and B cells [146] that are directed to the liver through the portal system. T cells, therefore, could participate directly not only to the autoimmune injury, but also to its amplification and perpetuation [43]. B cells, on the other hand, could secrete AMA, particularly of the IgA type. AMA-IgA could be then transported to the vascular side of biliary epithelial cells where they could recognize PDC-E2-like molecules located on the luminal surface cell membrane. AMA-IgA/PDC-E2-like molecules engagement could initiate apoptotic signaling cascade. Ultimately, the immune complexes of post-apoptotic PDC-E2 and IgG-AMA and the direct cytopathic effects of autoreactive T cells (and possibly AMA) lead to the selective bile duct destruction.

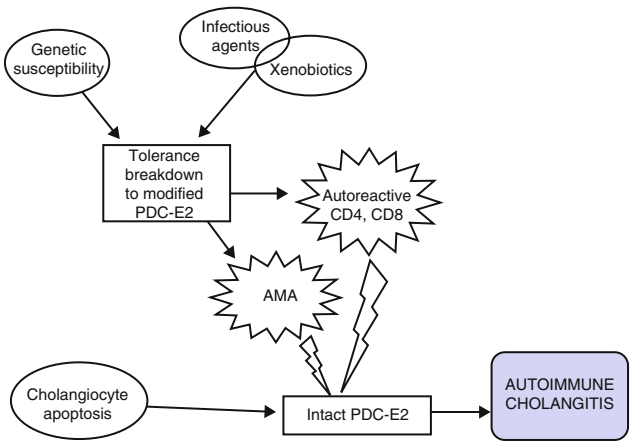

Fig. 49.2 Schematic view of the proposed hypothesis for PBC (i.e., autoimmune cholangitis) pathogenesis

Open Issues in PBC Pathogenesis

As we discussed in this chapter, numerous issues remain to be determined in PBC pathogenesis and we are convinced that solving this enigma would significantly enhance our knowledge on autoimmunity per se. Future efforts should be dedicated to overcoming some of these conceptual and logistic difficulties. First, only the collection of large series patients and the replication of data from the genome-wide analysis on thousands of SNPs will allow the determination of the genetic bases of PBC. Similarly, the collection of representative families should be encouraged to ultimately confirm reported associations. Second, the role of xenobiotics and bacteria in the onset of PBC should be further studied by means of new molecular multiplex tools. Third, we note that new potential mechanisms have been proposed and should be addressed in PBC, including microRNA and epigenetics. Fourth, it is time to prove the AMA pathogenic role in PBC and the proposed animal models may be a good starting point to achieve this goal. Finally, future developments in PBC pathogenesis will be possible only through a multidisciplinary approach, well illustrated by microarray data [147].

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Chapter 50
Primary Sclerosing Cholangitis

Marina G. Silveira and Keith D. Lindor

Introduction

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by fibrosing inflammatory destruction of the intrahepatic and/or extrahepatic bile ducts [1], leading to bile stasis, hepatic fibrosis and ultimately to cirrhosis, end-stage liver disease, and need for liver transplantation (LT). The majority of cases occur in association with inflammatory bowel disease (IBD), which often precedes the development of PSC [2]. The etiology of PSC is undefined, apart from an increasing body of evidence that points to an immunologic disturbance as a component of the disease. However, PSC lacks the features of a typical autoimmune disease and responds poorly, if at all, to typical immunosuppressive therapies [3]. No effective medical therapy for halting disease progression has been identified. A median duration of 12–18 years from the time of diagnosis before patients develop end-stage liver disease has been described. Among eligible patients, LT is currently the only life-extending therapy for patients with end-stage PSC, although the disease can recur in the allografted liver and be a cause of morbidity post-transplant [4].

Clinical Features

Epidemiology

PSC affects primarily young and middle-aged men, especially patients with underlying IBD. Population-based studies of disease frequency are available from Norway, Great Britain, and the United States, and indicate incidence of 0.9–1.3 per 100,000/year and prevalence of 8.5–14.2 per 100,000/year for these populations [5–7]. The prevalence of PSC is lower in Southern European, Asian, and African populations [8]. In the year 2000, national estimates in the USA were approximately 29,000 prevalent cases among the white population [5]. Approximately 70–80% of PSC patients in the USA have ulcerative colitis (UC) [9–14]. Conversely, approximately 2–7.5% of patients with UC [15] and 1.4–3.4% of patients with Crohn's disease [16] develop PSC. IBD can be diagnosed at any time during the course of PSC, and PSC can occur at any time during the course of IBD [17]. However, in general, IBD is diagnosed several years earlier than PSC [17]. PSC may also develop many years after proctocolectomy for colitis and IBD can develop many years after LT due to advanced PSC [15].

Pathophysiology

The precise pathogenesis of PSC is unknown, but immunologic, bacterial, viral, and toxic factors may play a role in a genetically susceptible host.

The inflammatory infiltrate of PSC is largely comprised of T cells; other cell types including NK cells, macrophages, B cells, and biliary epithelial cells are likely to play important roles in the immunopathogenesis of PSC [18]. Inflammation and liver injury is discussed in Chap. 27.The activation of the innate system as a primary inciting event of PSC has been proposed by several investigators [19]. Mucosal lymphocytes have also been implicated in the pathogenesis of inflammatory liver diseases complicated by IBD by demonstrating aberrant expression of (cytokines) CCL25 and MAdCAM-1 in the liver of patients with PSC associated with hepatic infiltration by CCR9+ and $\alpha 4\beta 7$+ T cells [20–22]. Lymphocytic infiltration and apoptosis have both been suggested to mediate the destruction of hepatocytes and biliary epithelium in primary biliary cirrhosis and to a lesser extent, in PSC [23–26]. Furthermore, inflammatory mediators are overexpressed in PSC. Induction of adhesion molecules and chemokines leads to the recruitment of intestinal lymphocytes. Bile duct injury results from the sustained inflammation and production of

K.D. Lindor (✉)
Department of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA
e-mail: lindor.keith@mayo.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5 ,
DOI 10.1007/978-1-4419-7107-4_50, © Springer Science+Business Media, LLC 2011

inflammatory cytokines. Biliary strictures may cause further damage as a result of bile stasis and recurrent secondary bacterial cholangitis.

Genetic Factors

The genetic predisposition to PSC is shown by familial occurrence, with an almost 100-fold increased risk of this disease among first-degree relatives [27]. However, inheritance of PSC appears to be complex, with one or more genes acting alone or in concert to increase or decrease risk [28].

Several genes have been associated with susceptibility to PSC. A human leukocyte antigen (HLA) association in PSC was first identified for the alleles HLA-B8 and DR3 [29]. The strongest reported association so far is with certain haplotypes in the HLA region, where HLA A1-B8-DR3, DR6, and DR2 confer susceptibility and HLA DR4 is associated with protection against PSC in Northern Europeans [30].

Some studies have also investigated the possible prognostic role of HLA, but with controversial results. The HLA DRB3*0101 locus, which may be present among 55–65% of patients with PSC [29], has been associated with reduced survival in one study [31]. The major controversy concerns the protective role of DR4, which in two studies was identified as a marker of rapid or aggressive disease progression [32, 33] and in one study, was associated with increased risk of cholangiocarcinoma but not accelerated disease progression [34]. No current guidelines include HLA typing in diagnosis and prognosis assessment of PSC.

Increasing evidence suggest that major histocompatibility complex (MHC) genes alone are unlikely to account for all the genetic risk in PSC [28]. A number of non-major histocompatibility class candidate genes may also influence susceptibility or resistance to clinical disease from PSC. An increased frequency of cystic fibrosis transmembrane conductance regulator (CFTR) gene abnormalities has been observed in patients with PSC and IBD. Chloride secretion was shown to be decreased in PSC patients when compared with healthy controls [35]. Therefore, the CFTR gene seems a plausible candidate gene for PSC. Several studies, all with a limited number of patients, have addressed the impact of CFTR variants on susceptibility to PSC, with conflicting results [30, 35–40].

Interestingly, studies comparing IBD susceptibility genes in PSC and IBD cohorts have failed to show any common genetic links including studies of NOD2/CARD15, TLR-4, CARD4, SLC22A4, SLC 22A5, DLG5, and MDR1 [18].

Clinical Manifestations

Asymptomatic patients represent about 15–40% of the patients at time of diagnosis in early studies [2]. More recently, more patients are identified at an earlier stage of the disease with fewer symptoms. One study showed that the majority of patients (greater than 55%) initially present with asymptomatically elevated liver enzymes [41]. Due to its close association to IBD, many cases come to medical attention when patients with IBD are screened for liver disease.

The clinical course of PSC is typically one of insidious worsening of cholestasis and eventual development of jaundice and end-stage liver disease [3]. As such, asymptomatic patients with PSC are at increased risk for developing symptoms over time. Fatigue and pruritus are reported as the most common symptoms. Jaundice, pain, fever and weight loss, cholangiocarcinoma, or manifestations of portal hypertension in advanced stages of liver disease are uncommon initial manifestations. Symptoms of bacterial cholangitis usually are not manifested until patients undergo endoscopic intervention or surgical exploration of the biliary tract [42]. Cholangiocarcinoma develops in up to 23% of patients [43] and can occur relatively early and before onset of cirrhosis [3].

Biochemical Features

A cholestatic picture of liver function with elevations in serum alkaline phosphatase values are the biochemical hallmark of PSC. Increases between 3–10 times the upper limit of normal occur in approximately 95% of cases. An elevated alkaline phosphatase is not a prerequisite for the diagnosis; up to 8.5% of patients in large studies have had normal alkaline phosphatase at diagnosis [13]. Serum alanine and aspartate aminotransferase levels are usually 2–3-fold higher than normal levels. The serum total bilirubin level is normal in 60% of individuals at diagnosis [2]. The synthetic function of the liver is intact in the majority of patients at the time of diagnosis, with normal levels of albumin and coagulation factors [8]. However, the liver biochemistries may be normal and can fluctuate during the course of the disease [44].

Several prognostic models for PSC have been developed, most of which include age, serum bilirubin, and histologic staging [9, 11, 13, 14, 45]. Most recently, a Mayo model for predicting the survival has been refined [46]. This uses the age of the patient, total serum bilirubin, aspartate aminotransferase levels, presence or absence of variceal bleeding and serum albumin as independent variables, and can be used in early stages of PSC, before onset of cirrhosis. The limitations of prognostic models include the inability to account for the development of cholangiocarcinoma and health-related quality of life [42]. Once decompensated cirrhosis is present, the Model for End-Stage Liver Disease (MELD) score [47] more accurately predicts survival and is more appropriately used in prioritizing patients for LT [3].

Serologic Features

Currently, testing for specific autoimmune antibodies does not contribute to the diagnosis of PSC. The prevalent autoantibody reactivity is perinuclear antineutrophilic autoantibodies (pANCA), present in approximately 80% of patients but lacking in diagnostic specificity [48–51]. The unidentified antigenic reactant is not the proteinase (myeloperoxidase) of conventional pANCA. Other autoantibodies such as antinuclear antibodies and smooth muscle antibodies occur in 20–60% of patients, usually in lower titers than those observed in autoimmune hepatitis [52]; their fine antigenic specificity has not been established. Antimitochondrial antibodies are rarely found in patients with PSC [10].

Radiographic Features

Cholangiography is considered to be the gold standard for the diagnosis of PSC [10]. In experienced hands, endoscopic retrograde cholangiopancreatography (ERCP) is successful in demonstrating the intra- and extra-hepatic biliary tree in 95% of the cases [3]. Segmental fibrosis of intrahepatic and/or extrahepatic bile ducts with saccular dilatation of normal intervening areas results in the characteristic beads-on-a-string appearance, as seen in Fig. 50.1.

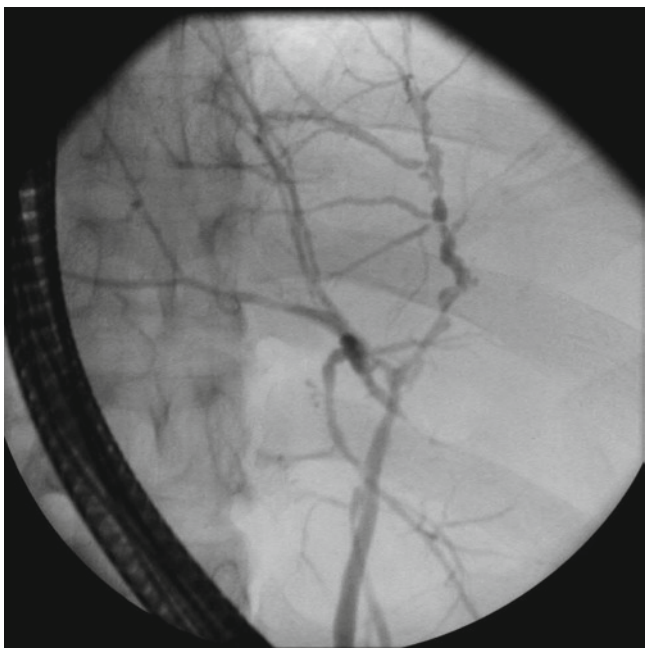

Fig. 50.1 Cholangiographic findings: Endoscopic Retrograde Cholangiogram demonstrating multifocal strictures with intervening saccular dilatation of both intrahepatic and extrahepatic bile duct characteristic of primary sclerosing cholangitis (PSC)

Intrahepatic duct involvement is nearly universal with most patients affected by intrahepatic and extrahepatic disease [42]. Procedure-related complications from ERCP can occur in 3–11% of patients and include abdominal pain, pancreatitis, bleeding, common bile duct perforation, biliary sepsis, and death [53–56]. The overall rate of complications in patients with PSC when compared to non-PSC patients undergoing ERCP does not appear to be significantly different, except that the rate of cholangitis is higher in PSC patients despite routine use of antibiotics pre-procedurally in these patients [56].

The use of magnetic resonance cholangiography (MRC) for detecting PSC has been evaluated as a rapid, noninvasive examination of the biliary tract. MRC has no significant morbidity when performed in appropriately selected individuals, and avoids the potential adverse effects of radiation exposure and contrast media associated with ERCP [57]. For the detection of PSC, MRC has been found to be accurate and comparable to ERCP [58–61], as seen in Fig. 50.2. Moreover, one study has suggested that it also results in cost savings when used as the initial test strategy for diagnosing PSC [55]. Factors that lead to difficulties in interpreting the MRC compared to ERCP include the presence of cirrhosis and PSC limited to the peripheral intrahepatic bile ducts [58]. The major disadvantage of MRC is that it is a purely diagnostic examination, although it can be used to identify patients who would benefit from subsequent therapeutic ERCP [42]. Although biliary tree changes on MRC aid in the diagnosis of PSC, they do not correlate with survival, as predicted by the Mayo Risk Score [60].

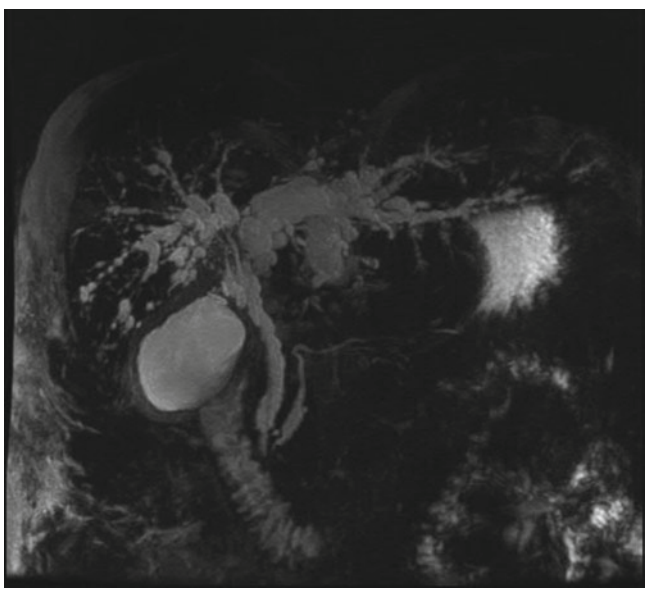

Fig. 50.2 Cholangiographic findings: Magnetic resonance cholangiogram demonstrating characteristic findings of PSC

Histologic Features

PSC is histologically characterized by damage, atrophy, and, ultimately, loss of medium- and large-sized bile ducts, within or outside the liver [62, 63]. These are not typically captured in a percutaneous liver biopsy. The histological picture is complicated by the fact that separation of the disease process itself from the effects of distal obstruction of bile ducts can be challenging [63]. The smaller ducts are affected by the resultant obstruction and gradually disappear (ductopenia). The characteristic pathologic features of PSC are concentric periductal fibrosis ("onion-skinning") that progresses to a narrowing and then obliteration of the small bile ducts leaving a bile duct scar, as seen in Fig. 50.3, but this is found in less than 15% of the patients with PSC [64]. Many of the biopsy changes, such as bile stasis, pseudoxanthomatous changes, Mallory bodies and copper accumulation, and lack specificity for diagnostic purposes [63], can occur with chronic extrahepatic bile duct obstruction from any cause [65]. Several stages can be recognized histologically, ranging from stages I–IV: cholangitis and portal hepatitis (stage I); periportal fibrosis or periportal hepatitis (stage II); septal fibrosis, bridging necrosis, or both (stage III); and biliary cirrhosis (stage IV) [66]. Sampling error is a significant limitation of liver biopsy [63].

Liver biopsy in patients with radiographic evidence of PSC is not needed for diagnosis, although it may help in excluding other diseases [67]. Histological staging may be complementary to ERCP evaluation, but most times is not necessary. Liver biopsy should probably be limited to patients with a challenging presentation, or those being investigated for small duct PSC or possible overlap syndrome with autoimmune hepatitis [41].

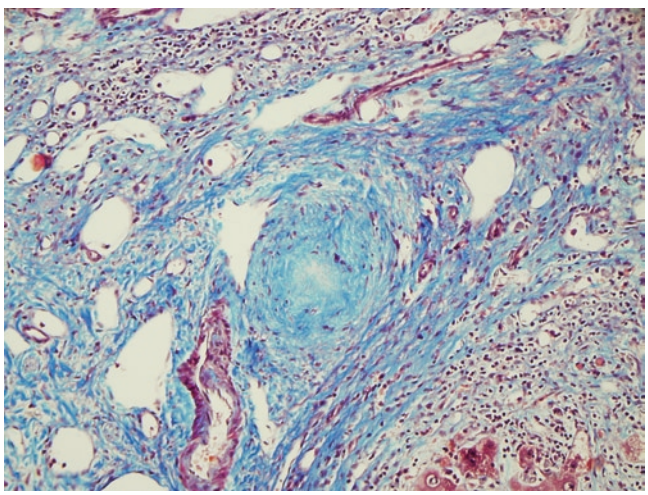

Fig. 50.3 Histological findings: Expanded portal area with distinct fibro-obliterative lesion in end-stage PSC (photograph courtesy of Dr. Schuyler Sanderson)

Disease-Modifying Treatments

Different forms of medical treatments have been studied, but to date, there is no clearly effective medical therapy that has been proven to cure or to delay the progression of PSC in randomized controlled studies.

Ursodeoxycholic Acid

Ursodeoxycholic acid (UDCA) has been the most widely evaluated drug in the treatment of PSC. UDCA, the 7-beta epimer of chenodeoxycholic, is a hydrophilic naturally occurring bile acid that appears to have fewer hepatotoxic properties than endogenous bile acids [68]. Its mechanism of action is not fully known. UDCA has several interrelated functions including expansion of the hydrophilic bile acid pool as well as direct choleretic, anti-inflammatory, and anti-apoptotic effects on hepatic epithelia [69, 70]. Bile-acid metabolism including their role as therapeutic agents is discussed in Chap. 12. In humans, naturally occurring UDCA accounts for up to 4% of the bile acid pool, and likely originates in the colon by bacterial epimerization of the primary bile acid, chenodeoxycholic [71]. Following its formation, UDCA is absorbed by the ileal and colonic mucosa to enter the portal circulation and subsequently the pool of bile acids. After oral administration in clinical practice, UDCA may reduce the ileal absorption of endogenous bile acids by competitive inhibition at the level of the terminal ileum [69]. Eventually, with therapy, UDCA will become the predominant bile acid, accounting for up to 40 or 50% of the total bile acid pool [70].

Several controlled and uncontrolled studies have consistently demonstrated that UDCA in a wide dose ranging from 10–30 mg/kg/day has beneficial effects on liver biochemistries [72–81]. A few studies have documented an improvement in liver histology [72, 75, 78] and cholangiographic features [78], but at doses lower than 20 mg/kg/day, UDCA does not seem to have a beneficial impact on clinically relevant findings such as the development of cirrhosis and it complications, need for LT and survival. In contrast, at doses higher than 20–25 mg/kg/day, some data is suggestive that UDCA may confer an advantage in survival free of LT [79], but larger studies have not shown this [81, 82].

Immunosuppressive Agents

Despite a presumed immunologic disturbance as a component of the disease, a classical response to immunosuppressive

agents has not been clearly demonstrated in PSC. Several immunosuppressive agents have been studied and, in addition to their limited clinical utility, many are associated with significant side effects [83–85]. Agents studied include azathioprine, budesonide [83], methotrexate [86], and mycophenolate mofetil [84, 87]. Tacrolimus was shown in small studies to cause significant improvement in serum-liver biochemistries including alkaline phosphatase [85, 88], but issues with toxicity and patient tolerance limit its clinical utility.

Other Agents

Several drugs with different mechanisms of action such as bezafibrate [89], colchicine [90], cladribine [91], cyclosporine [92], etanercept [93], infliximab [94], oral and transdermal nicotine [95, 96], penicillamine [97], pentoxifylline [98], pirfenidone [99], and silymarin [100] have been evaluated in the treatment of PSC. Despite encouraging results from a few studies [89, 100], none of them have demonstrated convincing evidence of benefit.

Combination Therapy

Drugs in monotherapy are often limited by efficacy and dose-related toxicity; combination therapy may hold the potential for improved efficacy through additive or synergistic effects, with the potential minimization of drug toxicities [101].

Positive, but very preliminary results were also observed in a few patients treated with the combination of cyclosporine and corticosteroid (methylprednisolone) [102], cyclosporine and sulfasalazine [103] and UDCA, prednisolone and azathioprine [104]. Similarly, studies including combination of low-dose prednisolone and colchicine [105], UDCA and corticosteroid (prednisone or budesonide) [106], UDCA and methotrexate [107], and UDCA and metronidazole [108], have not yet shown evidence supporting long-term use of any particular drug combination.

Innovative Approaches to Medical Therapy

Trials of antibiotics such as metronidazole and minocycline [109] have been promising but inconclusive. Minocycline has been found to exert biological effects independent of its antimicrobial properties, including anti-inflammatory activities such as inhibition of inducible nitric oxide synthase (iNOS), upregulation of interleukin 10, and direct suppressive effect on B and T cell function. Minocycline may also inhibit cell-death pathways by reducing both pro-apoptotic and pro-inflammatory enzyme activation [109]. A small study of docosahexaenoic acid (DHA) which improves CFTR function [110] is currently underway. Most promising for the near future are antifibrotic agents (such as angiotensin-converting enzyme [ACE] inhibitors and sirolimus/rapamycin), and inhibitors of formation of toxic bile (such as 24-norursodeoxycholic acid [norUDCA]) [111].

Recent animal studies show that disturbances in the renin-angiotensin system contribute to tissue injury and development of hepatic fibrosis [112]. Angiotensin-converting enzyme inhibitors (ACEs) and angiotensin-receptor blockers (ARBs) have both been shown in animal models of cholestasis and liver injury to have beneficial effects by both attenuating fibrosis and downregulating key inflammatory and fibrotic cytokines, making them promising potential agents for antifibrotic therapy in patients with PSC [113–124]. NorUDCA is a C23 homolog of UDCA with one less methyl group on its side chain. The unconjugated form of norUDCA is a weak acid that can be reabsorbed from the bile by cholangiocytes and resecretion by hepatocytes in a process termed cholehepatic shunting, which is associated with a bicarbonate-rich hypercholeresis from cholangiocytes [125]. NorUDCA had antifibrotic, anti-inflammatory, and antiproliferative effects that resulted in healing of the cholangitis and fibrosis in a mouse model of PSC [125, 126].

Endoscopic Therapy

Some patients present with clinical and biochemical deterioration and exhibit a dominant stricture that involves the larger extrahepatic biliary ducts. Such lesions may be amenable to endoscopic or radiologic dilatation with or without a biliary drainage procedure, such as sphincterotomy and stenting [67]. This leads to improvement of clinical symptoms, liver biochemistries and cholangiographic findings. However, the endoscopic treatment of PSC has generated controversy, not only with regard to optimal management, but also its overall influence on survival. The use of endobiliary stents has been compared to balloon dilatation alone in patients with PSC [127, 128], and a greater frequency of intervention-related complications including acute cholangitis was observed in patients with endobiliary stent placement. Repeated balloon dilatations of dominant biliary strictures resulted in improved actual survival rates compared to survival rates predicted by Mayo risk score [129, 130]. Nonetheless, the number of complications related to therapeutic ERCP in patients with PSC appears to be increased over diagnostic studies, particularly when more than five interventions are performed [56].

Biliary Surgery

Dominant strictures can also be managed surgically by dilatation or choledochojejunostomy, but this treatment has become uncommon with more recent advancement of endoscopic techniques and growing success of LT. At present, biliary surgery in patients with PSC, other than simple cholecystectomy, should be minimized and reserved for selected noncirrhotic patients who have marked cholestasis or recurrent cholangitis caused by a dominant extrahepatic or hilar stricture not amenable to endoscopic or percutaneous dilatation [67]. In patients who may undergo LT, prior biliary surgery has been associated with a significantly longer operation time, greater intraoperative blood loss, and a higher incidence of biliary complications post-liver transplantation compared with those patients with no history of biliary surgery [131–135].

Liver Transplantation

Although PSC is an uncommon disease, advanced-stage PSC remains among the most common indications for LT in the USA and in Europe [43]. Unique circumstances that require evaluation for possible LT include recurrent bacterial cholangitis despite intensive medical and endoscopic therapy, severe extrahepatic biliary obstruction that precludes operative repair, and uncontrolled peristomal variceal bleeding. Intractable pruritus may also be an indication for LT. LT should be considered before the disease is too advanced, in order to enhance the long-term survival rates post-liver transplantation [136]. Prognostic models can aid in the timing of LT. Reports from single centers performing LT in PSC patients have demonstrated excellent survival rates of 90–97% at 1 year and 83–88% at 5 years [137, 138]. However, retransplantation rates seem to be higher for patients with PSC than other diagnoses [3].

Recurrence of PSC in the liver graft has been documented. Diagnosis of recurrence can be challenging, as non-specific bile duct injuries and strictures caused by allograft reperfusion injury, ischemia, rejection and recurrent biliary sepsis can mimic the findings of PSC post-transplantation and need to be carefully excluded before the diagnosis of recurrence can be established [139, 140]. The frequency of recurrent PSC after LT remains controversial. The frequency of recurrent disease is estimated between 10–20% of patients [141], but a recent systematic review has indicated that publication bias might be a concern regarding this topic [4]. PSC might recur earlier at a higher ratio after living donor LT, particularly when the liver graft is obtained from a biologically related living donor [142]. Proposed risk factors for recurrent PSC include, IBD, prolonged cold ischemia time, number of cellular rejection episodes, previous biliary surgery, cytomegalovirus infection, and lymphocytotoxic cross-match [4] but these require further investigation. As more liver transplant recipients survive longer, the recurrence of disease may become the primary cause of morbidity and mortality in PSC [4].

Disease-Related Malignancy

Cholangiocarcinoma

PSC carries an increased risk of hepatobiliary malignancy, especially cholangiocarcinoma (CCA) [12, 143]. An independent account on CCA is available in Chap. 60. The development of CCA is the most lethal complication of PSC. CCA can arise at any stage of PSC, although, in general, the incidence is higher in more advanced disease [9, 144]. There are no clinical features that predict the diagnosis of CCA, and diagnosis can be challenging because the cholangiographic changes may look similar to those found in PSC without CCA.

The cumulative life-time incidence of CCA is estimated at 6–23% [43]. The reported prevalence of CCA in explanted livers and autopsy is much higher, approximately 30–42% [13, 145]. Overall, up to 50% of CCA cases are detected synchronous with the PSC diagnosis or within 1 year of diagnosis of PSC [143, 146–149]. Based upon the same reported series, the incidence of CCA during follow-up, starting at 1 year after the diagnosis of PSC, can be calculated as being between 0.5–1.5% per year. The malignancy usually develops in the fourth decade of life, whereas CCA in patients without PSC usually develops much later in life, in their seventh decade of life [43].

Risk factors for the development of CCA in PSC have not been clearly identified, but older age, longer duration of IBD, and smoking behavior have been associated with an increased risk for development of CCA in patients with PSC. Finding of biliary dysplasia on liver histology has also been proposed as a precursor in the development of cholangiocarcinoma [150, 151].

The diagnosis of CCA can be challenging. The role of serum CA19–9 level in the diagnosis of CCA is controversial. There are no tumor markers which are specific for cholangiocarcinoma. In the context of PSC, a serum CA19–9 level greater than 100 U/mL has been reported to have a sensitivity of 75% and a specificity of 80% for presence of cholangiocarcinoma [152, 153]. A recent study from Mayo Clinic found that a serum level greater than 129 U/mL provided a sensitivity of 78.6% and specificity of 98.5% for CCA in PSC [154]. Even though these studies suggest CA 19–9 is an accurate test to diagnose

cholangiocarcinoma, CA 19–9 was only found to identify patients with advanced, unresectable CCA, and thus its use is not appropriate as a screening test [154]. Ultrasonography, computed tomography, and magnetic resonance have inadequate sensitivity to distinguish CCA from PSC. Endoscopic biopsy and biliary brushing for cytology, digital image analysis, and fluorescent in situ hybridization are noted for good specificity, but poor sensitivity in detecting CCA [42].

Patients with PSC and CCA have a very poor outcome, with median survival of approximately 5–11 months [43, 148, 155]. Even though survival of patients in whom CCA was found incidentally by histological examination of the explanted liver has been reported to be good [9], in general, LT for patients with CCA results in a low success rate [156–159]. However, more recent data from investigational protocols have suggested better outcomes in highly selected individuals. The use of pretreatment radiotherapy and subsequent capecitabine for 2–3 weeks prior to LT at Mayo Clinic has yielded a 3- and 5-year actuarial survival of 82% [160]. The use of brachytherapy and continuous 5-fluoracil infusion before LT in Nebraska resulted in 45% long-term cancer-free survival after follow-up for a median of 7.5 years [161]. Curative resection among individuals with early-stage CCA may also be of benefit in PSC [146], although recent data suggest that transplant with neoadjuvant chemoradiation with localized, node-negative hilar CCA may achieve better survival with less recurrence than conventional resection [160, 162].

Tumor serology combined with cross-sectional liver imaging may be useful as a screening strategy for patients with PSC, followed by cholangiography with cytology for diagnostic confirmation [163]. The digital imaging analysis (DIA) and fluorescence in situ hybridization (FISH) techniques to detect chromosomal alteration of individual cells in malignant pancreatobiliary strictures, when used as an adjunct to biliary cytology, can improve sensitivity and positive predictive value for detecting cancer, while maintaining an acceptable specificity [164]. Screening and surveillance for CCA in patients with PSC at Mayo Clinic had a great impact on patient survival, as two-thirds of cancers in patients undergoing surveillance were detected at an early stage of disease and the majority was treated with LT after neoadjuvant chemoradiotherapy.

Recent studies have suggested that the incidence of CCA in patients with PSC treated with UDCA is lower than expected and decreases with time of therapy [156, 165]. Further studies are needed to confirm this finding.

Hepatocellular Carcinoma

Although patients with cirrhotic stage PSC may also be at risk for developing hepatocellular carcinoma, this malignancy occurs infrequently [144, 149, 166]. For more details on the molecular basis of HCC, please see Chap. 56.

Colonic Dysplasia and Carcinoma

Whether the presence of PSC increases the risk of colonic dysplasia and carcinoma in UC is controversial [167, 168]. Patients with PSC and UC have been found to have an increased incidence of colonic carcinomas compared to patients with UC alone in a few studies [149, 169–171]; however, contradictory results have also been presented [172, 173]. The size, design, end-points, and populations involved in these studies have varied, and critical review suggests that colorectal cancer is more common in the setting of PSC [168]. Furthermore, PSC patients with UC remain at an increased risk for developing colorectal dysplasia and carcinoma after they have undergone LT [15, 174]. The immunosuppressive treatment after LT may have an impact on the development of cancer.

Two studies have indicated that UDCA reduced the incidence of colonic dysplasias and/or carcinomas [175, 176].

Gallbladder Neoplasia

Dysplasia, adenomas, and carcinoma of the gallbladder have been described in PSC, but are less common than cholangiocarcinoma. PSC is recognized as one of the major risk factors for both gallbladder and bile duct carcinoma [177]. A recent study reported statistically significant association between hilar/intrahepatic biliary neoplasia and gallbladder neoplasia, suggesting a "field effect" in the intrahepatic and extrahepatic biliary tree in PSC [178]. Identification of gallbladder polyps on cross-sectional imaging should lead to consideration for cholecystectomy [179]. Large studies on this subject have not been performed. For more information on carcinoma of gall bladder, please see Chap. 61.

Conclusions

PSC is a presumed immune-mediated liver disease of young men associated with significant morbidity and mortality. However, there is no proven medical treatment available for it. Further studies are needed for better understanding of the pathophysiology of the disease and for development of an optimal therapeutic strategy for patients with PSC to improve health related quality of life and halt progression of disease, thereby decreasing incidence of complications of advanced liver disease, and the need for transplantation.

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Chapter 51
Biliary Atresia

Jorge A. Bezerra

Introduction

Biliary atresia is a disease of *mosts*. It is the *most* common cause of neonatal cholestasis and the *most* frequent indication for liver transplant in the pediatric population worldwide, accounting for 40–50% of all liver transplants in children [1]. The health care costs associated with biliary atresia are significant, reaching $65 million/year in the USA alone [1]. Despite the obvious adverse impact to children's health, advances in understanding of the etiology and pathogenesis of biliary atresia have not kept pace with the progress in other cholestatic disorders of childhood [2]. Biliary atresia has been the *most* challenging pediatric liver disease to understand and treat. The lack of progress reflects the multifactorial nature of the disease, which has challenged physicians since it was recognized early in the nineteenth century [3].

Biliary atresia is also a disease with a *most* intriguing pathology that targets primarily the bile ducts, producing a destructive and rapidly fibrosing process that blocks its lumen and disrupts bile flow. Yet, the associated pathological findings of giant-cell transformation of hepatocytes, the persistence of the ductal plate postnatally, the rapid progression to hepatic fibrosis, and the coexistence of non-hepatic malformations point to a heterogeneous pathogenesis of disease. The clinical presentation shares features with other causes of neonatal cholestasis; therefore, the initial task is to make a specific diagnosis in a timely fashion so that a surgical relief by hepatoportoenterostomy can be pursued promptly. If the diagnosis is delayed or if surgical intervention is not pursued, persistent cholestasis will lead to biliary cirrhosis, portal hypertension, and end-stage liver disease at a rapid pace. Therefore, this chapter will present an overview of the epidemiology, clinical features, and treatment of biliary atresia, and focus on an in-depth discussion of the cellular and molecular mechanisms regulating pathogenesis of the disease.

J.A. Bezerra (✉)
Department of Gastroenterology, Hepatology and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA
e-mail: jorge.bezerra@cchmc.org

Epidemiology

Biliary atresia occurs worldwide and affects 1 in 8,000–15,000 live births [4, 5]. Studies have suggested a time-space clustering of cases and seasonal variation [6–9], associations with advanced maternal age and increased parity, and a tendency for early fetal losses [7, 8]. A slight female predominance (1.25:1) may be present among affected infants, particularly in the "embryonic" form of the disease [4, 10]. The majority of cases of biliary atresia are sporadic; the disease rarely recurs in families and twin studies have shown that most sets are discordant for the disease [11–15].

Clinical Features

At Presentation

Infants with biliary atresia share the cardinal features of jaundice, acholic stools, and hepatomegaly in the first few weeks of life. They often appear well and develop acholic stools, but in the early stages of disease, the stools may have some bile pigment; fever and other systemic signs or symptoms are not common. Family history of cholestasis is almost always negative in biliary atresia. On physical examination, hepatomegaly is frequently present. In a subset of patients, the liver is mostly palpable at midline (below the xyphoid process) and associated with pathologic heart murmurs, dextrocardia, or other congenital malformations. The presence of lethargy should trigger evaluation of clotting function and of the status of the central nervous system because coagulopathy due to vitamin K deficiency may result in intracranial hemorrhage. Further, poor feeding and recurrent emesis may signal the coexistence of intestinal malrotation or a severe cardiac defect (Table 51.1).

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_51, © Springer Science+Business Media, LLC 2011

Table 51.1 Main clinical features of biliary atresia

Typical triad at the time of diagnosis	Comment
Jaundice	Secondary to direct or conjugated bilirubin
Acholic stools	
Hepatomegaly	±Splenomegaly
Non-hepatic complications	
Easy bruising, CNS hemorrhage	Coagulopathy due to vit. K deficiency
Poor feeding, vomiting	Coexistence of cardiac defects
Recurrent (bilious) emesis	Coexistence of intestinal malrotation
Features of advanced disease	
Moderate to severe splenomegaly	Due to progressive fibrosis
Growth failure	Poor caloric intake, decreased absorption
Ascites, umbilical/inguinal hernia	Increased metabolic needs and Portal hypertension
Caput medusae	Portal hypertension
Coagulopathy	Not responsive to vit. K

During Progression of Disease

The liver disease will progress in most infants with biliary atresia, potentially at a slower pace if portoenterostomy is performed at an early age. With progression of disease, infants may develop ascites, bacterial peritonitis, and obstruction of portal blood flow (secondary to portal vein thrombosis). Sometimes, the presence of ascites is suggested by inappropriate weight gain or the development of inguinal or umbilical hernia. The first onset of ascites may be due to spontaneous bacterial peritonitis, even in the absence of fever or other systemic signs. Variable signs of malnutrition may develop, and infants may show dilated vascular collaterals ascending from the anterior abdominal wall toward the chest in a distended abdomen (caput medusae) because of intra- and/or extra-hepatic portal hypertension.

Recognizable Phenotypes of Disease

Perinatal Form of Biliary Atresia

Most infants have a jaundice-free interval after birth and develop jaundice, acholic stools, and dark-colored urine in the first few weeks of life. The development of jaundice following a jaundice-free period after birth suggests that the disease results from a perinatal or early postnatal insult that targets the bile ducts, leading to an inflammatory and rapidly fibrosing obstruction of the duct lumen. Thus, this form is commonly referred to as "perinatal" or "acquired" biliary atresia.

Embryonic Form of Biliary Atresia

About 10–15% of affected infants have non-hepatic malformation and an earlier onset of jaundice, often present since birth. This clinical variant is referred to as "embryonic," "congenital," or "fetal" form of biliary atresia. These infants may have complete absence of extrahepatic bile ducts, with no sign of a fibrous cord at the time of exploratory laparotomy. The genetic basis for this form may involve developmental defects in laterality (the asymmetric positioning of single organs). Analysis of the nonhepatic malformations identified laterality defects in 29% of the patients, isolated gastrointestinal or cardiovascular anomalies in 59%, and intestinal malrotation (with or without preduodenal portal vein) in 12%. Splenic abnormalities (asplenia, double spleen, and polysplenia) were present in ~8% of the infants, occurring in isolation or in combination with one or more additional defects in a variant known as biliary atresia splenic malformation (BASM) syndrome [10, 16]. Although infants with the embryonic form of biliary atresia have no obvious clinical or biochemical differences from the more common perinatal form at the time of diagnosis, 15% are born to mothers with diabetes [10], and as a group may have worse outcome following portoenterostomy, with decreased transplant-free survival by 2 years of age [10, 17, 18].

Cystic Variant of Biliary Atresia

Biliary cysts are present in anatomic variants in which atresia is restricted to the common bile duct and may be associated with improved bile drainage after portoenterostomy [19]. Some of the infants with biliary cysts are detected prenatally during routine ultrasound examination of the fetus; jaundice and acholic stools may present soon after birth or after a variable period of time. A recent review of a large cohort with biliary atresia reported the presence of biliary cysts in ~10% of patients [20]. In this series, infants with this cystic variant were younger at presentation, but a delay in performing a portoenterostomy beyond 70 days of age was associated with poor long-term survival with the native liver [20]. The anatomical details of this variant and the differences in outcome raise the possibility that it differs in pathogenic mechanisms of disease.

The application of molecular techniques to study patients that are carefully cataloged into individual clinical forms or variants may provide insight into the physiological basis for clinical/anatomical variations. They may also make it possible in the future to revise disease definitions to take into account the patient's biological make-up.

Diagnosis

The timely diagnosis of biliary atresia in the neonate with cholestasis is a high priority because the success of portoenterostomy to restore biliary flow rapidly declines with age at surgery [21–23]. This becomes a challenge due to the lack of clinical signs and laboratory tests that are specific for the disease, making it difficult to reliably distinguish between biliary atresia, other causes of extrahepatic obstruction, and intrahepatic cholestasis (Table 51.2). Therefore, the clinician needs to develop a diagnostic algorithm that incorporates ancillary tests with high predictive value for biliary atresia, but tailored to the expertise available in the center.

Laboratory Studies

Routine indicators of liver function and injury are helpful but not diagnostic. Common markers of hepatocellular injury, serum alanine and aspartate aminotransferases, are moderately elevated, whereas the serum levels of alkaline phosphatase and gamma-glutamyl transpeptidase (GTP) progressively increase, indicating more profound biliary injury. GTP levels may have some discriminatory value, with low GTP rarely seen in infants with biliary atresia [24–26]. If infants are evaluated before 4 months of age, conjugated or direct hyperbilirubinemia is present, but rarely above 7 mg/dL despite the existence of an obliterative fibrosis and severe impairment of bile drainage. Serum albumin levels are usually normal. Although some infants may have prolonged prothrombin time, normalization of coagulopathy following administration of vitamin K and serum levels of albumin above 3 g/dL indicate normal synthetic function.

Table 51.2 Differential diagnosis of neonatal cholestasis: Main clinical syndromes

Extrahepatic diseases
Biliary atresia
Choledocal cyst
Spontaneous perforation of the common bile duct
Neonatal sclerosing cholangitis
Biliary sludge and cholelithiasis
Intrahepatic diseases
Alpha-1-antitrypsin deficiency
The Alagille syndrome
Canalicular transport defects
FIC1 deficiency: Low GTP-form of familial intrahepatic cholestasis (type 1)
BSEP deficiency: Low GTP-form of familial intrahepatic cholestasis (type 2)
MDR3 deficiency: High GTP-form of familial intrahepatic cholestasis (type 3)
Cystic fibrosis
Defects in bile acid synthesis
Hypopituitarism/hypothyroidism
Infection – examples: Cytomegalovirus, enterovirus, herpes simplex virus

FIC1 Familial intrahepatic cholestasis type-1 protein; *BSEP* bile salt export pump; *MDR3* multidrug resistance protein type 3

Radiological Studies

A sonographic examination of the upper abdomen is particularly useful in the search for potential causes of anatomic obstruction or cystic abnormalities of the biliary system and to survey for congenital malformations, such as midline liver, polysplenia, and vascular malformations. Absence of the gallbladder suggests biliary atresia, but its presence does not rule it out. Examining the hilar structures, the ultrasonographic appearance of a "triangular cord" is suggestive of biliary atresia, with a negative predictive value of over 95% for intrahepatic cholestasis ("neonatal hepatitis"), but its identification varies considerably according to the level of expertise of the examiner. An evolving technology with a great potential to identify the extrahepatic ductular structures is magnetic resonance cholangiography, but its value remains unproven to date [27, 28]. Other techniques include hepatobiliary scintigraphy, visualization of extrahepatic bile ducts by endoscopic retrograde cholangiopancreatography, and aspiration of duodenal fluid [29–35]. Their use may be limited by false positives or the availability of experienced endoscopists for the careful examination of the neonate [32–35].

Histopathology

Microscopic examination of a liver biopsy sample is a critical component of the diagnostic approach to the neonate with cholestasis. The initial biopsy is obtained percutaneously and typically shows preservation of basic lobular organization, with prominent abnormalities in the portal tracts and, to a lesser extent, in the lobule. Portal tracts are expanded by edema, proliferation of bile ducts, and fibrosis (Fig. 51.1). When present, bile plugs within proliferated ducts are highly suggestive of biliary atresia but may not be always present. Inflammation in the portal space and giant cell transformation of hepatocytes may be seen, but are not the dominant features and can also be found in other causes of intrahepatic cholestasis [36]. Canalicular cholestasis, lobular disarray, and extramedullary hematopoiesis do not have discriminatory value between biliary atresia and other causes of neonatal cholestasis.

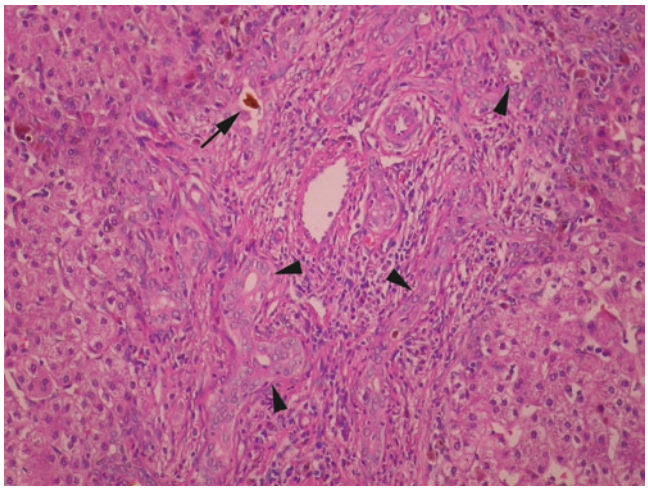

Fig. 51.1 Hematoxylin and eosin staining of a liver section obtained from a 2-month old infant at the time of diagnosis of biliary atresia depicting an expanded portal tract with increased profile of bile ducts (*arrowheads*), one of which containing intraluminal accumulation of bile ("bile plug"; magnification: 200×). Courtesy of Dr. Kevin Bove, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

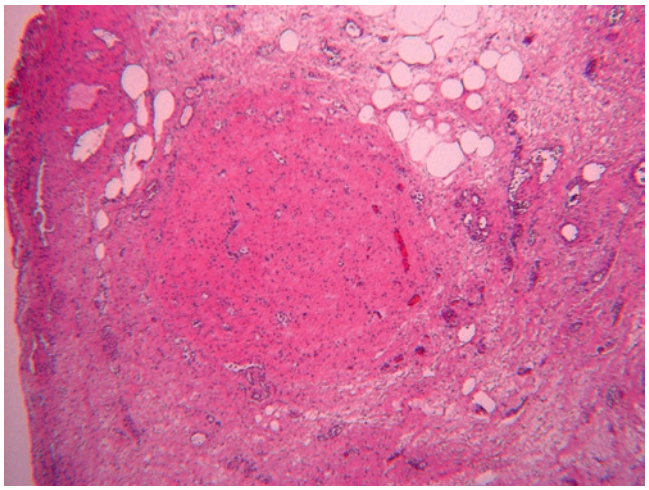

Fig. 51.2 Cross-section of an extra-hepatic bile duct remnant showing marked fibrosis, minute ductules, and vascular channels in a surgical specimen from an infant with biliary atresia. Courtesy of Dr. Kevin Bove, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

Exploratory Laparotomy and Cholangiography

When histopathologic features are suggestive of biliary atresia or the diagnostic workup is inconclusive, a direct examination of extrahepatic bile ducts must be performed in a timely manner, either through an exploratory laparotomy or via a laparoscopic approach. In most cases of biliary atresia, the gallbladder is small and fibrotic; if it has a lumen it may be filled with clear mucoid secretions. The extent of fibrosis along the extrahepatic system varies and has been grouped in three main types: type 1 atresia involves the common bile duct, type 2 extends up to the common hepatic duct, and type 3 atresia (the most common type) encompasses the entire length of extrahepatic bile ducts [37].

Histologically, the extrahepatic biliary remnants show the most typical finding of complete fibrous obliteration of the bile duct at one or more levels (Fig. 51.2). In some patients, small single-lumen ducts that are present in biliary remnants close to the hilum may show active cholangitis. In other patients, the main duct may be reduced to smaller channels surrounded by moderate chronic inflammatory infiltrate.

Pathogenesis of Disease

Any proposed pathogenic mechanisms of disease must take into account important clinical features that are exclusive to biliary atresia: (1) the onset of disease is restricted to the neonatal period; (2) the biliary system is the primary target; and (3) the lack of recurrence of hepatobiliary lesions typical of biliary atresia following liver transplantation. Although inflammation and fibrosis of the intra- and extrahepatic bile ducts are common in biliary atresia, conflicting evidence exists with regard to triggering events, pre- and postnatal timing of onset, and the factors that promote ongoing hepatobiliary inflammation. Based on patient-based studies, five mechanisms have been proposed: (1) a defect in morphogenesis of the biliary tract, (2) a defect in fetal/prenatal circulation, (3) exposure to environmental toxins, (4) viral infection, and (5) immunologic/inflammatory dysregulation (Table 51.3) [38].

Defect in Morphogenesis

The presence of ductal plate malformation in some livers at the time of diagnosis suggests that the pathogenesis of disease involves, at least in part, abnormalities of cell fate of the developing bile duct [39]. During normal development, the ductal plate undergoes remodeling to form tubular structures surrounded by thick mesenchyme between 11–13 weeks of gestation [40]. These findings share morphological similarities to abnormal ductules within the porta hepatis of livers from infants with biliary atresia, and suggest that abnormal mesenchymal support and improper remodeling of hilar ducts may be important pathogenic factors in early stages of disease development.

In addition to these anatomic considerations, the presence of poly- or asplenia, cardiovascular defects, abdominal situs inversus, intestinal malrotation, and anomalies of the portal vein and hepatic artery point to potential defects in embryogenesis and asymmetric left-right determination of visceral organs [41]. In support of this concept, abnormalities

Table 51.3 Cellular and molecular mechanisms linked to pathogenesis of biliary atresia

Mechanism	Human evidence	Animal-based data
Developmental defect	Onset of jaundice at birth Persistence of ductal plate[a] Coexistence of other embryonic abnormalities Gene polymorphisms in JAG1 and CFC1 genes	Extrahepatic biliary obstruction and situs inversus in the *Inv* mouse
Vascular defect	Vascular abnormalities Hepatic artery hyperplasia and hypertrophy	None
Infectious insult	Detection of reovirus, rotavirus, CMV, HHV6, HPV in affected children	Rotavirus-induced model of biliary atresia Reovirus cholangiopathy
Immunologic dysregulation	Hepatic expression of intercellular adhesion molecules and pro-inflammatory cytokines Hepatic infiltration by CD4+, CD8+ and NK lymphocytes; activation of macrophages Increased frequency of HLA-B12 allele Maternal chimerism Oligoclonal expansion of lymphocytes	Prevention of the biliary atresia phenotype in neonatal mice by loss of interferon-gamma, CD8+ cells, NK cells Cholangitis by transfer of activated CD3+ cells

[a]In some children with biliary atresia

CMV cytomegalovirus; *HHV6* human herpes virus-6; *HPV* human papillomavirus

in organ symmetry and bile ducts were identified in the *Inv* transgenic mouse. In this mouse, a recessive insertional mutation of the *Inversin* gene results in complete abdominal situs inversus, jaundice, poor weight gain, and death in the first week of life [42]. Polysplenia is also occasionally present. There is complete obstruction of extrahepatic bile ducts and proliferation of intrahepatic bile ducts, but the absence of inflammation or fibrosis makes this model differ from the typical histological features of biliary atresia [43, 44]. Furthermore, mutational analyses in children with laterality defects and biliary atresia have failed to identify mutations in the *Inversin* gene [45]. The potential role of other genes involved in laterality determination and pathogenesis of biliary atresia remains possible as suggested by reports of mutations in the *CFC1* gene in affected patients [46, 47].

The use of gene-inactivation technologies have identified several genes that play key roles in the development of intra- and extrahepatic bile ducts and, as such, emerge as candidates for a role in disease pathogenesis (Table 51.4). For example, inactivation of the gene encoding the hepatocyte nuclear factor (Hnf)-6 produces ductal plate malformation, agenesis of the gallbladder, and abnormal extrahepatic bile ducts [48]. Another intriguing phenotype is produced by the inactivation of the *Hairy and enhancer of split 1 (Hes1)* gene, which results in agenesis of the gallbladder and hypoplasia of the extrahepatic bile duct [49]. Jag1-Notch signaling pathways define a fundamental mechanism controlling cell fate during embryogenesis by modifying the ability of a broad spectrum of precursor cells to progress to a more differentiated state. Although the developmental abnormalities reported for heterozygous *Jag1/Notch2* mice are not similar to those of infants with biliary atresia, the *Jag1* gene may be a modifier of liver disease, as demonstrated by the identification of a high frequency of single-nucleotide polymorphisms of the *JAG1* gene in infants with biliary atresia with poor outcome [50]. Taken together, these data suggest that genetic factors governing morphogenesis of the biliary system may play an important role in development and/or progression of liver disease in biliary atresia.

Table 51.4 Molecular circuits controlling morphogenesis of the biliary system [138–143]

Protein	Biliary phenotype in mutant mice
Jagged-Notch pathway	Abnormal morphogenesis of IHBD
Hes1	Hypoplasia of EHBD Agenesis of gallbladder
Hnf6	Ductal plate malformation and intrahepatic biliary cysts Dilated EHBD Agenesis of gallbladder
Hnf1β	Paucity of small IHBD and dysplasia of large IHBDConstrictions of EHBD Gallbladder with abnormal epithelium and dilated cystic duct
Foxf1	Small or absent gallbladder and no epithelial cells
Foxm1b	Agenesis of IHBD
Foxa1/Foxa2	Hyperplasia of IHBD
Sox17	EHBD with ectopic pancreas Absent gallbladder
Lgr4	Hypoplasia of gallbladder and cystic duct

IHBD intrahepatic bile ducts; *EHBD* extrahepatic bile ducts

Defect in Fetal/Prenatal Circulation

Early interruption of flow in the hepatic artery, which supplies the intra- and extrahepatic bile ducts, has been proposed to be an initiating factor in the fibroinflammatory injury of biliary atresia. This is an attractive concept based on the

presence of hepatic artery and portal vein abnormalities associated with biliary atresia and the arterial hyperplasia, and on hypertrophy described in liver specimens of affected infants [51, 52]. However, additional data from humans or the development of experimental models to study the impact of blood flow on biliary development are necessary to further validate the relationship between arterial blood flow and injury to the developing bile duct.

Exposure to Environmental Toxins

To date, the only supportive patient-based evidence for a role of a toxic insult as a causative factor of biliary atresia is the time-space clustering of cases. In animals, unusual outbreaks of hepatobiliary injury in lambs and calves in New South Wales, Australia, occurred in 1964 and 1988, with pathologic specimens displaying features akin to the pathology seen in humans with biliary atresia. Despite the localized geographic distribution of the outbreaks, an extensive investigation for causative phytotoxins or mycotoxins was unrevealing [53].

Viral Infection

The possible role of viral infection as an initiating event in the pathogenesis of biliary atresia was initially suggested by Landing, who proposed that the biliary atresia phenotype results from viral exposure and resides along a continuum with neonatal intrahepatic cholestasis and choledocal cyst [54]. Consistent with this concept, different viruses have been detected in the liver of infants with biliary atresia, including hepatitis A, B and C viruses, cytomegalovirus, retrovirus, human papillomavirus, human herpes virus 6, reovirus, and rotavirus in specific groups of patients, but recovery of individual viruses has been inconsistent in different populations [55–73]. This notwithstanding, studies of livers of patients with biliary atresia have shown a molecular signature typical of the innate and adaptive immune responses to a viral challenge [74]. Among the potential viruses, reovirus type 3 and rotavirus type C continue to emerge as potential agents for biliary atresia.

Reovirus type 3 IgG and IgM were found in infants with biliary atresia [62, 65, 66, 75], but at least two studies could not find such an association [71, 76]. Using reverse transcription-polymerase chain reaction, reovirus was found in hepatobiliary samples of 55% of patients with biliary atresia and 78% with choledocal cyst, whereas the virus was present in tissues of only 8–21% of appropriately matched controls [69]. The putative association between reovirus and biliary atresia was initially suspected based on studies in young mice, which showed that reovirus infection in the weanling period resulted in the "oily fur syndrome," which is marked by growth failure, jaundice, and oily fur. Histologically, reovirus induced hepatitis and intra- and extrahepatic biliary epithelial-cell necrosis with surrounding edema and inflammation [77–79]. With repeated intraperitoneal injections, weanling mice developed fibrosis of the extrahepatic biliary tree, but did not progress to irreversible luminal obstruction [77]. However, if reovirus is administered soon after birth, there is no evidence of lesions in extrahepatic bile ducts resembling biliary atresia [78].

There is limited evidence from human studies supporting a role for rotavirus in the etiology of biliary atresia due to the inability to identify the virus in affected livers in a consistent fashion [67, 70]. However, administration of rotavirus to newborn mice consistently produces an injury to extrahepatic bile ducts and the biliary atresia phenotype [80, 81]. Interestingly, rotavirus has tropism for the liver and bile ducts when administered in the immediate postnatal period, but cannot be recovered from either tissue after the atresia phenotype is fully established (by the second week after infection) [82]. Thus, the inability to consistently identify rotavirus in affected human livers and/or bile ducts does not rule out the contribution of rotavirus to the etiology of biliary atresia.

Immune-Mediated Injury

Human Studies

Several patient-based studies suggest that the inflammation observed in the liver and bile ducts plays a significant role in the pathogenesis of biliary injury and obstruction. For example, cholangiocyte pyknosis and necrosis have been associated with infiltration of mononuclear cells and lymphocytes into the walls of interlobular bile ducts, duct walls at the porta hepatis, and remnants of extrahepatic bile ducts [83–85]. Some of these cells were CD8+ T lymphocytes, although they did not express perforin or granzyme B, markers of activated cytotoxic T lymphocytes [86]. CD4+ cells also populate affected livers and are associated with markers of pro-inflammatory activation, such as interferon-gamma (IFNγ), interleukin-2, the interleukin-2 receptor CD25, tumor necrosis factor-alpha (TNFα), and the transferrin receptor CD71 [87–90]. Additional evidence for an effector role of T lymphocytes was recently suggested by the findings that CD4+ and CD8+ T cells from liver and bile duct remnants displayed expansions of specific subsets of the T cell receptor variable regions of the β-chain (Vβ), with oligoclonal populations of T cells [91].

Further evidence of active Th1 proinflammatory immunity was provided by large-scale gene expression analyses of liver tissue from infants with biliary atresia and neonatal intrahepatic cholestasis. The approach identified a genetic footprint in which genes involved in lymphocyte differentiation (including the regulators of Th1 response osteopontin and IFNγ) are activated at early stages of biliary atresia, with simultaneous but transient suppression of markers of humoral immunity [92]. In addition, a potential role for Kupffer cells (resident hepatic macrophages) in promoting inflammation and fibrosis has been inferred from their increased number and size in the livers of infants with biliary atresia, as well as from their expression of the MHC class II antigen HLA-DR [88, 93, 94].

Cholangiocytes, which normally express MHC class I but not class II antigens, have been shown to aberrantly express human leukocyte antigen (HLA)-DR (a major MHC class II molecule) and may act as antigen-presenting cells [87, 95, 96]. They also express intercellular adhesion molecule-1, whereas one of its ligands, leukocyte functional antigen-1, is expressed on mononuclear cells infiltrating the duct epithelium [88, 97]. Interaction between these two molecules may facilitate inflammatory cell recruitment and foster amplification of the immune response.

Animal Studies

Greater support for a role of the immune system in the pathogenesis of biliary atresia derives from the use of the rotavirus mouse model in mechanistic studies that directly examined the role of specific genes and cell types in the expression of the disease phenotype. The applicability of this animal model is supported by the clinical, cellular, and molecular features that it shares with the human disease (Table 51.5). Initial studies used the animal model to directly explore whether the increased expression of IFNγ in human livers was a nonspecific response or part of the mechanisms of biliary injury. This was done by investigating the development of the atresia phenotype in mice carrying the genetic loss of IFNγ. Deficiency of IFNγ prevented the obstruction of bile ducts, improved symptoms, and fostered long-term survival of newborn mice infected with rotavirus [82]. Histological analysis showed inflammation along the wall of extrahepatic bile ducts, but there was no obstruction of the lumen, suggesting that this cytokine may promote lumenal obstruction by amplifying the inflammatory response. The same impact on the duct phenotype cannot be assumed for all cytokines. For example, deficiencies of interleukin-12 and TNFα have been shown not to prevent duct obstruction or the atresia phenotype after rotavirus infection [98, 99].

Following reports that CD4+ and CD8+ T cells populate livers at the time of diagnosis, individual cell types were depleted from neonatal mice soon after challenge with rotavirus [100]. The loss of CD4+ cells decreased the Th1 signature, but had no impact on the development of the biliary atresia phenotype. In contrast, the loss of CD8+ cells prevented duct obstruction after rotavirus infection while allowing the development of cholangitis. The similarities between the improved phenotypes produced by the loss of CD8+ cells or IFNγ suggest that both factors work in synergy to promote duct obstruction [82, 100]. Perhaps a more dramatic effect on the atresia phenotype was recently produced by depletion of NK cells [101]. In this work, investigators first quantified the mononuclear cells in the extrahepatic bile ducts of neonatal mice. Among these cells, NK lymphocytes were the most abundant. When primed by rotavirus, NK cells were able to recognize and injure cholangiocytes by direct contact via the Nkg2d receptor. Most notably, depletion of NK cells or antibody blocking of Nkg2d immediately after birth rendered mice resistant to rotavirus-induced biliary atresia. Histological analysis of the duct showed no cholangitis and the mucosa appeared intact. Without duct obstruction, mice had adequate growth and long-term survival despite viral infection [101].

Table 51.5 Similarities in clinical, histological, and immunological features of biliary atresia between humans and the experimental mouse model

Feature	Human disease	Mouse model
Onset of symptoms	Restricted to neonatal period	Restricted to neonatal period
Jaundice	Yes	Yes
Acholic stools	Yes	Yes
Virus etiology	Rotavirus, reovirus, HPV, CMV, others	Rotavirus
Bile duct proliferation	Yes	Yes
Portal inflammation	Yes (variable intensity)	Yes
Fibrosis	Common at presentation	Not present until late in disease
Epithelial injury in EHBD	Yes	Yes
Obstruction of EHBD	Yes	Yes
Non-hepatic anomaly	Yes (in the embryonic form)	No
Hepatic CD4+ cells	Increased + Th1 program	Increased + Th1 program
Hepatic CD8+ T cells	Increased, activated	Increased, activated
Hepatic macrophages	Increased, activated	Increased, activated
Apoptosis	Biliary epithelium	Biliary epithelium
Molecular signature	Liver: Th1-like network EHBD: Not studied	Liver: Th1-like network EHBD: Th1-like network

HPV human papilloma virus; *CMV* cytomegalovirus; *EHBD* extrahepatic bile ducts

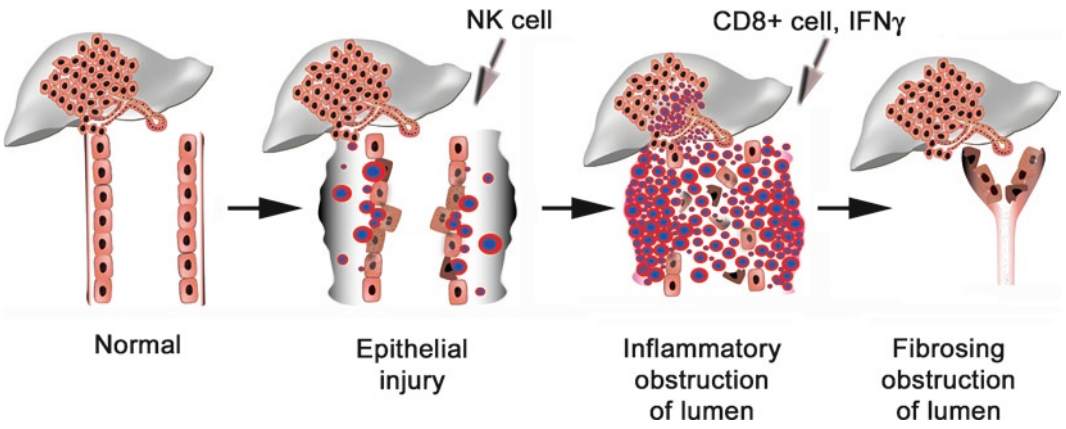

Fig. 51.3 Biological continuum of pathogenesis of biliary atresia based on studies in the rotavirus-induced mouse model. In early stages of disease, an exogenous insult (e.g.,: virus) infects cholangiocytes and triggers a NK lymphocyte-based epithelial injury to the duct mucosa. This is followed by an exuberant inflammatory response driven by CD8+ T lymphocytes and pro-inflammatory cytokines (example: IFNγ), and progression to atresia by fibrosis (modified from [102])

The combined genetic and cell depletion studies suggest that the pathogenic mechanisms of biliary atresia may obey a biological continuum previously not recognized. The mechanisms involve an environmental exposure (a "trigger"; example: rotavirus) that injures the bile duct epithelium directly and through NK cell-mediated cytotoxicity ("initiating" phase). This is followed by an expansion of the inflammatory response by the expression of proinflammatory cytokines (such as INFγ) and the activation of CD8+ cells that occlude the lumen ("obstruction" phase), and the deposition of extracellular matrix with rapid progression to atresia of bile ducts ("fibrosing" phase; Fig. 51.3) [102].

Treatment

The lack of progress in deciphering the pathogenic mechanisms of biliary atresia in humans has limited the development of effective medical therapies. Undoubtedly, recent studies using the rotavirus-induced mouse model identified specific cellular and molecular effectors that are required for the development of the atresia phenotype. However, these effectors need to undergo validation in humans before they can be targeted in trials designed at blocking progression of the liver disease. Currently, the treatment of infants includes portoenterostomy and post-surgical supportive care to optimize bile drainage and long-term outcome.

Portoenterostomy

The only therapeutic choice to increase biliary flow and improve jaundice is portoenterostomy. This surgical procedure, designed by Kasai and Suzuki in late 1950s, consists of a careful dissection of the biliary tract to the level of the porta hepatis, removal of (rudimentary) gallbladder and biliary remnants, and the anastomosis of a jejunal segment to create a conduit in a Roux-en-Y fashion to drain any bile that flows into patent ducts at the porta [103] (For more detailed description of the operation, see [104].).

Medical Treatment Following Portoenterostomy

The goals of postoperative management of infants with biliary atresia are threefold: (1) prevention of cholangitis, (2) stimulation of choleresis, and (3) nutritional support. Infants typically receive parenteral broad-spectrum antibiotics perioperatively and for 2–5 days after surgery, followed by oral prophylaxis with trimethoprim-sulfamethoxazole (TMP-SMX; 5 mg TMP/kg/day) or another antibiotic for 3–12 months [37, 105]. Ursodeoxycholic acid (UDCA; 10–20 mg/kg/day), a more hydrophilic bile acid, has been used by many centers to improve choleresis. Although a few studies have documented the efficacy of UDCA in promoting choleresis, weight gain, and decreased pruritus [106, 107], it has no obvious impact on long-term survival or the need for transplant between treated and nontreated patients [108]. Lastly, corticosteroids are used post-operatively in some centers, with dosing regimens and duration of treatment varying remarkably among published reports [109–113]. However, more recent reports failed to show benefit from this form of therapy [114, 115]. Therefore, future multi-center randomized controlled clinical trials are required to objectively determine the role of corticosteroids in the treatment of infants following portoenterostomy.

Infants should receive approximately 125% of the Recommended Dietary Allowance based on weight for height at the 50th percentile, with additional calories often needed if biliary drainage is marginal. If cholestasis is present, infants require supplementation and close monitoring to prevent the consequences of vitamin deficiencies: (1) vitamin A dosed at 5,000–25,000 IU/day, (2) vitamin D at 1,200–4,000 IU/day, (3) vitamin E at 25 IU/kg/day (in a miscible form: D-alpha-tocopheryl polyethylene glycol 1,000 succinate/TPGS), and (4) vitamin K at 2.5 mg 3 times/week. Doses must be adjusted based on serum levels of specific vitamins and prothrombin time/international normalized ratio (for vitamin K). Unfortunately, malnutrition frequently develops with persistent cholestasis, liver disease progresses despite adequate nutritional support, and coagulopathy not responsive to vitamin K supplementation develops late in the course of the liver disease.

Complications and Sequelae

Most infants with biliary atresia have successful bile drainage after portoenterostomy, but adequate bile flow may be transient. If bile drainage is not achieved, ongoing cholestasis and rapid progression to end-stage liver disease are the rule, at which time, orthotopic liver transplantation offers the only chance for long-term survival. Unfortunately, the inflammatory and fibrosing processes continue in the liver in most patients, even when there is adequate bile drainage (i.e., no jaundice). In these patients, the clinical course is variable and is often marked by episodes of cholangitis and progressive fibrosis, which lead to sinusoidal obstruction and portal hypertension.

Cholangitis occurs in up to 60% of infants with successful bile drainage after portoenterostomy [37, 116]. One strategy of unproven efficacy, but in widespread use, is the prophylactic administration of TMP-SMX during the first year of life to prevent cholangitis. Surgical modifications of the Roux-en-Y have not consistently decreased the incidence of cholangitis [37]. Infants present with the triad of fever, acholic stools, and increased levels of serum bilirubin. Individual symptoms can also occur in isolation; therefore, the clinician must keep a high degree of suspicion so that prompt diagnosis is made and antibiotic therapy is initiated. Biochemically, conjugated bilirubin rises and levels of serum aminotransferases, GTP, and alkaline phosphatase may increase above baseline values; leukocytosis with or without immature cells ("left shift") may also be present. Antibiotic treatment should be initiated promptly.

With progression of liver fibrosis, portal pressure gradually increases and significant portal hypertension develops in 34–76% of infants with biliary atresia [117, 118], with esophageal bleeding occurring in approximately 19% of all long-term survivors [119, 120]. Primary therapy for children with biliary atresia and hemorrhage from esophageal varices is endoscopic injection sclerotherapy (in the very young infant) or variceal band ligation [121–123]. Intractable bleeding requires prompt surgical intervention directed at ligation of varices and creation of a porto-systemic shunt; for those children with recurrent bleeding, prompt evaluation for liver transplantation is in order. Beta-blockers represent another therapeutic option for primary prophylaxis or after an episode of variceal hemorrhage (secondary prophylaxis). However, although their use is well accepted in adults, there are few data regarding the efficacy of beta-blockers in children. Other complications include hepatopulmonary syndrome, cystic dilatation of intrahepatic bile ducts (forming bilomas), hepatocellular carcinoma, and portopulmonary hypertension (a rare, severe complication that may develop in those patients that survive into adulthood with the native liver).

Long-Term Outcome and Liver Transplantation

Long-Term Outcome

Actuarial survival with native liver has been estimated at 32–61% at 5 years and 27–54% at 10 years of life [37, 124, 125]. Among the several factors that may influence long-term outcome, age and the size of the ductules in biliary remnants at the time of portoenterostomy have been systematically examined. Studies point to better outcome when portoenterostomy is performed in infants <60 days of age and when the size of ductules within the biliary remnant measures ≥150 μ(mu)m. Other factors that influence long-term outcome include episodes of cholangitis after surgery, the decade when surgery was performed, and the experience of the surgical team [124, 126]. An effect of the "center experience" in achieving high success rates has been emphasized by an improved survival rate to 78% in centers in which a large number of portoenterostomies are performed by the surgical team [6, 127, 128].

Liver Transplantation

Access to transplantation by a greater number of children has been driven by remarkable improvements in surgical techniques, which have led to the development of reduced-size and living-related donor transplant and to improved immunosuppression

[129–131]. As a consequence, biliary atresia has become the most frequent indication for liver transplant in children [132–136]. For example, biliary atresia has been the primary indication for liver transplantation at Cincinnati Children's Hospital Medical Center (July 1985 to August 2009), accounting for 50.5% of 395 transplanted children. The primary indications for transplant evaluation are: (1) persistent cholestasis associated with severe malnutrition, growth failure, and hepatocellular dysfunction and (2) decompensated cirrhosis, as evidenced by intractable ascites and hemorrhage. The use of liver transplantation has increased the overall long-term survival to >80%. For all children, the overall goals of liver transplantation include restoration of hepatic function, improved nutritional status, adequate growth and development, and improved quality of life with full social reintegration [136, 137].

Acknowledgment This work was supported by the NIH grants DK64008 and DK83781. Dr. Bezerra is the Cincinnati Principal Investigator of the NIDDK-funded Childhood Liver Disease Research and Education Network (NIH grant DK62497).

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Part VI
Molecular Pathobiology of Neoplastic Hepatobiliary Diseases

Chapter 52
Benign Liver Tumors

Jessica Zucman-Rossi

Introduction

Focal nodular hyperplasia (FNH) and hepatocellular adenomas (HCA) are the two major types of hepatocellular benign tumors. They are defined by a benign proliferation of hepatocytes, but in the clinical practice, these lesions may be sometimes difficult to diagnose from well-differentiated hepatocellular carcinomas (HCC) [1, 2]. Recently, different molecular pathways, specifically altered in FNH and HCA, have been identified. Moreover, analysis of the genotype-phenotype correlation in HCA also enabled the identification of well-defined sub-types of adenomas leading to propose a new molecular classification of these tumors. Actually, this new molecular classification provides robust foundations to better understand bases of the benign hepatocellular tumorigenesis including its relationship with the malignant transformation. It is also an important step in the search of novel markers specific to these tumor subtypes that could be used in clinical practice for diagnosis or prognosis. In this chapter, we will review the recent progress we performed in the molecular characterization of FNH and HCA according to the clinical and pathological features of each defined subgroup.

Focal Nodular Hyperplasia

Clinical and Pathological Characteristics

FNH was first described by Edmondson [3] and is the second most frequent benign liver tumor after hemangioma. It usually occurs in women between 20–50 years old [4]. In most of the studies, oral contraceptive use is not significantly associated with a significant increased risk of FNH occurrence; however, estrogens could increase the size of the nodules [5–7]. Typical FNH are characterized by a central stellate fibrous region containing malformed vascular structures [8] (Fig. 52.1). The solitary central artery with high flow and the absent portal vein give the lesions their characteristic radiological appearance in more than 80% of the FNH cases [9, 10]. However, some true FNH at pathological examination may have no detected scar, particularly small nodules measuring less than 3 cm in diameter. In all cases, the treatment of FNH is conservative because there is very low risk of secondary effect and malignant transformation has not been clearly reported [11].

FNH usually occurs in normal or sub-normal liver [8]. The lesion is multinodular composed of normal hepatocytes arranged in 1–2-cell-thick plates. Bile ductules are usually found at the interface between hepatocytes and fibrous regions [1, 12]. It is thought that increased arterial flow hyperperfuses the local parenchyma leading to secondary hepatocellular hyperplasia. FNH is, therefore, considered the result of a hyperplastic response to increased blood flow rather than a neoplastic process [8, 13, 14]. Various vascular abnormalities such as hereditary hemorrhagic telangiectasia, arteriovenous malformation, and abnormal venous drainage may be associated especially in patients with multiple FNH.

Epigenetic and Genetic Features in FNH

Clonal analysis using either the HUMARA test, or analyzing chromosome gains and losses demonstrated the reactive polyclonal nature of hepatocytes in FNH in 60–100% of the cases, depending on the series [15–25] (Table 52.1). In the remaining cases, a possible clonality of the hepatocytes may be related to local clonal patch effects. In the search for gene mutations, altering oncogenes or tumor suppressor genes known to be involved in liver tumorigenesis, FNH, did not demonstrate somatic gene mutation either in CTNNB1, TP53, HNF1A, GP130 or adenomatous polyposis coli (APC) [19, 23, 26–28]. Recently, specific patterns of miRNA expression have been identified in benign hepatocellular tumors [29]. Among the 250 tested miRNA, we observed a

J. Zucman-Rossi (✉)
Department of Oncology, Inserm U674, Université Paris Descartes
27 rue Juliette Dodu, Paris 75010, France
e-mail: zucman@cephb.fr

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5 ,
DOI 10.1007/978-1-4419-7107-4_52, © Springer Science+Business Media, LLC 2011

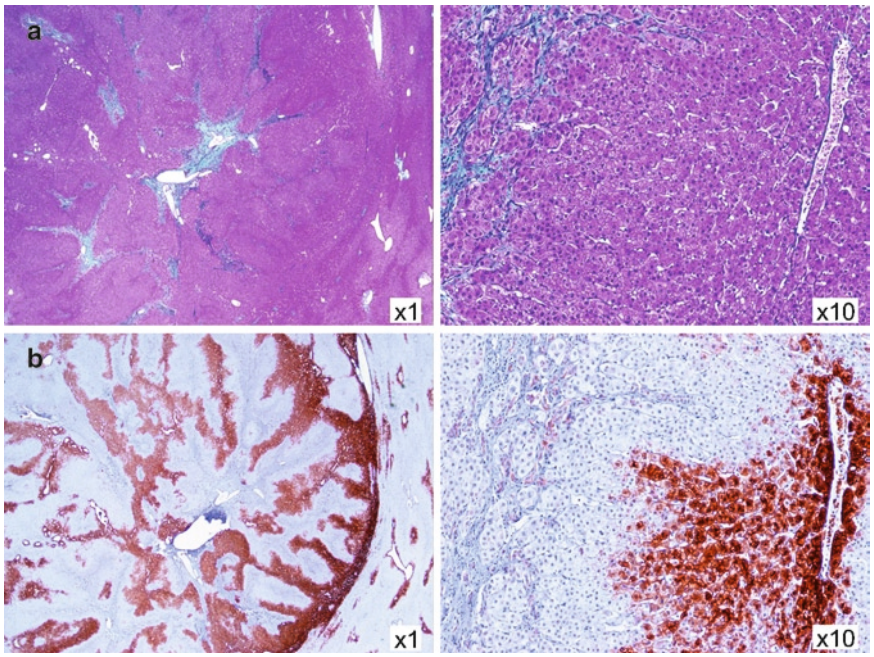

Fig. 52.1 β-catenin activation in FNH. (**a**) Serial sections of FNH stained with Masson's trichrome (**a**) and immunohistochemistry using GS antibody (**b**). Typical aspect of GS staining in a "map-like" pattern (*lower left*); this large GS+ area is centered on a central vein and remains at a distance from the fibrous band colored in *blue* by trichome

Table 52.1 Summary of FNH clonality analyses (adapted from Rebouissou et al [27].)

Published studies	Number of analyzed cases	Monoclonal lesions (%)
Gaffey et al. [15][a]	8	6 (75)
Paradis et al. [18][a]	13	0
Chen et al. [16][a]	1	1
Bioulac-Sage et al. [19][a, b]	18	7 (38)
Zhang et al. [17][a]	1	0
Chen et al. [23][b]	6	3 (50)
Raidl et al. [20][a]	3	1 (33)
Kellner et al [21][a]	7	1 (14)
Nakayama et al. [25]	1	0
Heimann et al. [22][c]	1	1
Gong et al. [24][a]	9	0
Overall studies	*68*	*20 (29)*

[a]HUMARA test

[b]Gain or loss of chromosome by CGH or allelotype

[c]Karyotype

global down-regulation of the miRNA expression in FNH when compared to normal livers or HCC [29].

Signaling Pathways Altered in FNH

A recent study identified that ANGPT1 and ANGPT2, two genes coding for angiopoietins, had an altered mRNA expression in FNH suggesting that angiopoietins may be involved in the regulation of vasculogenesis in FNH [30]. Moreover, the ANGPT1/ANGPT2 ratio of expression is highly increased in FNH compared with normal liver, HCC, or adenoma. However, this ratio of expression is also slightly increased in cirrhosis, regenerative, and dysplastic nodules [28]. In contrast, the overexpression of the NTS transcript, which encodes neurotensin, was observed specifically in FNH. The ratio of gene expression NTS/HAL (Histidine Ammonia-Lyase) was fully specific and sensitive to discriminate FNH from the other benign tumors [28].

Genome-wide transcriptomic analysis enabled to identify new signaling pathways altered in FNH [28]. First, activation of the TGFβ pathway activation was identified in cells located around the central fibrous scar. We also identified β-catenin activation with a heterogeneous distribution along the hepatocellular nodules; in FNH nodules, β-catenin activation follows a characteristic architectural distribution over relatively large areas, sometimes centered on visible veins and usually remaining at a distance from fibrous bands (Fig. 52.1). Recently, β-catenin was identified as a main actor of hepatocyte proliferation, liver regeneration, hepatocyte metabolism, liver development, and as a master regulator of liver zonation [31–36]. In normal hepatocytes, β-catenin and its known target genes, such as glutamine synthetase (GS), are expressed exclusively in the immediate perivenous areas [37, 38]. The specific β-catenin expression pattern observed in FNH may be interpreted as the result of an altered hepatocyte zonation in this tumor leading to increased β-catenin gradient starting from the veins. Because FNHs are most frequently polyclonal tumors, it is not surprising to find a heterogeneous distribution of β-catenin pathway

activation in the absence of gene mutation contrasting to that observed in β-catenin–mutated HCA or carcinoma, which usually shows more diffuse cytoplasmic GS staining and nuclear β-catenin localization [15, 17–19, 39].

Hepatocellular Adenomas

Clinical and Pathological Features of HCA

Hepatocellular adenoma (HCA) is a rare benign monoclonal tumor occurring in 85% of the cases in women between 20–50 years [40, 41]. Its occurrence is closely associated with oral contraception and 80% of HCA are developed in women taking oral contraceptives (OC) during more than 2 years [41–44]. This relationship may contribute to explain how HCA prevalence varies according to countries: HCA are more frequent in Europe and North America than in Asia where OC use is infrequent. HCA development can also be rarely related to other risk factors such as: androgenic-anabolic steroids intake [45–47], glycogen storage disease type 1 [48–50] and familial polyposis coli [26, 51, 52]. Proliferating hepatocytes usually resemble normal cells that may be steatotic or show glycogen storage. The tumor is also characterized by the lack of frequent mitosis, portal tract and cholangiolar proliferation [1, 12]. HCA nodules are more frequently solitary, but two or three nodules occasionally develop simultaneously. The development of more than ten HCA nodules is rare and has been specifically defined as adenomatosis by Flejou and collaborators in 1985 [53].

Two major complications of HCA may arise: (1) the risk of bleeding (ap. 25% of HCA cases) is related to the large size of the nodule (>5 cm) [42, 54]; (2) malignant transformation into HCC is estimated to 5–7% of the cases [55–57]; it is particularly high in glycogenosis and in patients using androgenic-anabolic steroids [46, 58–60]. The risk of malignant transformation also varies according to the HCA subtype (see below).

Recently, HCA has been described as a heterogeneous disease including at least three main subtypes of tumors in which histologic phenotypes are closely related with specific genetic alterations and clinical features [61–63].

First Subtype of Adenoma: HNF1α Inactivated HCA (H-HCA)

The most frequent group of HCA is defined by the biallelic inactivating mutations of the Hepatocyte Nuclear Factor 1α (*HNF1α*) gene representing about 35% of all HCA [64]. These tumors are phenotypically characterized by a marked steatosis [61, 63, 64]. In 90% of the cases, H-HCA are sporadic lesions displaying somatic mutations. However, in rare families with an inherited mutation in one allele of *HNF1α*, MODY3 (maturity onset diabetes of the young type 3) patients are predisposed to develop familial liver adenomatosis which could be clinically difficult to manage [53, 64–66]. Thus, *HNF1α* meets the genetic criteria of a tumor suppressor gene [64]. However, liver adenomatosis is a rare complication of MODY3 and its development may be promoted by heterozygous germline mutation inactivating CYP1B1, a key metabolism enzyme responsible for the formation of hydroxylated and genotoxic metabolites of estrogen [67].

HNF1α is an atypical homeodomain-containing protein that was originally identified as a hepatocyte-specific transcriptional regulator [68]. In vivo and in vitro models of HNF1α inactivation demonstrated that this transcription factor plays an important role in hepatocyte differentiation and is also crucial for metabolic regulation and liver function [69, 70]. Interestingly, and consistent with the identified tumor suppressor function of HNF1α in humans, *hnf1α*-deficient mice develop a dramatic liver enlargement that has been associated with increased hepatocyte proliferation, sometimes accompanied by dysplasia [69, 71]. However, the role of HNF1α in the control of cell proliferation and/or survival in hepatocytes is still poorly understood.

Transcriptomic analysis of H-HCA enabled us to identify a specific induction of glycolysis and lipogenesis in these tumors [72]. In H-HCA, this aberrant promotion of lipogenesis that is linked to HNF1α inactivation and that is independent of both SREBP-1 and ChREBP activation is the major mechanism of the tumor steatotic phenotype [72]. In a more recent study, we showed that H-HCA also displayed overexpression of several genes encoding growth factor receptors, components of the translation machinery, cell cycle and angiogenesis regulators with particularly, an activation of the mTOR pathway. Moreover, estradiol detoxification activities were shutdown suggesting a "hyper-sensitivity" of H-HCA to estrogenic stimulation. In cell model, inhibition of HNF1α recapitulated most of these identified transcriptional deregulations demonstrating that they were related to HNF1α inhibition [73]. Finally, a miRNA expression profiling enabled to identify a down-regulation of miR-107 specifically in H-HCA [29]. In addition, using a cell model, we showed that the expression of miR-107, which is implicated in lipid metabolism, is controlled by the level of HNF1α expression.

Second Subtype of Adenoma: β-Catenin Activated Adenomas (βHCA)

The sub-group of β-catenin activated adenoma (βHCA) is less usual, representing about 15–20% of the adenoma cases [39, 40, 61, 63]. HCA are rare in males; however, in βHCAs,

we observed an over-representation of males when compared to the other HCA subtypes. This group of βHCA presented other specific characteristics: βHCA frequently showed cytological abnormalities and pseudo-glandular formation since they were observed in 69% of the cases, whereas steatosis was very unusual. Importantly, for the clinical practice, βHCA was frequently diagnosed as borderline lesions between adenoma and HCC or associated with HCC [63]. Whereas true benign adenoma activated for β-catenin does exist, the highest risk of malignant transformation has been confirmed in other studies [40, 61, 74].

β-catenin mutations and the subsequent activation of the Wnt signaling have been first identified in a wide variety of human tumors and they are found in about 25–40% of HCC. In β-catenin mutated HCA, the spectrum of β-catenin mutations is similar to that observed in HCC and hepatoblastomas with frequent small in-frame deletions of exon 3 that exclude the amino acids used to be phosphorylated by GSK3β [27, 39, 61, 63]. No, or very infrequent, mutations of other genes involved in the WNT/β-catenin pathway, such as APC or the Axin family genes, have been identified in sporadic adenomas [39, 75]. In contrast to the other subtypes of HCA, βHCA were often associated with malignant transformation [61, 63]. We did not find any HCA cases with both β-catenin mutations and biallelic inactivation of HNF1α, suggesting that these two tumorigenic pathways are mutually exclusive. Moreover, most of the genes known to be targeted by β-catenin in hepatocyte were found overexpressed in βHCA, and some of them have been suggested as possible important drivers of hepatocarcinogenesis. Particularly, in accordance with previous findings in HCC, βHCA demonstrate several deregulations in amino acid metabolism suggesting an increased glutamine synthesis rate and a reduced amino acid catabolism (unpublished data). By immunohistochemistry, most of the βHCA cases demonstrate a homogeneous overexpression of GS, usually in a strong and diffuse pattern, associated with an aberrant cytoplasmic and nuclear expression of beta-catenin [1, 40].

Third Subtype of Adenoma: Inflammatory Adenoma (IHCA)

In the search for genotype-phenotype correlations in HCA, we defined a homogeneous subgoup of tumors, the inflammatory HCA (IHCA), characterized by the presence of inflammatory infiltrates within the tumor [76]. IHCA represent about 35–45% of the adenoma cases, they predominately occur in women and they are frequently associated with obesity and more rarely with alcohol intake [61, 63]. These observations suggest that alcohol intake and obesity could have a direct role in the initiation of tumorigenesis of IHCA. Tumor hepatocytes in IHCA express elevated levels of serum amyloid A (SAA) and C-reactive (CRP) proteins, two members of the acute-phase inflammatory response, whereas SAA and CRP are not expressed in inflammatory cells, Kupffer cells, or other sinusoidal cells in IHCA. Inflammatory infiltrates are largely localized to arterial vessels, but are also found within the sinusoidal lumens of IHCA. Overall, inflammatory infiltrates observed in IHCA were highly polymorphous including T and B lymphocytes, histiocytes, and Kupffer cells [62]. Interestingly, in patients, activation of the inflammatory response in the tumor hepatocytes may be responsible for a peripheral inflammatory syndrome that can regress after a curative resection of the IHCA [77]. IHCA are also frequently telangiectatic and this subgroup of lesions included most of the adenoma previously termed “telangiectatic FNH” or “telangiectatic adenoma” [40, 61, 63, 78].

Recently, in the search for new oncogene altered in HCA, microarray transcriptomic analysis of IHCA permitted to identify an activation of the IL6/STAT3 and Interferon type1/2 pathways in IHCA [62]. A subsequent search for gene alterations has identified recurrent somatic mutations of IL6ST (Interleukin 6 Signal Transducer) activating gp130 in 60% of the IHCA cases [62]. IL6ST encodes the cell surface signaling receptor gp130 shared by at least six different cytokines including IL6, IL11, LIF, OSM, CTNF, and CT-1 to activate the JAK/STAT oncogenic pathway [79, 80]. We further demonstrated that the gp130 mutants identified in IHCA were able to activate STAT3 independently of the interleukin signaling. Then, we searched for possible interaction and cooperation of gp130 activation with other pathways altered in the overall series of HCA. *HNF1α* inactivation and gp130 activation were mutually exclusive in HCA. On the contrary, in half of the β-catenin activating HCA, we identified a gp130-activating mutation. For two IHCA associated with a malignant transformation, both an activating gp130 and β-catenin mutations were found. Further, analysis of 220 HCCs revealed rare gp130 alterations in all cases accompanied by β-catenin-activating mutations, suggesting a cooperative effect of these signaling pathways in the malignant transformation of hepatocytes [62].

Conclusion

Our knowledge of the benign hepatocellular tumorigenesis has considerably increased during the last years. FNHs are hyperplastic responses to a hemodynamic disturbance related to vascular abnormalities. It is characterized by a zonated activation of the β-catenin and TGFβ pathways that are probably at the origin of the limited hepatocyte proliferation. On the other hand, for HCA, we defined different homogeneous

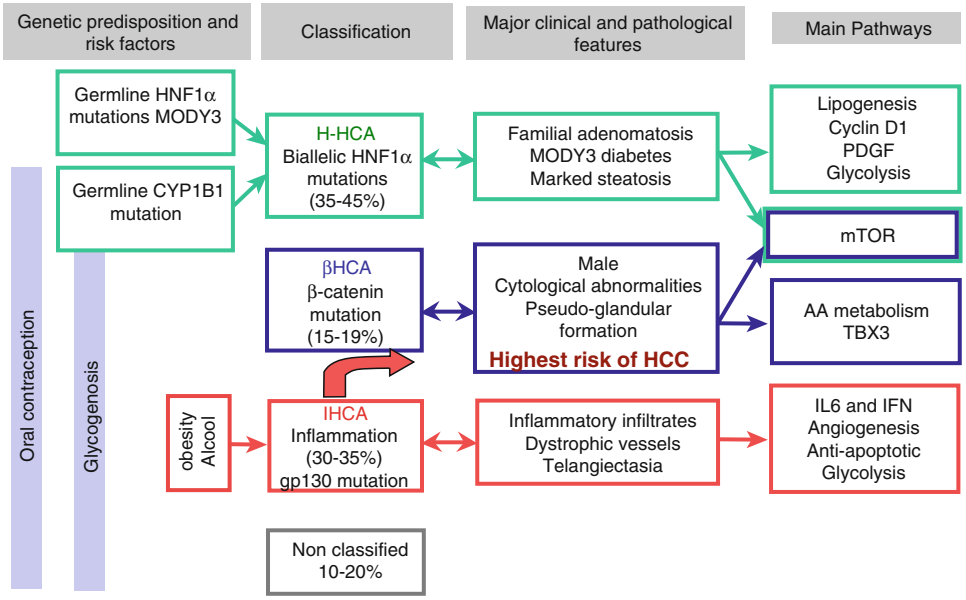

Fig. 52.2 Molecular classification of hepatocellular adenomas

sub-groups of adenomas driven by oncogene and tumor suppressor gene mutations (Fig. 52.2). Each sub-group of tumors is closely associated with specific risk factors, genetic susceptibility, pathological features and prognosis. Altogether, these observations revealed the broad diversity of the different sub-types of adenomas.

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Chapter 53
Hepatoblastoma

Marie Annick Buendia and Monique Fabre

Introduction

Hepatoblastoma (HB) is the most frequent malignant liver tumor in infants and young children, and it represents the third most common intra-abdominal pediatric malignancy following neuroblastoma and Wilms' tumor [1, 2]. This tumor usually develops between 6 months and 3 years after birth, accounting for more than 90% of primary hepatic malignancies in this age group [3]. HB development during the intrauterine life and neonatal period has also been documented [4]. In less than 10% of cases, HB occurs in children over 4 years and it has been detected exceptionally in adults [5, 6]. Higher tumor incidence in males has been reported in some studies [7]. HB is usually diagnosed in patients presenting with enlarged liver mass and elevated levels of serum alpha-fetoprotein (AFP) [8]. Significant advances in histopathological approaches have fostered the use of diagnostic biopsies for accurate staging of the tumor [9, 10].

With an annual incidence around one in a million children under 15 years of age, HB is a very rare cancer accounting for approximately 1% of all pediatric malignancies [2, 3, 6]. However, its incidence appears to be rising in different countries, and increased risk of HB in children with very low birth weight (<1,500 g) has been found in Japan, the United States, and more recently, the United Kingdom [11–15]. Interestingly, the association between low birth weight and subsequent tumor development has been clearly established for HB but not for any other pediatric or adult cancer. Perinatal treatments such as erythropoietin given to highly immature newborns might have oncogenic effects on the liver [16]. Alternatively, events during intrauterine life might increase the tumor risk, as recent studies have suggested potential links between maternal eclampsia or pre-eclampsia, prematurity, low birth weight and HB occurrence [17]. In this chapter, etiological factors, clinical and pathological tools for diagnosis and management of HB, as well as recent molecular biology and genetic data will be presented.

Etiological Factors and Associated Conditions

The etiology of HB has not been fully elucidated. HB differs from hepatocellular carcinoma (HCC) by the absence of underlying liver disease induced by chronic infection with hepatitis B or C virus, ethanol consumption, or hepatocarcinogenic factors such as aflatoxin B1. By contrast, early studies have correlated HB occurrence with parental exposure to metals, petroleum, and paints [18], and more recently, with adverse events associated to prematurity and low birth weight [17]. The rapid development of HB on apparently normal hepatic background suggests a genetic or epigenetic origin of the tumor, as for other pediatric neoplasms. Whereas most HBs are sporadic, 5% are associated with congenital abnormalities and overgrowth syndromes including the Beckwith-Wiedemann syndrome (BWS), hemihypertrophy, and occasionally trisomy 18, Prader-Willi, and Simpson-Golabi-Behmel syndromes [8]. BWS patients have an increased risk of developing intra-abdominal childhood tumors (Wilms' tumors, adrenocortical carcinomas, rhabdomyosarcomas, and HBs), suggesting common genetic pathways. In particular, BWS is characterized by genetic and epigenetic abnormalities at the chromosome 11p15 band, and loss of imprinting (LOI) in this region leading to biallelic expression of IGF2 has been implicated in increased tumor susceptibility [19]. In HB, loss of maternal 11p15.5 alleles has been associated with overexpression of IGF2 and alteration of the IGF axis [20–22]. The important role of IGF2 in liver development and its growth-promoting and anti-apoptotic activities strongly implicate this factor in HB pathogenesis.

Highly elevated risk of HB has been found for children with a family history of familial adenomatous polyposis (FAP), a cancer predisposition syndrome associated with

M.A. Buendia (✉)
Oncogenesis and Molecular Virology Unit,
Institut Pasteur, Paris, France
e-mail: marie-annick.buendia@pasteur.fr

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_53, © Springer Science+Business Media, LLC 2011

germline mutations of the adenomatous polyposis coli (*APC*) gene. Biallelic inactivation of *APC* has been found in HBs from FAP kindreds [23]. In recent studies, more than 5% of HB cases had family background suggestive of FAP, and the deduced risk was increased more than 700-fold for such patients [24, 25]. Mutations of the *APC* gene leading to constitutive activation of the Wnt/β-catenin pathway have been found also in sporadic HBs, illustrating the pivotal role of Wnt signaling in this embryonic liver tumor.

Presentation, Clinical Staging and Treatment

In most cases, children present with abdominal distension, abdominal pain, and gastrointestinal disorders [2]. Anemia and paraneoplastic syndromes like virilization are not uncommon. Liver function tests such as alanine aminotransferase, total bilirubin, albumin, and alkaline phosphatase remain generally normal. Elevated level of serum AFP represents a fairly constant marker, as it has been found in 90% of HB cases. However, AFP levels must be interpreted with caution because AFP is commonly elevated in normal neonates until 6 months of age, and it may be elevated also in association with other tumors including hemangiomas/hemangioendotheliomas and mesenchymal hamartoma of the liver [26]. Conversely, tumors that fail to express AFP at diagnosis commonly include a small cell undifferentiated component and are thought to be more aggressive [27].

The location of the tumor within the liver, its size, and extent of spread are usually determined by abdominal ultrasonography (US), computed tomography (CT) scan, and magnetic resonance imaging (MRI) [28]. US identifies the liver as the organ of origin, while CT and MRI outline the anatomic extent of the tumor, clarify its relationship to the central venous structures, and evaluate for multicentricity. Chest CT allows detecting lung metastases at diagnosis in about 20% of cases.

Due to the rarity of this tumor, clinical studies have been conducted by large, national, or international cooperative groups. Different staging systems have been used worldwide, including modified TNM staging, the Children's Oncology Group (COG) system mainly used in North America [29], and the PRETEXT (PRETreatment EXTent of disease) system, which now is widely used and represents a common international criteria for risk stratification [27]. Several groups involved in clinical trials such as the Children's Cancer Group (CCG), the Pediatric Oncology Group (POG) and the COG in the United States, the German Cooperative Pediatric Liver Tumor Group (GPOH), and the Japanese Pediatric Liver Tumor (JPLT) Group have traditionally used a staging system based on surgical findings and outcome of a primary operation together with histological features defining the possible radicality of resection (stage I: primary complete resection; stage II: microscopic residual tumor; stage III: nodes positive, or tumor spillage, or macroscopic incomplete resection; stage IV: metastatic disease). A different preoperative staging system based on the anatomy of the liver and radiological findings at diagnosis has been developed by the Liver Tumor Strategy Group (SIOPEL) of the International Society of Pediatric Oncology [30]. SIOPEL has defined two broad categories of risk: standard-risk (SR) for patients with HB confined to the liver and involving no more than three hepatic sectors (PRETEXT stages I, II, and III), and high-risk (HR) for those with HB extending into all four sectors (PRETEXT IV) and/or with lung metastases or intra-abdominal extra-hepatic spread [31]. Tumors with AFP <100 ng/mL at diagnosis have also been assigned to HR in different studies.

The foundation of HB management is surgical resection, and tumor resectability is considered the single most important prognostic determinant of this neoplasm. When the lesion is confined within the liver, complete tumor resection is the only curative treatment, achieved either with standard partial hepatectomy or orthotopic liver transplantation (OLT) [32]. Prognosis is excellent after complete surgical removal with no evidence of residual disease, while conversely, the outcome is generally poor for those who have kept residual disease. The introduction of chemotherapy treatment using cisplatin in the 1980s has markedly improved patient outcome, mostly for patients presenting with initial unresectable tumor. In Europe, the 5-year survival rose from 28% in 1978–1982 to 66% in 1993–1997 [6], and similar trends have been reported in United States and Japan [29, 33]. Preoperative chemotherapy causes the tumor to shrink, allowing removal of a previously unresectable tumor. For HR cases, similar survival has been found in patients treated with either cisplatin/5-fluorouracil/vincristine or cisplatin/continuous infusion doxorubicin.

In patients with unresectable disease following neoadjuvant chemotherapy or with recurrent disease, OLT has led to improved cure rates [34–36]. OLT may be considered for tumors that involve all four sectors of the liver or those localized in close proximity to major vascular structures. In such cases, OLT offers 80% long-term disease-free survival compared to 30% following rescue transplantation for incomplete resection. The persistence of viable extrahepatic deposits after CT, not amenable to surgical resection, is considered to be the only absolute contraindication to liver transplantation [37]. For 135 pediatric patients with HB in the UNOS database from 1987–2004, the 1-, 5-, and 10-year survival was 79, 69, and 66%, respectively [38]. The main adverse event was metastatic or recurrent disease, accounting for 54% of deaths. The level of serum AFP may be used to determine response to therapy and to detect recurrence. Beside surgery and OLT, new methodologies

have been applied for local control, including trans-arterial chemoembolization (TACE) [39] and radiofrequency ablation (RFA) [40]. However, new therapeutic regimens are urgently needed for advanced-stage and metastatic tumors. It will be important to develop novel targeted therapies through the identification of molecular targets for which drugs are available.

Pathological Classification

Macroscopically, HB consists of a large multinodular expansile mass that may show areas of necrosis and hemorrhage, well demarcated from the normal liver but not encapsulated, and with a variegated cut surface. Tumors that are resected following chemotherapy are usually firm to hard depending on the presence of fibrosis and/or osteoid component. Due to frequent morphological heterogeneity, a minimum of one section per cm in the largest tumor diameter should be examined for correct evaluation of all histological types.

Histopathological classification of HB has been considerably improved during the last decades. Ishak and Glunz [9] have first described two tumor types: pure epithelial and mixed epithelial and mesenchymal. Microscopically, HB may be composed entirely of epithelial elements (56%) or of a combination of epithelium and mesenchyme (44%) [8]. Epithelial HBs are composed of varying proportions of cells that resemble early embryonal hepatocytes, fetal hepatocytes, or small undifferentiated cells, evoking various stages of hepatocyte development [41]. Some tumors show macrotrabecular areas where the cells are arranged as 5–20 cell thick trabeculae. In completely resected tumors, pure fetal histology confers a better prognosis, while small-cell undifferentiated (SCU) histology is associated with poor outcome [42]. The most common mesenchymal elements are osteoid and chondroid (cartilage). Usually, the osteoid component makes up a minor component in about one third of untreated HBs, but it can increase up to 90% of the tumor area after chemotherapy treatment [43]. The presence of mesenchymal elements has been associated with improved prognosis in patients with advanced disease [42]. The following observations form the basis of current histological classification of HB.

Epithelial Types

The most common type is the *fetal form,* in which cells resemble hepatocytes and grow in a pattern mimicking the fetal liver. The cells are smaller than normal hepatocytes, have abundant cytoplasm, and distinct boundaries. There are arranged in 2–3 cell thick trabeculae and present a "light and dark" pattern. Foci of extramedullary hematopoiesis may be seen (Fig. 53.1a). Two sub-types are recognized: well-differentiated (<2 mitoses per 10 high power fields [HPF]) and mitotically active, also called crowded fetal with frequent nuclear pleiomorphism and more than 2 mitoses per 10 HPF (Fig. 53.1b). Criteria for this definition do not include architectural arrangements in cords or compact sheets, nor cytoplasmic aspects that may be either rich in lipid or glycogen (clear cells), eosinophilic or amphophilic. Pure fetal HBs consisting uniquely in fetal cells are found only in 5% of cases and they are endowed with favorable prognosis.

The *embryonal form* is composed of less mature cells with predominant solid growth pattern. Some structures are lined by tall columnar cells with high nucleo-cytoplasmic ratio, and pseudo-rosettes and acinar formations may occur (Fig. 53.1c). Embryonal tumors may also be arranged as sheets of irregular, angular cells with large nuclei, dense nuclear chromatin, and indistinct cell membrane.

The *small cell undifferentiated (SCU) form,* formerly anaplastic type, is composed of monomorphous populations of small blue cells, suggesting that it might represent a tumor of hepatic stem cells [44]. Cells may be either round, ovoid or fusiform, with a scant cytoplasm, round lightly chromatic nuclei with inconspicuous nucleoli, high nucleo-cytoplasmic ratio, and frequent mitoses (Fig. 53.1d). They can be found as discrete nests in frequent association with embryonal patterns in otherwise typical HBs. In different studies, adverse outcome has been correlated with SCU histology associated with low AFP levels in HB patients [27, 45]. Recent data indicate that some of these tumors may be more appropriately classified as rhabdoid or rhabdoid-like tumors, on the basis of cytogenetic findings and negative immunostaining for INI1 [46, 47]. Because rhabdoid tumors can share clinical and histologic features with HB, rare cases with hepatic localization may be misdiagnosed as HB. However, the *rhabdoid cell form* can be occasionally found in HB. Neoplastic cells have an abundant eosinophilic cytoplasm containing paranuclear inclusions, and vesicular nuclei with a centrally located prominent nucleolus. Among other rare HB components, the *cholangioblastic cell form* is thought to originate from the ductules/canals of Hering, where hepatic progenitor cells are located. Some tumor areas, usually in close contact with SCU patterns, show strands/trabeculae of small, uniform, oval-shaped cells with scant cytoplasm and hyperchromatic nuclei embedded within a thick, desmoplastic stroma. Finally, the *endocrine cell form* is composed of small, monomorphous, cohesive cells that stain positive for endocrine markers and show ribbon-like arrangement resembling an islet cell tumor.

In the *macrotrabecular form,* cells are arranged in 5–20 cell thick trabeculae (Fig. 53.1e). Cell nuclei are vesicular with prominent nucleoli, while cytoplasmic features such

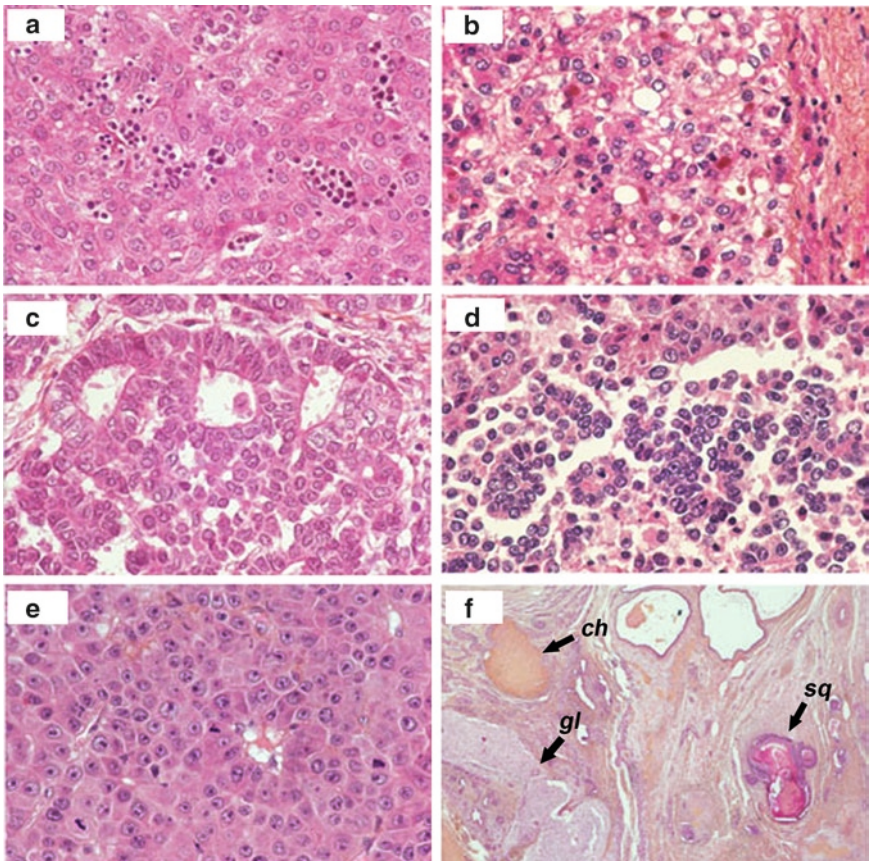

Fig. 53.1 Histologic HB subtypes. (**a**) Well-differentiated fetal type with abundant eosinophilic cytoplasm and distinct boundaries, and extramedullar hematopoietic foci; (**b**) crowded fetal type with pleiomorphic cells and nuclei; (**c**) embryonal type with irregular, angular cells and acinar formation; (**d**) small cell undifferentiated (SCU) type. Note small to medium-sized round cells with focal spindling, lightly chromatic nuclei with inconspicuous nucleoli, and high nucleo-cytoplasmic ratio; (**e**) compact macrotrabecular type with vesicular nuclei, prominent nucleoli and mitoses; (**f**) mixed epithelial and mesenchymal HB with teratoid features including primitive mesenchymal spindle cells, chondroid (ch), neuroglial (gl), and squamous (sq) components. H&E staining. Magnification: (a-e) × 250;(f) ×125

as lipid or glycogen storage, eosinophilic or amphophilic characters are not retained as criteria for defining this subtype. While many authors refer to this subtype only when the macrotrabeculae are comprised of hepatocytes appearing similar to those in HCC, others also consider this subtype when the cells are of the fetal or embryonal type. It is noticeable that tumors with features of both HB and HCC have been described in school-aged children, and they have been named transitional liver-cell tumors [48].

Mixed Epithelial and Mesenchymal Types

Mixed HBs include, in addition to any epithelial type, stromal elements such as a spindle-cell component resembling the primitive mesenchyme, and frequent osteoid or chondroid components (Fig. 53.1f). Some mixed tumors also include teratomatous elements such as neuro-ectodermal tissues (embryonal tubules, ganglion cells, neuroglia, melanocytes, and endocrine cells), or mesenchymal (striated muscle and bone), and epithelial derivates (respiratory, enteric, and squamous cells). These features have led to surmise that HBs derive from a primitive cell endowed with the ability to differentiate into multiple cell types.

It is worth noticing that many reports point to the clinical impact of the stage of tumor cell differentiation in HB. However, the high morphological heterogeneity frequently found in a majority of tumors has hampered the use of histological classification for HB management.

Immunohistochemical Markers for HB Diagnosis and Prognosis

Differential expression of a number of protein markers between tumors and normal liver and between various HB elements can be used to firm up diagnosis and stratify HB subtypes, particularly in small biopsies that may pose

problems to the pathologist. Moreover, some markers have been endowed with prognostic value. Widely used immunohistochemical (IHC) markers are often liver-specific proteins that may help recognize the stage of hepatic differentiation within a tumor compartment. Indeed, tumor cells in HB frequently express proteins that are abundant in the prenatal liver and strongly downregulated after birth, as shown in the following examples.

AFP is a marker of liver-progenitor cells that is expressed at varying levels in HB, mostly in embryonal and small undifferentiated tumor cells, while its expression is low or undetectable in fetal, or mesenchymal tumor cells [49, 50] (Fig. 53.2b). Thus, absence of AFP immunostaining does not exclude a malignant liver tumor. The heparan sulfate proteoglycan glypican 3 (GPC3) is another oncofetal protein that is strongly expressed in Wilms' tumors and HCCs, and it is considered as a promising diagnostic marker. Cytoplasmic immunoreactivity for GPC3 with strong, diffuse positivity has been reported in more than 90% of HB cases [51]. All epithelial subtypes are stained, more strongly for the embryonal and small-cell undifferentiated patterns (Fig. 53.2c). Delta-like 1 homolog (DLK1/Pref-1), an imprinted, paternally expressed gene, is expressed during embryonic development, notably in hepatic progenitor cells. It is aberrantly activated in different carcinomas and the DLK1 membrane protein represents a sensitive HB marker that is expressed in all epithelial subtypes [52].

The cytokeratin (CK) immunophenotype that reflect liver developmental processes can be extremely informative for discriminating HB from other solid tumors and for stratifying different HB subtypes. Expression of CK8, CK18, and CK19 has been found in most HB cases, with important variations among subtypes; while CK8 shows positive staining mostly in fetal areas, CK18 staining is detected in all epithelial components and CK19 staining is uniformly strong in embryonal and ductular differentiation areas, while it decorates only cholangiocytes in the normal liver after birth [49]. Thus CKs represent reliable markers for differentiating fetal and embryonal cell types in HB.

Other commonly used hepatic markers such as alpha-1-antitrypsin (AAT or SERPINA1) and Hepatocyte Paraffin 1 (Hep Par 1) are also widely expressed in HBs, albeit more intensely in fetal areas [53]. The carcinoembryonic antigen (CEA), a cell-adhesion molecule, first described as a biliary glycoprotein, is expressed on the canalicular surface of fetal and embryonal cells. Claudin-1 and claudin-2, two major constituents of tight junctions, are expressed preferentially in more differentiated tumor cells in the fetal component and their expression is inversely correlated with cell proliferation [54]. Similarly, fetal HBs show increased immunostaining of glutamine synthetase (GS), the prototype member of hepatic perivenous (PV) markers that are activated by the Wnt/β-catenin pathway [55]. In the normal liver, GS is physiologically expressed in one or two hepatocyte plates located immediately beside the central veins [56]. It is homogeneously over-expressed in fetal cells of HB, generally forming large positive areas occasionally centered by central veins, whereas it is faintly expressed in less differentiated epithelial cells. Interestingly, GS expression can also be detected in mesenchymal components showing intense cytoplasmic and nuclear accumulation of β-catenin (Figs. 53.2 and 53.3).

While the β-catenin protein localizes at the membrane of hepatocytes and cholangiocytes in the normal liver, IHC analysis of β-catenin in HBs has shown nuclear/cytoplasmic accumulation of the protein in a large majority of tumors [50, 52, 57, 58]. Membranous β-catenin localization was preserved only in fetal-type tumoral hepatocytes and was associated with E-cadherin expression. Strong nuclear accumulation was noted predominantly in areas showing active proliferation and in poorly differentiated HB components. Illustrative examples showing activation of β-catenin in epithelial cells as well as in immature mesenchymal tissues such as the neuroectodermal component are shown in Figs. 53.2 and 53.3. Strikingly, the intensity of β-catenin immunostaining is inversely correlated with that of its hepatic target GS. Finally, immunolabeling of β-catenin represents a valuable marker at diagnosis, and it might also be a useful clinical tool for estimating the patients' prognosis [59].

The proliferation markers Ki-67 and PCNA show strong immunoreactivity in poorly differentiated epithelial HBs, but they are barely expressed in fetal cells and in mesenchymal derivatives [60], and their labeling indexes are thought to have prognostic relevance [61].

Thus, a combination of immunohistological markers allows distinguishing HB from other tumor types and stratifying different HB components, and can provide prognostic information in clinical practice.

Genetic Alterations in Hepatoblastoma

Extensive genetic studies using conventional cytogenetic approaches, loss of heterozygosity (LOH) analysis, and comparative genomic hybridization (CGH) analysis have led to define the major chromosomal abnormalities in HB. Analysis by flow cytometry has shown that tumor cells are often diploid, particularly in the fetal type, or hyperdiploid, and aneuploidy was found mainly in the embryonal subtype [60, 62].

Abnormal karyotypes were seen in 50–70% of tumors, with recurrent abnormalities affecting mainly the chromosomes 1, 2, 8, and 20 [63–68]. Chromosome gains are more frequent than losses. The most salient features are trisomies of chromosomes 2 (or 2q) and 20, often seen as the only abnormality [69, 70]. Other common structural changes

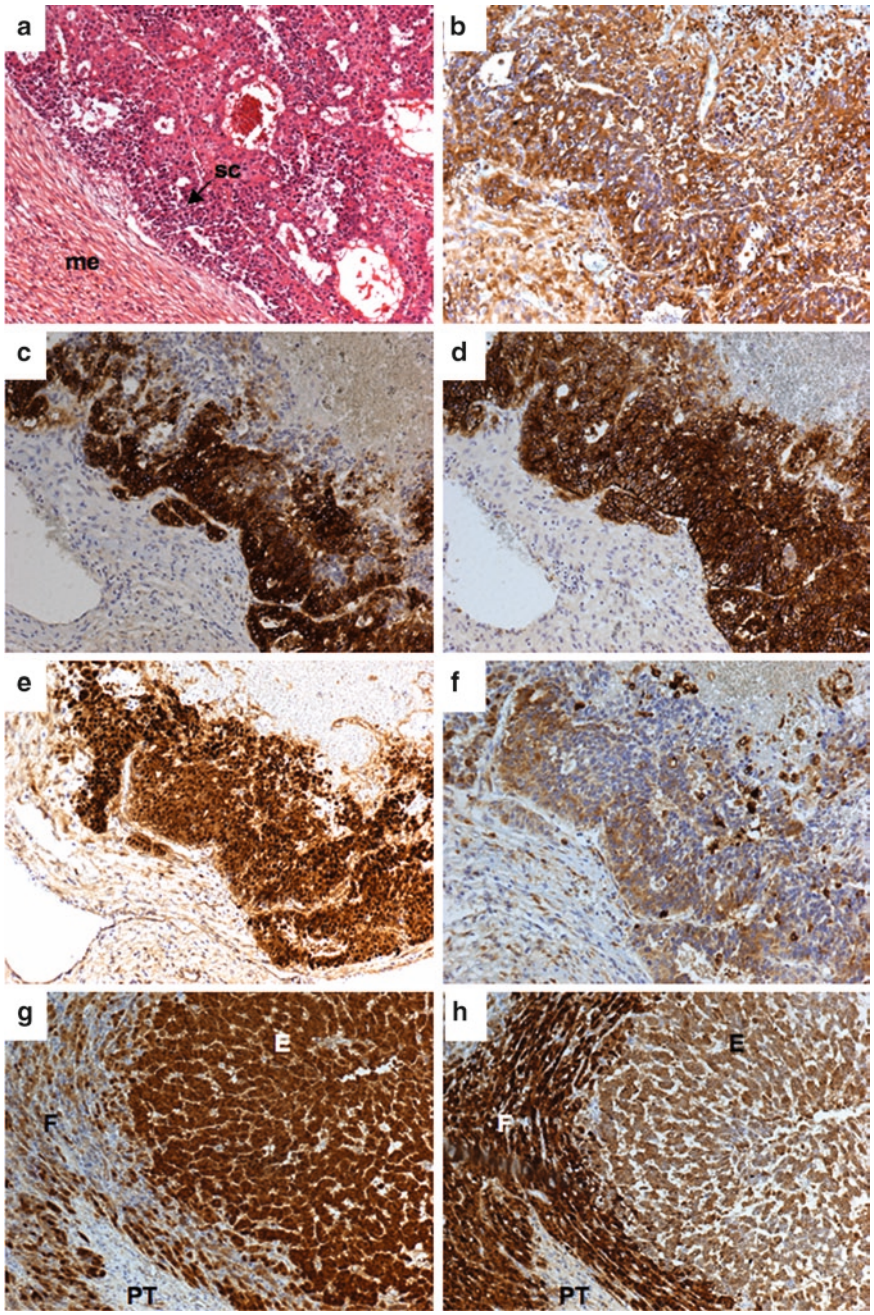

Fig. 53.2 Immunohistochemical (IHC) markers in various HB patterns. (**a**–**f**) Serial analysis of a mixed epithelial-mesenchymal HB with small cell undifferentiated component. (**a**) H&E staining. The epithelial component consisting mainly of small cells (sc) compresses the neighboring mesenchymal element (me). (**b**–**f**) Heterogeneous immunostaining of the oncofetal proteins AFP (**b**) (×160), GPC3 (**c**) and EpCAM (**d**), and of β-catenin (**e**) and its hepatic target GS (**f**). In all cases, higher labeling intensity with frequent diffuse positivity is seen in small cells compared with stromal cells. In the SCU component, note intense cytoplasmic immunoreactivity of GPC3, strong membranous and cytoplasmic reactivity of EpCAM, and nuclear and cytoplasmic reactivity of β-catenin, while AFP and GS show more variable labeling patterns. (**g**, **h**) Serial analysis of an embryonal and fetal epithelial HB. (**g**) β-catenin immunostaining showing strong and diffuse cytoplasmic and nuclear reactivity in embryonal tumor cells (E), membranous and focal cytoplasmic labeling in fetal tumor cells (F), and no reactivity in the portal tract (PT). (**h**) GS immunostaining with strong cytoplasmic labeling of fetal cells but no reactivity in the portal tract, and weak labeling of embryonal cells. Magnification: (a, c-f) × 125, (b) × 160, (g-h) × 250.

consist in rearrangements involving 1q and 2q. Breakpoints at 1q12-q21, often associated with an unbalanced translocation der(4)t(1;4)(q12;q34), result frequently in trisomy of the long arm of chromosome 1 [71, 72], while chromosomal breaks on 2q generally involve 2q35-q37 [65, 73]. Cytogenetic analysis of a large series of HBs has revealed that tumors with translocations involving 1q12–21 harbor more numerical chromosome aberrations (mostly trisomies) than tumors

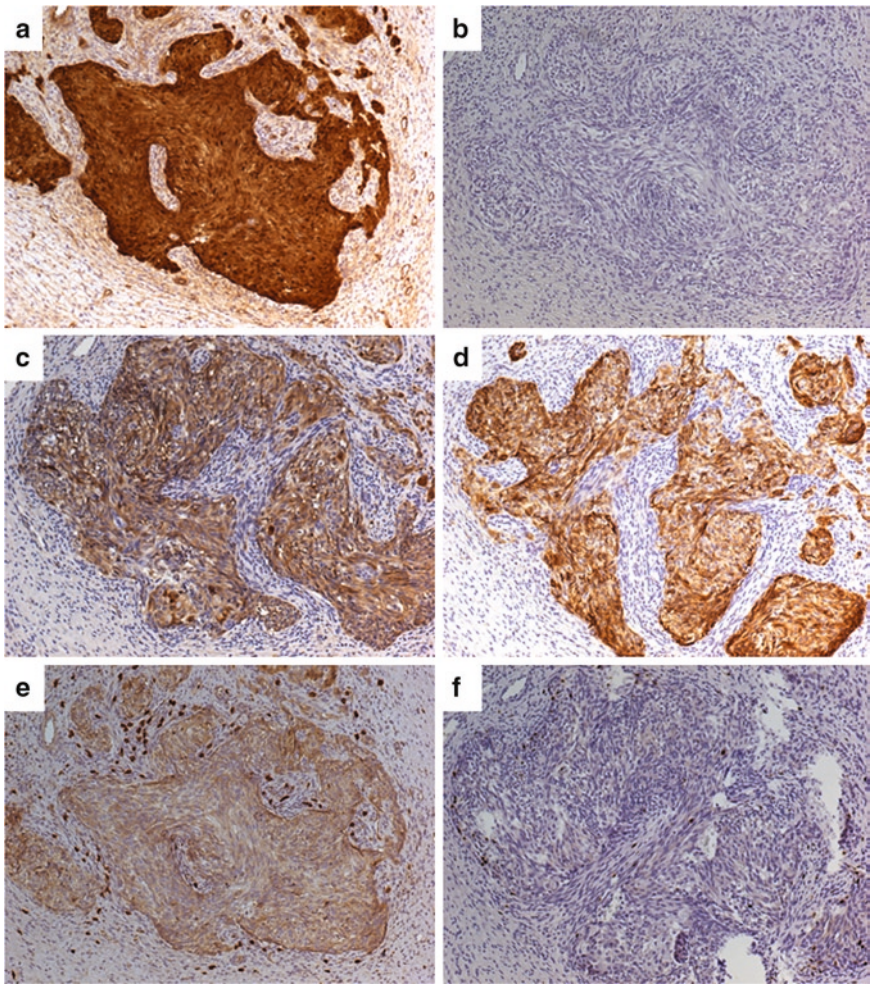

Fig. 53.3 Representative IHC markers in stromal patterns with teratoid features. (**a**) β-catenin immunostaining showing strong and diffuse cytoplasmic and nuclear reactivity in spindle neuroectodermal cells. Note some positive, isolated stromal stem cells. (**b**) Absence of AFP immunostaining in stromal and neuroectodermal components. (**c**) GS immunostaining highlighting the cytoplasm of neuroectodermal cells surrounded by negative mesenchyme. (**d**) CD56 (N-CAM) immunostaining with strong and diffuse cytoplasmic reactivity in the neuroectodermal component. (**e**) CD117 (C-Kit) decorating strongly the cytoplasm of several isolated stem cells in the stroma and less intensely the neuroectodermal component. (**f**) Ki67 immunostaining (MIB-1 antibody) showing a low proliferation index (<5%) in both compartments. Magnification: ×125

with other structural rearrangements [74]. Overall, the cytogenetic profile of HB evokes features also seen in embryonal rhabdomyosarcoma.

CGH analysis of HB has detected common alterations with adult-liver tumors (HCC), including gain of chromosomes 1q, 8q, and 17q and loss of 4q. However, the overall profiles are strikingly different between the two tumor types. HBs usually harbor a low rate of chromosomal imbalances (mean: three changes per tumor) and 22% of these tumors show no gross abnormality. CGH analysis confirmed the predominance of gains at chromosomes 2/2q, 20, and 1q. Gains also involved chromosomes 7, 8, 17, 22, and X, while losses were found at lower rate, mainly on chromosomes 1p and 4q [75–80]. Recent studies using high-density single-nucleotide polymorphism genotyping microarrays evidenced high-grade amplifications at chromosomes 7q34, 14q11.2, and 11q22.2 [81]. Interestingly, two alterations were found to be significantly associated with poor prognosis: gain of 8q and gain of 20 [78]. However, in these studies, no specific correlation has been reported between alteration profiles and tumor histotypes. By contrast, a recent study of 24 HB tumors by array CGH has revealed that gains of chromosomes 2p and 8/8q are predominantly found in immature, highly proliferative tumors, with robust correlation with shorter survival [50]. Noticeably, the *MYCN* and *MYC* oncogenes are located at the chromosomal loci 2p24.1 and 8q24.21. Gain of these regions was associated with enhanced expression of *MYCN* and *MYC* genes, and activated Myc signaling in poorly differentiated HBs [50].

Search for allelic losses has been carried out mainly at chromosomes 1p and 11p, showing frequent LOH at 1p36 and 11p15.5. In the latter, loss of the maternal allele is

consistent with imprinting of the IGF2-H19 locus at this region [20, 82, 83]. It has been reported that the number of chromosomal imbalances was significantly higher in HBs with LOH on 11p [78]. Recent comprehensive allelotyping of HB has outlined frequent allelic losses on 1q, 4q, 13q, 17p, and 17q, with commonly deleted regions at 1q44, 4q21–22, 13q14, 17p13, and 17q11.2 [84]. Interestingly, two of these chromosomal loci contain the tumor suppressor genes *RB1* and *TP53*. However, the mutation rate in these genes appears to be very low in HB.

Overall, genetic studies of HB have revealed a relative genomic stability with few distinctive chromosomal alterations. These observations are in agreement with the notion that childhood solid tumors display fewer cytogenetic defects than adult tumors, probably because they require fewer events to progress [85]. Accordingly, HB shares common genetic features with other intra-abdominal pediatric tumors such as Wilms' tumor and rhabdomyosarcoma, but a limited number of common alterations with adult HCC. Genetic studies of HB also provided some clues for abnormal activation of oncogenic pathways such as those of IGF2 and Myc.

Mutational Activation of β-Catenin

Although a number of cytogenetic and molecular abnormalities have been described, HB pathogenesis remained poorly understood until the first evidence for mutations in the *CTNNB1* gene encoding β-catenin, a key effector of the canonical Wnt pathway. This multifunctional protein plays important roles in intercellular adhesion and in cell growth, survival, and differentiation [86]. In normal epithelial cells, β-catenin is localized at the plasma membrane, where it forms a complex with E-cadherin and α-catenin at the sites of adherent junctions. Excess β-catenin is phosphorylated at four N-terminal serine-threonine residues by GSK3- β and CK1 within a cytoplasmic complex including APC and Axin, and phosphorylated β-catenin is degraded by the ubiquitin-proteasome pathway. Upon Wnt signaling, GSK3- β activity is inhibited, β-catenin is stabilized and translocated to the nucleus where it interacts with the transcription factors Tcf/Lef and activates the transcription of target genes. In different types of human cancers, constitutive activation of β-catenin is achieved either by loss-of-function mutations in the APC or Axin genes, or by activating mutations in the N-terminal domain of β-catenin [87, 88].

Wnt signaling plays important role in liver development and tumorigenesis. In early somitogenesis, suppression of Wnt signaling is required for endodermal pattern formation, while at later stages increased Wnt activity alters endodermal fate by enhancing liver growth [89–91]. Following hepatic specification, the Wnt/β-catenin pathway stimulates the growth of hepatoblasts during morphogenesis of the expanding liver bud [92]. In the adult liver, β-catenin and its negative regulator APC are complementary localized in the PV and periportal (PP) areas where they control liver-specific metabolic programs such as glutamine synthesis, urea cycle, and glucose metabolism [93, 94]. Several recent studies have highlighted the key role of Wnt/β-catenin signaling in the compartmentalization of liver functions defined as metabolic zonation. Moreover, it has been shown that convergence of HFN4α and Lef1 onto hepatic target gene promoters leads to activation of PV genes and repression of PP genes [95]. Interestingly, c-Myc, one of the major downstream targets of the Wnt/β-catenin pathway in different tissue contexts, has not been involved in this process [96, 97].

Following the initial report of frequent mutations of β-catenin in HCC [98], several studies have demonstrated a high rate of β-catenin mutations in HB, providing evidence for abnormal reactivation of the developmental Wnt pathway in this tumor [57, 58, 99–101]. Sequence analysis of the β-catenin N-terminal domain revealed interstitial deletions or missense mutations in the GSK3β phosphorylation motif in 50–90% of cases. This is among the highest rates of mutation described so far in human neoplasms. The finding of identical β-catenin gene alterations within different histotypes of the same tumor suggested that mutational activation of the oncogene occurred in a common precursor cell. Because β-catenin mutations were found at similar rates in all HB histotypes and at different tumoral stages, they are not endowed with prognostic ability.

Activation of the Wnt/β-catenin pathway may also be achieved by loss-of-function mutations of Axin in about 5–10% of HB cases [102–104]. Moreover, the increased incidence of HB in FAP has led to evaluate sporadic tumors for alteration in the APC gene. While different studies failed to find mutations in the APC mutation cluster region [57, 99], others found a mutation rate around 10% [23, 25, 105]. Thus, genetic alterations in the APC/β-catenin pathway have been detected in a vast majority of HB cases analyzed, leading to consider this pathway as a hallmark of HB, and an essential player in HB pathogenesis.

The Wnt/β-catenin pathway governs cell proliferation and differentiation, and these biological processes play major roles in tumorigenesis. Therefore, identification of Wnt target genes that are activated in HB may provide important clues on the mechanisms underlying HB pathogenesis. One of the critical targets of Wnt in intestinal tumorigenesis is c-myc, which is able to rescue the APC phenotype in mice [106]. Curiously, different reports converge to demonstrate that c-myc is not activated in response to Wnt in the hepatic context, where it does not mediate the proliferative effects of Wnt signaling [96, 107]. Activation of MYC and MYCN has been associated to genetic alterations on chromosomes 2p and 8q in immature and aggressive HBs [50]. Other targets

such as cyclin D1 and the transcriptional repressor TBX3 might be involved in the growth-promoting and anti-apoptotic effects of Wnt in the liver and both factors have been associated with unfavorable prognosis [58, 108, 109].

One of the most salient features of Wnt-related profiles in HB is the significant overexpression of hepatic PV markers. These factors are also activated in adult HCCs carrying mutant β-catenin [110, 111]. They are functionally involved in metabolic pathways such as ammonia and glucose metabolisms, and detoxification pathways [55, 94, 112, 113]. The prototype member of these factors is the gene encoding GS that shows increased immunostaining in almost all HBs, with strong predominance in well-differentiated cells of the fetal pattern [50, 52].

Additionally, among genes activated in both immature and differentiated tumors, negative regulators of the Wnt pathway such as Conductin (AXIN2) and the secreted factor Dickkopf-1 (DKK1) that binds to the Wnt receptor LRP5/6 are strongly overexpressed [50, 104, 114]. Increased expression of the naked cuticle (NKD-1) and β-TrCP genes has also been noted [115]. Similar activation of Wnt antagonists has been reported in different tissue contexts, and may be interpreted as an attempt to activate a negative feedback loop that serves to restrict the range or duration of the signal. However, it should be kept in mind that these factors might deserve other functions, and they should not be able to inhibit the activation of Wnt target genes in cells expressing a mutant, constitutively active allele of β-catenin. More studies are clearly needed to elucidate the role of Wnt/β-catenin signaling in HB pathogenesis.

Oncogenic Pathways in HB

Although pediatric tumors are thought to require less oncogenic events, owing to the immaturity of the cells of origin, a variety of signaling pathways including growth factor and developmental pathways have been implicated in HB etiology [85]. One of the earliest changes detected in HB was upregulation of the imprinted gene IGF2 associated to imprinting errors at chromosome 11p15.5, in a locus linked to BWS [20, 116, 117]. Different studies argued for uniparental disomy with loss of the maternal allele, abnormal methylation status of the IGF2 promoter, or persistence of monoallelic expression of IGF2 in tumor cells [81, 118, 119]. The IGF2 regulator PLAG1, a developmentally regulated gene located on chromosome 8q11, was also found to be overexpressed [120]. Activation of hepatocyte growth factor (HGF) signaling in HB has been inferred from the finding of abnormal cytoplasmic staining of the HGF receptor Met [121]. Interestingly, a marked downregulation of Met following chemotherapy has been observed in this study.

More recently, analysis of the PI3K/AKT pathway in HB has shown that the phosphorylated forms of AKT, GSK3-β, and m-TOR were overexpressed in most tumors. Furthermore, mutation of the PIK3CA gene was seen in one case [122]. Inhibition of PI3K/AKT signaling represents an interesting approach for halting proliferation and inducing apoptosis in the pediatric tumor. Analysis of gene expression profiles in 13 primary HBs has led to propose that the oncogenic MAPK signaling cascade was activated in aggressive epithelial HBs harboring a SCU component [123]. Activation of Myc signaling associated with upregulation of MYC and MYCN was identified in poorly differentiated HBs carrying DNA copy gains at chromosomes 2p and 8q [50].

Telomerase reactivation and telomere maintenance play a crucial role in carcinogenesis. Telomere length and expression levels of the human telomerase reverse transcriptase (hTERT) are considered significant prognostic biomarkers in cancer [124]. In HB, the levels of hTERT mRNA, or telomerase activity have been correlated with patient prognosis, and telomerase reactivation might be a useful prognosis-associated factor [125]. Similar studies in HCC have concluded for significant correlation with clinicopathological data such as differentiation, multipolar mitosis, anaphase bridge, and patient outcome [126].

Besides the canonical Wnt/β-catenin signaling, evidence has been provided for activation of two developmental pathways in HB. Notch signaling is known to regulate cell-fate decisions and morphogenesis in the fetal liver by enhancing stem-cell differentiation into biliary epithelial cells and tubular formation [127]. In the Alagille syndrome, mutation or deletion of the Notch ligand Jagged1 (JAG1) is associated with bile duct paucity [128]. Moreover, Notch signaling has been implicated in the pathogenesis of different human cancers. Activation of the Notch pathway was measured in HB through the expression level of HES1, a major Notch target gene [52]. In this study, HES1 expression was highly enhanced in pure fetal HBs, and to a lesser extent in the less differentiated embryonal and SCU components, while no correlation with other biliary markers has been reported. A second evolutionarily conserved pathway that plays a crucial role in embryonic development is Hedgehog signaling. Ligand-independent activation of the Hedgehog (Hh) pathway through genetic alterations has been involved in medulloblastomas and basal-cell carcinomas, while few studies have investigated ligand-driven activation in other tumor types [129]. Recent studies have shown increased expression levels of the Hh target genes glioma-associated oncogene homolog 1 (GLI1) and Patched (PTCH1), and downregulation of the Hh interacting protein (HHIP) in a significant fraction of HB samples [130].

It is probable that these signals are coordinated to control HB pathogenesis. However, the precise cross-talks between these pathways and whether they act in hierarchy remain

unknown. Better understanding of the complex oncogenic networks operating in HB initiation, maintenance, and progression will help assess prognosis and may help guiding the use of cytotoxic and targeted therapy.

Molecular Classification of Hepatoblastoma

During the last decade, gene expression profiling has been developed to examine the biological phenotypes of different human cancers. This approach has demonstrated indisputable power for identifying new malignant subtypes and predicting clinical outcome. In addition, similar techniques have been used to predict sensitivity or resistance to specific chemotherapeutic drugs.

In different studies, specific gene expression profiles have been recognized in HB tumors compared to normal livers, fetal livers, or HCCs [117, 123, 131]. Major differences were found in genes regulating cell cycle, cell division, and apoptosis. The observed strong deregulation of genes involved in the Wnt and BMP/TGF-β pathways is consistent with the view that common mechanisms may underlie organ development and tumor formation. Moreover, recent analyses have led to characterize specific expression patterns in different HB subclasses that reflect different phases of normal liver development [50] (Table 53.1). In comparative analysis of human HBs and normal mouse livers at different days of gestation, poorly differentiated tumors co-clustered with mouse livers at early stages (E11.5 and E12.5), whereas differentiated tumors gathered with mouse livers at late fetal and perinatal stages [50]. Despite marked morphological heterogeneity in a majority of tumors, these two molecular subclasses were mostly associated with the main epithelial component (fetal vs. other less differentiated patterns such as embryonal, crowded-fetal, and macrotrabecular types) and with proliferative rate. In this regard, it was not unexpected to find that activation of Myc signaling and the MAPK cascade were specifically linked to poorly differentiated HBs [50, 123].

As immature cells are frequently endowed with intrinsic capacities of proliferation, motility, and mobility, poorly differentiated tumors display aggressive and invasive phenotypes. Accordingly, the two molecular HB subclasses were tightly associated with features of tumor stage, such as vascular invasion and extrahepatic metastasis, with strong impact on patient survival rates (Table 53.1). These studies allowed identifying a genomic signature of 16 genes that demonstrated strong prognostic relevance both in diagnostic biopsies and in post-treatment tumor specimens [50].

Further work integrating molecular and genetic data with clinical information in larger tumor sets will be crucial for accurate classification of tumors, risk stratification, and successful development of new therapies for pediatric HB patients.

Table 53.1 Major biological and pathological features of two HB subtypes

Characteristic features	Poorly-differentiated HBs (C2)	Well-differentiated HBs (C1)
Epithelial components	Embryonal, crowded fetal, macrotrabecular, SCU	Fetal, pure fetal
Rate of proliferation	High (1–10% of the cells)	Low (<1% of the cells)
Tumor stage	Invasive and metastatic tumors	Localized, indolent tumors
Risk treatment	Mostly high risk	Mostly standard risk
Chromosomal alterations[a]	Average: three affected chromosomes (chrs 1, 9, 11)	Average: seven affected chromosomes (chrs 1, 2, 6, 8, 12, 17, 20)
Specific chromosomal changes[a]	Gain of 2p, gain of 8q	None
Mutations of β-catenin, Axin and APC genes	77%	85%
Activation of Wnt target genes[a]	*AXIN2, DKK1, TBX3, LGR5, MYCN, BIRC5NPM1, HDAC2*	*AXIN2, DKK1, TBX3, LGR5,GS, RHBG, LECT2, AQP9* *CYP2E1, CYP1A1, CYP2C9*
Hepatoblast markers	*DLK1/Pref1, ITGA6, GJA1*	*DLK1/Pref1, ITGA6, GJA1*
Markers of liver progenitor cells	High expression of *AFP, CK19* and *EPCAM*	Low expression of *AFP, CK19* and *EPCAM*
Activation of imprinted genes	*IGF2, DLK1, PEG3, PEG10, BEX1, MEG3, NDN*	*IGF2, DLK1, PEG3, PEG10, BEX1, MEG3, NDN*
Stage of hepatic development	Embryonic and early fetal	Late fetal/perinatal
Activated pathways and networks[a]	Cell cycleMitotic checkpoint Myc signaling	Metabolic activity of the hepatic perivenous area

Studies were carried out on 102 tumor samples from 85 HB patients as previously reported [50]

SCU small cell undifferentiated tumors

[a]Analysis of 25 tumors [50]

Conclusion

Although current treatments have considerably improved the disease outcome, children suffering from advanced stage, relapsed or metastasized HB show a poor survival rate, and alternative therapeutic options are under investigation. Recent studies have shown potential benefits of suicide gene therapy using adenovirus-mediated transfer of gene-directed enzyme-prodrug combinations, which might lead to improve the delivery of cytotoxic drugs [132]. Importantly, recent advances in our understanding of the specific oncogenic networks implicated in tumor development provide a valuable basis for designing adapted therapies. It has been shown that specific alteration patterns implicated in the development of a given malignancy can determine the response to conventional chemotherapy, and confer susceptibility to targeted therapies [133]. In this regard, identifying gene mutations associated with exploitable therapeutic approaches is gaining considerable value. This is apparently the case for HB, where the finding of high rate mutations in β-catenin or in the Axin and APC tumor suppressor genes has opened the way to novel therapies aimed at inhibiting the Wnt pathway in tumor cells.

In conclusion, the integration of clinical, morphologic, IHC, molecular, and genetic data provides a strong basis for approaching a precise diagnosis and for applying appropriate treatments to HB childhood patients. It may be anticipated that the identification of key genomic alterations involved in tumor-cell proliferation and invasiveness will considerably improve treatment outcomes for patients with advanced stage HBs that are resistant to conventional chemotherapy.

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Chapter 54
Hepatocyte Growth, Proliferation and Experimental Carcinogenesis

Giovanna Maria Ledda-Columbano and Amedeo Columbano

Introduction

Adult liver is normally quiescent and has a very low level of hepatocyte cell division. However, most hepatocytes rapidly proliferate in response to a reduction in liver mass caused by physical, chemical, nutritional, vascular, or virus-induced liver injury. In spite of the several studies aimed to understand the molecular mechanisms responsible for hepatocyte proliferation, the exact mechanisms responsible for the exit from the quiescent state and the re-entry into the cell cycle remain unknown.

As recently reviewed [1], the original idea that a single humoral agent could function as a key, capable of unlocking all the events required for liver regeneration is no longer acceptable; indeed, no single genetically modified mouse model demonstrates a complete blockage of both DNA replication and cell proliferation after 2/3 partial hepatectomy (PH) (see Chap. 18). Thus, using criteria established by genetic studies in other organisms, no single gene can be considered "essential" for liver regeneration. A more recent hypothesis predicts that the activity of multiple interconnected pathways is required for liver regeneration; this hypothesis is supported by the existing literature showing that the ablation of genes involved in different pathways can inhibit liver regeneration, leading to the notion that this process requires the activation of dozens of different pathways [2–4]. Finally, an alternative view was proposed [1], suggesting that the essential circuitry required for liver regeneration is encompassed by three types of pathways: those activated by cytokines, growth factors, and c-metabolic networks that link liver function with cell growth and proliferation. As redundancy exists among the intracellular components of each network, loss of an individual gene rarely leads to complete inhibition of liver regeneration, but rather to a change in the timing of hepatocyte DNA replication or mortality in only a fraction of the animals carrying the defect. The latter view, although attractive, may apply for the relevant pathways required for the metabolic readjustment of the liver needed to allow regeneration of the organ, but may not necessarily identify the events critically involved in the re-entry into the cell cycle. Perhaps, to precisely distinguish and separate the changes elicited by the metabolic and circulatory perturbations imposed by the removal of 2/3 of the organ mass (adaptive changes), and those specifically leading to a transition of liver cells from a quiescent to a replicative state (mitogenic changes), other experimental models need to be carefully explored. In the recent years, an important contribution for the understanding of the critical events responsible for the G0-G1 transition of the hepatocytes has come from studies on liver hyperplasia induced by several xenobiotics or endogenous molecules, able to induce the entry of hepatocytes into the cell cycle in the absence of previous cell death/loss, and therefore defined as primary mitogens [5, 6]. In this chapter, we will discuss the most relevant differences between liver regeneration and direct hyperplasia in terms of (1) timing of proliferation, (2) spatial distribution of proliferating cells, (3) the ploidy state resulting from the different modes of proliferation, and (4) the difference in the pathways involved in direct hyperplasia vs. liver regeneration. We will also elucidate the possible therapeutic importance of primary mitogens in conditions where liver regeneration is impaired (i.e., aging and liver related transplantation). Finally, in view of the well established association between cell proliferation and hepatocellular carcinoma (HCC) development, we will also discuss some of the results arising from studies focused on alterations in genes involved in cell proliferation, and also the "unconventional" effect of direct hyperplasia induced by some primary mitogens on the growth of preneoplastic lesions and on HCC development.

From a functional point of view, the liver is an extraordinary organ which plays a central role in the metabolic functions of the body, being able to carry out over 5,000 functions; indeed, hepatocytes (the main functional cells of the liver) are deputated to detoxification of both endogenous and exogenous molecules (see Chap. 11), synthesis of secreted proteins (albumin, most coagulation factors, plasma carrier proteins), and metabolism of amino acids (see Chap. 9), lipids (see Chap. 10), and carbohydrates (see Chap. 8). These functions

A. Columbano (✉)
Department of Toxicology, School of Medicine,
University of Cagliari, Cagliari, Italy
e-mail: columbano@unica.it

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases,* Molecular Pathology Library 5 ,
DOI 10.1007/978-1-4419-7107-4_54, © Springer Science+Business Media, LLC 2011

are not identical among the hepatocytes, as they are highly dependent on the cell location within the hepatic lobule (metabolic zonation) (see Chap. 2). Periportal hepatocytes, for example, express urea cycle enzymes and convert ammonia to urea while, in contrast, pericentral hepatocytes express glutamine synthase and utilize ammonia to generate glutamine. As a major regulator of plasma glucose and ammonia levels, liver is also essential for optimal function of the brain, for bile synthesis and therefore, for absorption of fat and lipophilic nutrients.

Due to its astonishing capacity to perform so many essential functions, it is not surprising that the liver is endowed with a remarkable capacity to adapt to metabolic perturbance. Liver regeneration after 2/3 PH typifies one such capacity, in that the organ changes instantly from an essentially quiescent state to a rapidly growing one [7]. This experimental model consists in the removal of specific lobes (three of the five liver lobes of rodents), without causing damage to the residual two lobes. The latter rapidly expand and in 5–7 days achieve the recovery of a liver mass equivalent to that present prior to surgery. Thus, liver regeneration is more a process of compensatory growth triggered to restore the original mass than a true organ regeneration, defined as the reconstitution of a structure that has been excised. Notably, while true regeneration is carried out by dedifferentiated cells, liver regeneration after PH, under normal conditions is carried out by the remaining fully differentiated cells, with no involvement of stem/progenitor cells [8]. Only when the response is impaired, as is the case of chronic liver injury induced by chemicals in rodents or in chronic liver diseases in humans, the hepatocytes exhaust their ability to replicate. In this context, hepatic progenitor cells (or "oval cells," as they are called in rodents) appear as a rich population of small round cells spreading from the periportal area into the parenchyma and will be discussed elsewhere in the textbook (see Chaps. 16 and 17.) [9, 10]. Similar to rodents, the resected parts of the human liver do not regrow, but the remaining segments enlarge to compensate for the loss of tissue and the principles governing hepatic growth in rodents appear to be similar to those in humans. Thus, understanding the mechanisms of initiation of liver regeneration in rodent models should not only provide new important insights in the understanding of the molecular mechanisms responsible for the entry of hepatocytes into the cell cycle, but also greatly contribute to the establishment of clinical procedures to accelerate the growth of liver transplants and might also provide new approaches for gene therapies involving the liver. However, liver regeneration is the result of a complex interplay between at least two distinct sets of rapidly evolving changes; those elicited by the dramatic metabolic and circulatory perturbations imposed by the removal of 2/3 of the organ mass (adaptive changes), and those specifically leading to a transition of liver cells from a quiescent to a replicative state (mitogenic changes). For example, PH promotes rapid transcriptional changes of more than 100 genes [4, 11]. However, whether these genes are directly related with the entry into the cell cycle or are responsible for specific adjustments that hepatocytes require to deliver all essential hepatic functions while going through cell proliferation, is still unclear. Unfortunately, while the generation of transgenic mouse models initially produced the belief that the identification of the genes critically involved in liver regeneration would have soon been possible, from the recent literature it is now evident that the single ablation of several genes involved in different pathways can influence liver regeneration; this finding led, on one hand to the notion that no single gene can be considered responsible for liver regeneration, probably due to the existence of redundant pathways, which complement for the loss of any given gene, and on the other hand, regeneration of the liver requires the activation of dozens of different pathways. How many of these pathways are directly related to the triggering of cell cycle again remains an unsolved question. Thus, the question arises whether other models of hepatocyte proliferation are available that could be used to identify the critical genes specifically involved in the entry of hepatocytes into the cell cycle.

In recent years, an increasing number of agents (primary out causing liver injury has been identified [5]. Opposite to liver regeneration after cell loss/injury, the proliferative process induced by primary mitogens, which is now the initial event, generates an increase in liver size and an excess of cells (Fig. 54.1). After mitogen withdrawal, regression of the initial hyperplasia occurs via apoptosis. The final result, in both circumstances is a return to the normal mass of the organ indicating a strict regulatory control of the organ size. On the other hand, if the mitogen is continuously present, no regression or further increase of liver mass occurs, suggesting that liver cells become refractory to the proliferative stimuli, once the organ has doubled its size. The potency of the mitogenic stimulus and the peaks of the S phase varying according to the nature of agents. Primary mitogens include a broad spectrum of chemicals with little structural similarity, both exogenous and endogenous, such as peroxisome proliferators (PPs) [12], the halogenated hydrocarbon TCPOBOP [13], retinoic acids (RAs) [14], triiodothyronine (T3) [15, 16], lead nitrate (LN) [17], ethylene dibromide (EDB) [18], cyproterone acetate (CPA) [19] α-hexachlorocyclohexane (α-HCH) [20] and others (Table 54.1). Among these agents, it is remarkable that PPs, retinoic acid, T3, and the halogenated hydrocarbon TCPOBOP, are all ligands of nuclear receptors of the steroid/thyroid superfamily, namely peroxisome proliferator-activated receptor-alpha (PPARα), retinoic acid receptor (RAR), thyroid hormone receptor (TR) and constitutive androstane receptor (CAR). All of them, functioning as ligand-activated transcription factors, regulate the expression of genes involved in lipid metabolism, adipogenesis,

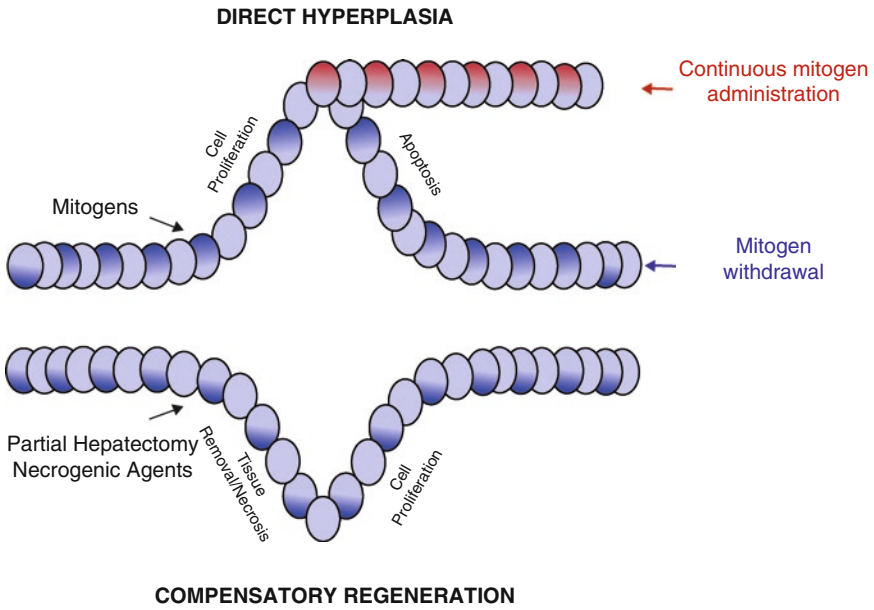

Fig. 54.1 Schematic representation of liver regeneration and direct hyperplasia. Liver regeneration is triggered to compensate cell loss, due either to surgical removal or necrosis induced by hepatotoxicants. Proliferation stops once the liver has regained its original mass. In direct hyperplasia, liver-cell proliferation is not preceded by cell loss and generates an increase in liver mass and an excess of cells. After mitogen withdrawal, regression of the initial hyperplasia occurs, via apoptosis. If mitogen administration is continued, apoptosis is inhibited (no regression takes place), but the liver becomes refractory to the mitogenic stimulus (no further growth occurs)

Table 54.1 Primary mitogens in rodents

Nuclear receptors ligands	Type of nuclear receptor	Species	Single/repeated treatment
TCPOBOP	CAR	Mouse only	Single
Phenobarbital	CAR	Rat-mouse	Multiple
9-cis Retinoic acid	RXR	Rat-mouse ND	Single
All-trans retinoic acid	RAR	Rat-mouse	Single in rats – multiple in mice
Triiodothyronine	TR	Rat-mouse	Single in rats – multiple in mice
GC-1	TR	Rat-mouse ND	Single in rats
Methylclofenapate	PPARα	Rat-mouse	Single in rats – multiple in mice
Ciprofibrate	PPARα	Rat-mouse	Single in rats – multiple in mice
WY-14,643	PPARα	Rat-mouse	Single in rats – multiple in mice
BR931	PPARα	Rat-mouse ND	Single in rats
Cyproterone acetate	GR	Rat-mouse	Single in rats – multiple in mice
Other liver mitogens			
4-Acetylaminoflourene		Rat-mouse ND	Single
α-Hexachlorocyclohexane		Rat-mouse	Single in rats – multiple in mice
Lead nitrate		Rat only	Single
Ethylene dibromide		Rat-mouse	Single

ND not determined

xenobiotic detoxification, differentiation, etc. Upon ligand binding, PPARα, RAR, TR, and CAR, heterodimerize with the 9-cis-retinoic acid receptor, RXRα, and bind to specific DNA elements consisting of direct repeats of the consensus motif TGACC separated by one (DR1) or more base pairs, depending on the receptor [21–23].

While the molecular mechanisms responsible for the proliferative response of hepatocytes to administration of primary mitogens are still largely unknown, studies on this model of hepatocyte proliferation revealed that many "dogmas" deriving from work performed on liver regeneration do not apply to liver hyperplasia. These include, for example, the kinetics of the proliferative process, the spatial distribution of replicating hepatocytes, the changes in ploidy and the early modifications of gene expression. Notably, while liver regeneration is almost always directly associated with the carcinogenic process, hyperplasia induced by some primary mitogens does not, or on the opposite, exerts an anti-tumoral effect.

Autonomy and Timing of the Proliferative Process in Liver Hyperplasia

The extent and timing of liver regeneration are known to vary according to circadian rhythms [24]; some hints on the mechanisms by which these rhythms control hepatocyte proliferation after PH have recently been achieved [25, 26]. However, it is also known that the timing of DNA replication, which is not under the control of circadian rhythms, appears to be an intrinsic property of hepatocytes. Rats and mice differ in the timing of DNA replication after PH; while in rats, S phase begins 16–18 h after PH, in mice, no DNA synthesis occurs before 36–42 h [27]. In an elegant study, Weglarz and Sandgren transplanted rat hepatocytes into the livers of mice after PH and found that rat hepatocytes replicated earlier than mouse hepatocytes in the resultant chimeric livers [28]. These results indicate that the timing of hepatocyte DNA replication after PH is an autonomous process, primarily guided by intrinsic signals, and that distantly produced cytokines or growth factors may have a permissive, but not an instructive role in progression to "S" phase and would not override the internal hepatocyte "G" clock. However, the model of a "hepatocyte G clock" has been challenged by studies on mitogen-induced direct hyperplasia, which demonstrated that a delayed induction of DNA synthesis in mice does not reflect an intrinsic longer duration of G1 in the mouse hepatocyte; indeed, the CAR agonist TCPOBOP triggers a fast DNA synthesis response which occurs 12 h before that observed after PH (24 vs. 36 h) [29]; in rat liver, DNA synthesis starts 12 h after T3 or Nafenopine, a PPARα ligand, a time when no evidence of DNA synthesis occurs after PH [16, 30]. Thus, the kinetics of induction of hepatocyte DNA synthesis are specific to the signaling pathway initiating DNA synthesis. Whether the anticipated entry into S phase observed after TCPOBOP or T3 or Nafenopine is the consequence of the rapid upregulation of genes critical for hepatocyte proliferation, or downregulation of checkpoint genes in G1 remains to be determined. Notably, ablation of *c-Jun* further accelerates the entry of mouse hepatocytes into S phase (18 h) in response to a single dose of TCPOBOP, suggesting that the duration of G1 phase in mouse liver can be further shortened [31].

Spatial Distribution of Replicating Hepatocytes

The spatial distribution of proliferating hepatocytes in the regenerating liver has a predictable pattern that varies according to the time after surgery. Initially, cell proliferation is highest in cells located in zone I of the lobule. In mouse liver, for example, at the maximum of ^{3}H-thymidine incorporation, about 80% of all labeled nuclei are in zone 1, 15% in the middle zone (zone 2) and 5% in zone 3 [32]. Similarly, rat hepatocytes with the lowest level of replication are located in zone 3 of the lobule, surrounding central veins, but, as regeneration proceeds there is a more random distribution of proliferating cells throughout the lobule [33, 34]. To justify such a zonal distribution of proliferating cells, a "streaming liver model" has been postulated [35]; however, evidence against the theory of the streaming hepatocytes was provided by other studies [36, 37]; in particular, Bralet et al. [36], using *Escherichia coli* 3-galactosidase as a marker gene, were able to show that the distribution pattern of the positive cells was the same at different time points of the study, thus demonstrating that the β-galactosidase-positive cells had not moved inside the lobule during the 15 months of the experiments. Lobular zonation, therefore, could be explained by metabolite-induced gene regulation or by a shorter G1 interval in zone 1 compared to that of cells in zone 2 and 3. The zonation of induction of enzymes by various mitogenic xenobiotics has been extensively demonstrated [38, 39], but the zonal induction of DNA synthesis is less well characterized, although reliable methods have been established [40]. Overall, the conclusion of these studies is that the type of zonation observed following PH does not apply to many ligands of nuclear receptors. Indeed, both in mice and rats, several studies have reported that BrdU incorporation is observed predominantly in zone 2, rather than zone 1 hepatocytes; for instance, it was reported that the hepatocytes proliferating at 18 or 24 h after T3 alone were predominantly in the midzonal region of the hepatic lobule, whereas those after PH alone were predominantly in the periportal region of the liver [41]. When T3 was administered at PH, the labeled cells occupied both the midzonal and periportal regions of the liver. Thus, within the first 24 h of T3 administration or PH, cells in different parts of the hepatic lobule (midzonal, T3; periportal, PH) are recruited into the cell cycle to induce proliferation. The cells proliferating at 72 h after PH alone, T3 alone, or the combination were predominantly in the midzonal region. A similar midzonal localization of rat hepatocytes undergoing DNA synthesis was obtained with nafenopine (Fig. 54.2), although a recent report showed that a distinct PPARα ligand, ciprofibrate, stimulates DNA synthesis in periportal hepatocyte [42]. In mouse, the difference in the zonation of replicating hepatocytes between direct hyperplasia and liver regeneration is even more evident. Indeed, midzonal and not periportal hepatocytes are the first to respond to CAR ligands (Fig. 54.2), while a panlobular distribution is seen after PPARα ligands [42]. It is still unclear whether the response of midzonal hepatocytes is dependent upon a zonal distribution of the receptors and/or receptor-associated coactivators; however, it should be noted that midzonal hepatocytes, and not periportal are the first to

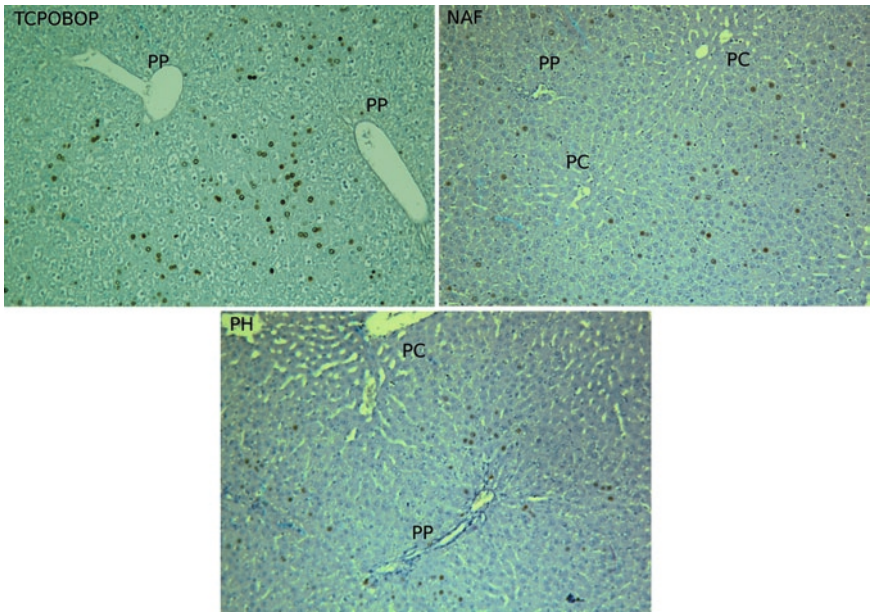

Fig. 54.2 BrdU-staining showing that midzonal hepatocytes are the first to proliferate following a single dose of TCPOBOP (24 h, mouse liver) or Nafenopin (18 h, rat liver). On the opposite, mostly periportal hepatocytes are in S phase 18 h after PH (rat liver)

respond also to a single dose of LN. From these results, it is clear that different stimuli may trigger DNA synthesis in different populations of hepatocytes. This notion may be of some importance in certain clinical conditions when a rapid and synchronous proliferation of hepatocytes is required.

Liver Ploidy

Given that replicating hepatocytes may not be the same in liver regeneration and direct hyperplasia, one relevant question is whether the different proliferative stimuli may also result in a different ploidy state of the liver. It is well known that while a newborn liver (rat or mouse) contains only mononucleated diploid cells, polyploidization and binuclearity develop rapidly [43, 44]. Hepatocytes become polyploid by physiological failure of cytokinesis once diploid hepatocytes undergo mitosis, but do not form a contractile ring. Therefore, cytokinesis aborts, and a binucleated tetraploid liver cell is generated [45]. Insulin signaling through Akt has been reported to control a specific cell division program which leads to the physiologic generation of binucleated tetraploid liver cells [46]. Independently of the mechanisms responsible for liver polyploidization, rat liver of young adult animals (2–3 months of age) is constituted predominantly by tetraploid cells (85–90%) with a small percentage of diploid (8–10%) and octoploid cells (1–3%). Among the tetraploid cells, there is a 65% of mononucleate and a 20% of binucleate hepatocytes (BN) which are absent immediately after birth and appear at about 3 weeks after birth, preceding the occurrence of mononucleate tetraploid (MT) cells. All these cells are capable of replicating after PH; however, after surgery, there is a rapid decrease in the number of BN cells, with their complete disappearance 3 days after operation. At the same time, an increase in the number of MT and mononucleate octoploid (MO) cells is also observed [47, 48]. What about direct hyperplasia? The ploidy state appears to be dependent from the nature of the mitogenic agent; ligands of nuclear receptors, such as phenobarbital (PB) and TCPOBOP (CAR agonists), or methyl clofenapate, Wy-14,643, ciprofibrate and nafenopine (PPARα-agonists) cause liver polyploidisation with a striking increase in mononucleated 8n cells within the liver [48, 49]. However, the most striking effect observed after PH, namely the disappearance of binucleate hepatocytes is not observed after treatment with PPs [48]. Other mitogens such as 4-acetylaminofluorene (4-AAF) suppress binucleation (similar to PH), but cause an increase in the fraction of diploid hepatocytes (differently from PH) [50]. Liver hyperplasia induced by LN leads, similar to liver regeneration, to an increase in ploidy state, but unlike PH, is associated with an increase of binucleate hepatocytes ($4\times2c$ and $8\times2c$) [48]. A disturbance in cytokinesis seems to be the major phenomenon occurring during LN-induced cell proliferation. At the present, it is not known whether this is due to LN induction of factors that specifically inhibit cytokinesis [51], or to alterations in the expression and/or phosphorylation/dephosphorylation state of proteins which play a crucial role in the cytokinetic process [52], or both. The possibility that LN-induced disturbance of cytokinesis can be due to PI3K/Akt pathway, downstream from insulin signaling [46] may also be envisaged.

Although more studies are needed to determine the causes of the different patterns of ploidy seen after different proliferative stimuli, it is clear that not only zonation and kinetics, but also the ploidy state seen after PH, is different in mitogen-induced liver hyperplasia (Table 54.2). More detailed studies on the ploidy state of the liver during direct hyperplasia are also needed in order to carefully establish whether and how the increase in DNA content induced by primary mitogens correlates with increase in cell number. Hyperplasia induced by LN, for example, results in doubling of DNA content; however, because of the increase in ploidy due to induction of binucleate hepatocytes, the increase in cell number seems to be limited to only about 25% [48]. It is possible that increased nuclear ploidy, and not an absolute increase in cells, entirely accounts for the hepatomegaly induced by PPs [53].

Gene Expression During Liver Hyperplasia

A large number of genes are activated after PH at the transcriptional, posttranscriptional, or posttranslational level [54, 55]. Involved in this process are cell cycle genes, metabolic genes, genes coding for extracellular matrix proteins, growth factors, cytokines, and transcription factors. Generally, gene expression during liver regeneration is divided into phases that include the expression of immediate early genes (IEGs) and delayed early genes (DEGs). Expression of these genes is controlled by transcription factors, cytokines, and growth factors, which themselves are modulated by signal transduction pathways that receive and transduce stimuli necessary for cell replication and tissue regeneration [56, 57].

Immediate Early Genes/Transcription Factors

Within minutes after PH, hepatocytes in the remnant liver undergo transition from the quiescent G0 state into G1 phase of cell cycle. While the precise mechanism(s) responsible for triggering this transition are not known, enhanced expression of genes occurring within 15 min to 2 h after PH, probably mediates G0/G1 transition. A large number of genes were identified and defined as IEGs, including the *fos* and *jun* family, *egr-1, LRF-1, c-myc, IGFBP-1, PRL-1,* and others [54, 58]. As IEGs are induced in a protein synthesis-independent manner, their transcription must be activated by transcription factors that pre-exist in the liver cells, in a latent form; increased binding of NF-κB, AP-1, C/EBP occurs within minutes after PH [11], with STAT3 activation occurring shortly thereafter. Activation of AP-1 and NF- κB, as well as DNA synthesis, can be inhibited by pretreatment of the animals with antibodies against tumor necrosis factor-α (TNF-α), suggesting that this cytokine plays an important role in the initiation of liver regeneration by activating transcription factors [59]. Several lines of evidence indicate that TNF-α and other cytokines such as interleukin 6 (IL-6) may play a critical role in the G0-G1 transition of hepatocytes: (1) germ-free rats exhibit a delayed liver regeneration after PH when compared to normal animals [60]; (2) liver regeneration is severely impaired in mice lacking TNF-α receptor 1 (TNFR1) or IL-6 [61, 62]; (3) these defects are corrected after injection of IL-6 [61, 62], suggesting that the role of TNF-α is to regulate secretion of IL-6. The results from these studies suggest that after damage to the liver, either by PH or by hepatotoxicants, the following sequence of events is believed to be required for initiation of liver regeneration: TNF-α–NF- κB- IL-6-STAT3, cyclins D and E and eventually entry into S-phase. However, other reports showed only minor defects in hepatocyte proliferation or no difference in the regenerative capacity in IL-6 and TNF-α knockout mice [63, 64], suggesting that other factors, such as surgical trauma, might play a role in the impairment of liver regeneration. Thus, whether TNF-α and IL-6 are the exclusive triggers that initiate liver regeneration is still questionable. It is possible that cytokines play a major role during the inflammatory process that follows the surgical removal of part of the liver; loss of cytokines may therefore aggravate the effects of surgery leading to sickness of the animal, and, as a consequence to inhibition of the regenerative response. Whatever the explanation, it is plausible that a large proportion of IEGs and transcription factors are not directly correlated with DNA replication, but rather with a metabolic rearrangement of the liver following cell loss.

Notably, most of the changes observed after PH and considered essential for liver regeneration do not take place in liver hyperplasia induced by ligands of nuclear receptors. Indeed, no activation of transcription factors such as AP-1, NF- κB, STAT3, and C/EBP could be observed in rats or mice after treatment with agonists of CAR, PPARα, and TR [30, 65–67]. Since activation of latent pre-existing transcription factors is thought to induce the expression of IEGs, it is not surprising that in mitogen-induced direct hyperplasia, many of the early genes upregulated after PH (*c-fos, c-jun, c-myc, LRF-1*) are not, or minimally modified after treatment with mitogens such as T3 or TCPOBOP or Nafenopine (2–3 fold increase vs. 50–100 fold observed by RT-PCR after PH) [16, 30, 66, 67]. The results of these studies thus cast doubts about the assumption that many of the IEGs whose expression is increased immediately after PH are really critical for the cell-cycle progression. Interestingly, even in liver regeneration, it is sometime difficult to interpret the role of IEG. Increased expression of *c-fos* and *c-jun* was reported soon after PH or following treatment with several hepatotoxins. A terrific number of reports have been published supporting the

Table 54.2 Main differences between liver regeneration (LR) and direct hyperplasia (DH)

	Initial replication	Nuclear ploidy	BN cells	IEGs			Transcription factors			
LR				c-fos	c-jun	c-myc	AP-1	NF-κB	STAT3	CEBP-β
PH	Zone I	⬆	⇩	⬆	⬆	⬆	⬆	⬆	⬆	⬆
CC14	Zone I	⬆	ND	⬆	⬆	⬆	⬆	⬆	⬆	⬆
DH										
TCPOBOP	Zone II	⬆	ND	⇔	⇗⇘	⇔	⇔	⇔	⇔	ND
T3	Zone II	ND	ND	⇔	⇔	⇔	⇔	⇔	⇔	ND
NAF	Zone II	⬆	⇔	⇔	⇔	⇔	⇔	⇔	⇔	⇔
BR931	?	ND	ND	⇔	⇔	⇔	⇔	⇔	ND	ND
LN	Zone II	⬆	⬆	⇔	⬆	⬆	⬆	⬆	⬆	ND
4-AAF	?	⬆	⇩	ND	ND	ND	ND	ND	ND	ND
CPA	?	⬆	⇩		⇔	⇔				⇔

BN binucleate cells; *IEGs* immediate early genes; *ND* not determined

⬆ Increase

⇩ Decrease

⇔ Unchanged

⇗⇘ Very slight increase

role of these IEGs in liver regeneration (for a review see [54]). However, intriguingly, following injury with CCl_4, an increased transcription of *c-fos* and *c-jun* (together with *c-myc*) is observed in virtually all hepatocytes of zone III (more damaged cells), but not in zone I (where regeneration starts) [68]. Moreover, liver cell death rapidly occurring after PH in rats fed a Vitamin A deficient diet is associated with an increase in the expression of *c-jun* and *c-fos* much higher than that seen in PH animals fed a basal diet [69]. In addition, after portal branch ligation (PBL), which produces atrophy of the deprived lobes (70% of the liver parenchyma), whereas the perfused lobes undergo compensatory regeneration and *c-fos, c-myc,* and *c-jun* expression are similarly induced in atrophying and regenerating lobes [70]. These results suggest that many of these changes are nonspecific, possibly stress-induced, cellular responses triggered when liver cells are exposed to a potentially dangerous damage, rather than a "mitogenic" program. If so, it is possible that stress-activated pathways may play a checkpoint role delaying the progression to S phase, rather than being the triggers of hepatocyte proliferation. If the IEGs play a role as stress sensors, one may explain why during hyperplasia induced by primary mitogens, only minor changes in the expression of IEGs occur. In this context, it is interesting to note that mice lacking c-Jun in the liver display impaired liver regeneration after PH with a phenotype correlating with increased protein levels of the cdk-inhibitor p21 in the liver [71]; however, the same mice show an accelerated entry into S phase and an increase of replicating hepatocytes after a single treatment with TCPOBOP [31], suggesting that *c-jun* also possesses an anti-proliferative effect. *c-myc* encodes a transcription factor that has long been considered essential to liver regeneration since this process is associated with an increase in *c-myc* expression accompanying the synchronous entry of remaining hepatocytes into the cell cycle [58, 72, 73]. However, as already mentioned, we reported that increased levels of *c-myc* are not detected in liver hyperplasia stimulated by several primary mitogens, thus questioning the role of *c-myc* in hepatocyte proliferation. Notably, a recent study where reduced hepatic *c-myc* expression in adult male transgenic mice was generated by injecting into the tail vein adenovirus expressing Cre-recombinase (AdCre) showed that, despite a 90% decrease in hepatic expression of *c-myc,* restoration of liver mass 7 days later was not compromised, suggesting that hepatocytes deficient in *c-myc* are able to proliferate in response to PH [74].

Mitogen-induced direct hyperplasia is also IL-6-TNF-α independent. Indeed, CAR-mediated hepatocyte proliferation occurs at a high extent in IL-6 knockout mice [75], and proliferation induced by TCPOBOP occurs at a even higher rate in mice knockout for both TNFR1 and TNFR2 [76], clearly demonstrating that these cytokines do not play a major role in mitogen-induced hepatocyte proliferation, but rather may exert an anti-proliferative effect through a negative cross-talk with nuclear receptors. Our studies and work by others showed that also hepatocyte proliferation induced by the PPARα ligand, ciprofibrate, is TNF-α-IL-6 independent [75, 77–79].

Thus, it is evident that almost none of the early changes associated with "priming" of hepatocytes in liver regeneration occurs when hepatocytes are stimulated to divide by ligands of nuclear receptors possessing mitogenic activity (Table 54.2). It should also be noted that while hepatocyte growth factor (HGF) mRNA is frequently found increased in liver regeneration after PH or necrogenic conditions [3, 80], its levels are strongly decreased in direct hyperplasia induced by the PP BR931 [81]. Unfortunately, opposite to HGF and Transforming Growth Factor-α(TGF-α), treatment with T3, TCPOBOP or members of the PPs family stimulate hepatocyte proliferation in vivo, but is not effective in hepatocyte cultures, thus hampering the possibility of further mechanistic studies in in vitro conditions. Whether the inability of the latter hepatomitogens to induce DNA synthesis in primary cultures of hepatocytes is due to downregulation of nuclear receptors or to other causes (i.e., other cell types in the liver mediate the nuclear receptor-induced proliferative stimuli) remains to be determined.

Entry into S phase following PH in rats and mice occurs at around 18 and 36 h respectively; following mitogen treatment, DNA synthesis is anticipated by 6 and 12 h, respectively. The reader should be alerted that strain specific differences in these numbers have been reported in mice. The accelerated entry into S phase following treatment with TCPOBOP, nafenopine, and T3 correlates with a very rapid induction of cyclin D1 [16, 29], suggesting that early activation of cyclin D1 gene by ligands of nuclear receptors may be one of the most rapid effects of activated nuclear receptors; this implies that they bypass the priming phase which requires a series of gene activation, as seen in the PH model. Further studies to characterize molecular interactions between cyclin D1 gene and TRs, PPARs, CAR and possibly other nuclear hormone receptors may be important to verify whether this gene plays a critical role in mitogen-induced hyperplasia. However, it should be noted that similar to liver regeneration, no single gene can likely be considered essential for the entry into S phase in direct hyperplasia. Indeed, cyclin D1 knockout mice respond almost as efficiently as wild type to induction of proliferation by TCPOBOP [82]. This might be due to certain adaptive changes in these animals.

A critical question that remains to be answered is which among the many genes up or downregulated after a mitogenic stimulus are essential for hepatocyte proliferation. Although the number of genes modified in mitogen-induced hyperplasia is much lower than that seen after PH, a large proportion of IEGs are metabolic genes unrelated to or not directly connected

with DNA synthesis. A useful approach to this relevant question can be to investigate transcriptional targets regulated in a common fashion by 2 distinct proliferative stimuli, namely primary mitogens and PH. Recent studies have been performed by complementary DNA (cDNA) microarray technology to characterize a large set of gene responses to both stimuli. These showed new transcriptional responses, both upregulatory and downregulatory, in both the PH and CAR pathways [83]. Each treatment causes diverse effects other than cell proliferation, since PH induces injury responses and metabolic adaptations and CAR stimulates genes of phase I activation and detoxification. However, a set of common early response genes was detected by comparing the two treatments, showing that enhanced cell survival and early biogenesis of critical cell components complement the induction of proliferation. Among the genes characterized in our microarray analysis, *Gadd45β* was of particular interest; it is strongly and rapidly induced by both PH and TCPOBOP treatment [83]. Notably, early induction of *Gadd45β* is not species specific as enhanced levels of *Gadd45β* mRNA have been demonstrated also in rat liver following administration of T3 [84]. *Gadd45β* unlike two other homologs (*Gadd45α* and *Gadd45γ*), plays an anti-apoptotic role and is activated by TNF-α via NF-kB [85]. Thus, induction of *Gadd45β* coincides with entry into an active cell cycle, but its action might be to protect hepatocytes from apoptosis. Regardless of how it functions, *Gadd45β* is one of a few genes upregulated in both liver regeneration and direct hyperplasia. Whether this gene is critical for hepatocyte replication is unknown. Recent studies [86] have shown that *Gadd45β* is required for liver regeneration as *Gadd45β* mice display increased mortality and profoundly impaired hepatocyte survival and cell-cycle progression in response to PH. Whether *Gadd45β* also plays such a critical role in direct hyperplasia is not known and warrants further studies.

Termination of Liver Hyperplasia

A still unanswered question in biology is how the size of an organ is determined. Accumulating evidence suggests that a "size checkpoint" operates at the level of the organ's total mass, rather than of the size or the number of the constituent cells. As mentioned, in adult organisms, liver is in a quiescent state, but under certain conditions shows a remarkable regenerative capacity. Indeed, following a 2/3 surgical resection, most of its size is regained within 3–4 days. After the initial growth, no further enlargement of the liver is observed, suggesting the existence of pathways leading to termination of liver regeneration. While some studies have initially proposed transforming growth factor β (TGF-β) as the terminator of regeneration [87], no clear evidence for this has ever been achieved; moreover, how the liver senses this termination signal or what are the mechanism(s) involved is unknown. Even more striking is the capacity of the liver to modify its size in response to physiologic stimuli (i.e., hepatic enlargement during pregnancy) or in response to xenobiotics with mitogenic potency. Under the latter condition, the liver can double its size in a matter of few days, but it becomes refractory to further challenge by the same xenobiotic. Many xenobiotics able to induce liver enlargement are ligands of nuclear receptors, and interestingly are also non-genotoxic liver carcinogens [12, 13]. In spite of several studies, the key molecular events which govern the tumoral potency of ligands of nuclear receptors are still unclear. The breakthrough that many compounds which possess liver tumor promoting ability are also potent inducers of hepatocyte proliferation led to the hypothesis that the mechanisms by which these agents cause liver neoplasia are a consequence of their mitogenic capacity that ultimately results in an increased rate of mutation [88, 89]. However, it is now clear that the proliferative response of the liver to these mitogens is lost very shortly (1 or 2 weeks), indicating that the hyperplastic liver becomes refractory to mitogenic stimuli. This observation also suggests that the tumors arising in these enlarged livers may be the consequence of the "escape" of "selected cells" from the regulatory mechanisms governing the size of the organ. Thus, the identification of the molecular mechanisms responsible for the refractiveness of the enlarged liver to further mitogenic stimuli, is critical for improving our knowledge of the control of organ size, and also for determining whether dysregulation of these pathways is a possible mechanism for the clonal expansion of "resistant" hepatocytes and their progression to HCC. Recent studies showed that, in *Drosophila,* organ size regulation is orchestrated by the Hippo kinase cascade, a growth-suppressive pathway that ultimately antagonizes the transcriptional co-activator Yorkie (Yki); a single phosphorylation site in Yki mediates Yki inactivation and the relative growth suppressive output of the Hippo pathway [90]. It was also demonstrated that Hippo signaling pathway exists also in mammalian and leads to phosphorylation of YAP, the mammalian homolog of Yki. Thus, the Hippo signaling component YAP1 has emerged as an important regulator of organ size in mammals; moreover, as its overexpression was observed in many human tumors, it is now considered also as a candidate oncogene [91]. However, it is unknown whether the Hippo pathway is involved in the termination of the adaptive liver enlargement observed after administration of several hormones or drugs or once the liver has regained its original mass after PH.

Potential Efficacy of Primary Mitogens in the Context of Liver Regeneration in the Elderly and Living Related Transplantation

The loss of regenerative capacity is the most dramatic age-associated alteration in the liver. For learning more about

changes in senescent liver, please see Chap. 19. Several studies on the regenerative response that follows 2/3 PH have shown that the response is both delayed and reduced with aging in rats and mice.[92–94]. While telomerase shortening or increased polyploidy do not appear to play a major role, investigations of replicative senescence in cultured cells have shown that the inability of senescent cells to proliferate is caused by the alteration of chromatin structure, leading to epigenetic silencing of cell-cycle genes [95–97]. As reviewed elsewhere [98], the first evidence indicating epigenetic inhibition of liver proliferation in old mice was obtained by studies examining C/EBPα expression in old livers. C/EBPα is expressed at high levels in the liver and restrains proliferation of young livers by inhibiting cyclin-dependent kinase, cdk2 [99]; aging switches C/EBPα from complexes with cdk2 to high-molecular-weight complexes containing Rb, E2F4 and the chromatin-remodeling protein Brm [100, 101] (i.e., the C/EBPα–Brm complex). This C/EBPα–Brm complex occupies E2F-dependent promoters such as b-myb, cdc2, DHFR, and c-myc and inhibits expression of these genes after PH. The C/EBPα–Brm complex also inhibits expression of *Foxm1b,* which is required for proper liver regeneration. Indeed, while mice lacking *Foxm1b* gene have a greatly reduced proliferative response to PH [102], adenoviral delivery of Foxm1b to 12-month-old wild type mice augments liver regeneration to the level of young mice [103]. Aging also increases protein levels of histone deacetylase 1 (HDAC1), which binds to the C/EBPα–Brm complex. The recruitment of the HDAC1–C/EBPα–Brm complex to *c-myc* and *Foxm1B* promoters leads to deacetylation of histone H3 at K9, followed by trimethylation of K9 of histone H3 and silencing of transcription via interactions with heterochromatin protein 1a (HP1a) [104]. These studies also revealed that C/EBPα–HDAC1-mediated modifications of histone H3 lead to the epigenetic repression of *c-myc* and *Foxm1b* promoters.

The impaired liver regeneration observed in old rodents has led to the general idea that "old" hepatocytes have intrinsic defects in their capacity to enter the cell cycle. This notion was recently challenged by the finding that a single injection of the hepatomitogen TCPOBOP to aged mice resulted in a strong induction of hepatocyte proliferation [105]; DNA synthesis and M phase were nearly identical in both 1-year- and 8-week-old mice and stimulation of hepatocyte DNA synthesis was associated with increased expression of several cell-cycle associated proteins (cyclin D1, cyclin A, cyclin B1, E2F, pRb, and p107), all of which were comparable in aged and young mice. TCPOBOP treatment also increases expression of *Foxm1b,* to a similar degree in both groups. Hepatocytes therefore, retain their proliferative capacity in old age despite impaired liver regeneration. Overall, these results suggest that, unlike a wide-spread notion, the capacity of the hepatocytes to enter cell cycle is maintained during aging, if an appropriate proliferative stimulus is provided. This finding is of particular importance as it is still unknown whether the depressed replicative response observed in aged rodent liver results from lowered levels of growth factors and/or hormones, or from intrinsic changes within the cell. Perhaps, even more important, these results also have significant clinical relevance as they suggest a potential therapeutic approach to relieve the proliferative block after liver injury that is observed in the elderly. Indeed, while liver regeneration is severely impaired in old mice subjected to 70% PH (with virtually no BrdU-positive hepatocytes, 48 h after surgery), treatment with TCPOBOP 2 h prior to PH induces a very efficient proliferative response with a labeling Index of 21.3% ± 5.4 in TCPOBOP + PH mice vs. 1% ± 0.2 of mice subjected to PH alone, and a fourfold increase in the mitotic index [106]. The increased hepatocyte entry into cell cycle observed in the liver of mice receiving TCPOBOP prior to PH is associated with enhanced levels of cyclin D1 mRNA and protein, compared with those of mice subjected to PH alone and enhanced nuclear levels of p107. Similar results were found in rat liver after treatment with T3 [107]. Indeed, treatment of 1-year-old intact rats with T3 increases cyclin D1 expression that is followed by an enhanced proliferative response. In addition, T3 given before 70% PH stimulates regenerative response (LI was 10.8 vs. 2.28%), and expression of cyclin D1 PCNA 24 h after PH. Notably, pre-treatment with T3 also improves the regenerative response of the liver after 90% hepatectomy (LI was 27.9 vs. 14.2%) [107, 108]. Overall, studies with primary mitogens suggest that, in principle, mitogen-induced hyperplasia could be applied to human therapy in patients with reduced regenerative capacity or massive loss of hepatocytes. This aspect is of importance because major hepatectomy is primarily used to treat malignant liver disease. Old patients characterized by a low hepatic regenerative capacity are at special risk. Therefore, acceleration of the hepatocyte proliferation like that obtained after primary mitogens such as TCPOBOP or T3, may hopefully contribute to overcome the reduced regenerative response in the elderly.

Another condition where the proliferative capacity of xenobiotic mitogens could be applied to human therapy is living donor liver transplantation (LDLT). Indeed, the ratio of liver to body mass has emerged as a critical parameter in assessing the feasibility of living-related liver transplants, thus ensuring availability of an appropriate mass of liver to provide immediate function in the recipient, while maintaining the appropriate mass and function in the donor; given the relative scarcity of potential donors for living related transplantation, it follows that if the number of recipients is large, the requirement to find a donor who can safely provide adequate liver mass may further limit donor selection. Until recently, relatively little attention has been paid to factors and techniques that could increase the size of a donor liver

before transplantation [109]. This is unfortunate because while LDLT is a life saving procedure for the recipient, it is a potentially lethal operation for the donor. Therefore, very stringent criteria have to be fulfilled prior to hepatectomy to ensure donor safety [110–112]. For the donor, fast liver regeneration is imperative to reduce the probability of liver insufficiency [113]. Thus, an improvement in regenerative capacity would enhance donor safety and increase the possibility of including individuals who are not eligible for donation due to insufficient liver size [114, 115]; to develop such approaches in vivo requires investigation in animal models. In this context, it was shown that a single treatment with TCPOBOP induces a doubling of the liver mass and, more importantly, of the hepatic DNA content within 3 days. When 70% PH was performed in these large livers, a massive liver regeneration (LI was approximately 70%) occurred that was similar to that observed in mice subjected to PH alone [106]. These results clearly suggest that the regenerative capacity after 70% PH is independent of the liver size at the time of surgery. This, in turn, suggests that, on a theoretical basis, a drug capable of inducing the same hyperplastic effect in human liver as that observed in mice could be extremely useful in the field of living-related transplantation for the following reasons: (a) the donor will have a larger liver after partial resection and (b) the recipient will be transplanted with a larger portion of the liver possessing a full regenerative capacity. It is also important to note that regeneration of a larger liver is associated with improved liver function, as shown by the analysis of the two parameters of liver function, prothrombin time and albumin plasma levels, commonly used after hepatic surgery. Similar results were previously shown in rats treated with T3 [116] The fact that an increased liver mass could be obtained in two different species (rat and mouse) using two different ligands of nuclear receptors encourages the concept that the strategy of using primary mitogens could be developed to provide better outcomes from surgery. Whether interspecies differences (rodents vs. humans) will prevent application of this approach in man will require further experimentation. However, it is important to stress that techniques that could enhance liver mass might extend the possibility of surgery to a greater proportion of patients and that the use of primary mitogens may form part of such strategies.

Cell Proliferation and Hepatocarcinogenesis

HCC is a major health problem and one of the commonest malignancies with approximately 600,000 new tumors diagnosed annually [117]. Chapter 56 describes the molecular basis of HCC. Its incidence varies widely among the different geographic areas, reaching peak values in Southeast Asia and sub-Saharan Africa, where hepatitis B virus (HBV) infection is endemic; moreover, due to the spread of hepatitis C virus (HCV) (see Chap. 38), it is constantly increasing in the United States and Europe. Overall, it is the third cause of cancer-related death, behind only lung and stomach cancers. In Western countries, in 30–40% of patients, the disease is diagnosed at early stages, when it is amenable to potentially curative treatments. Five-year survival rates of up to 60–70% can be achieved in well-selected patients. However, HCC diagnosed at an advanced stage has a poor prognosis, owing to the underlying liver disease and to lack of effective treatment options. No systemic therapy has improved survival in patients with advanced HCC, although a recent study showed that median survival was nearly 3 months longer for patients treated with sorafenib – a multikinase inhibitor targeting the vascular endothelial growth factor receptor, the platelet-derived growth factor receptor, *c-kit,* and *Raf* – than for those given placebo [118]. Genetic events associated with the development of HCC, include mutations in β-catenin gene, inactivation of the tumor suppressor p53, overexpression of various HER (Human Epidermal Growth Factor Receptor) family members and of the *c-met* receptor; several cancer-relevant genes seem to be targeted at the epigenetic level (methylation), and, in addition, telomere erosion, chromosome segregation defects and alterations in the DNA-damage-response pathways contribute to generate genomic instability in human HCC. Comparative genomic hybridization studies so far have pointed to frequent chromosomal gains in 1q, 6p, 8q, 11q, and 17q, and losses in 1p, 4q, 8p, 13q, and 17p. Gene-expression analyses of human HCCs have led to their successful molecular classification on the basis of prognosis, etiology, and intrahepatic recurrence [119, 120]. In spite of an extraordinary improvement in our knowledge of the molecular alterations associated with HCC, there is still an absolute need of a more detailed and clinically grounded genomic characterization of human HCCs, of the identification of markers of early stage disease as well as the detection of patients at greatest risk of developing this tumor.

Development of HCC is a multistage process, involving initiation, promotion, and progression [121], characterized by the progressive sequential evolution of morphologically distinct stages, such as chronic liver injury, necro-inflammation and regeneration, small cell dysplasia, appearance of low-grade and high-grade dysplastic nodules, and, finally in the formation of fully developed HCC [122, 123]. In humans, most of the research on HCC is conducted on patients who have already developed the disease. This limits the scope of the investigation to tumor biology and does not allow extensive inquiry into the mechanisms of disease progression. On the contrary, relevant rodent models of liver carcinogenesis provide a unique opportunity to understand the role of the etiological factors and mechanisms of tumor development. A number of models for the study of hepatocarcinogenesis

have been developed over the years. The most widely used have been the initiation-promotion models of rat-liver carcinogenesis; much of what we know about tumor initiation, promotion, and progression in the liver has come from this model system. The classic model of carcinogenesis is an initiating single administration of a genotoxic agent – such as diethylnitrosamine (DENA) – followed by the application of promoting agents. This procedure produces multiple preneoplastic lesions some of which progress to a nodular stage and to adenomas; a few HCCs eventually arise from the nodules and adenomas over many months (usually 12–14 months). Three are the best known protocols of hepatocarcinogenesis: (a) the phenobarbital (PB) model generated by Peraino and further developed by Pitot; (b) the choline deficient (CD) model and, (c) the resistant-hepatocyte (R-H) model [124–127]. These experimental models contributed to clearly establish a few fundamental notions: (1) while the interactions between chemicals and cellular DNA may be essential, they are not sufficient, since at least one round of cell proliferation is required for initiation [128]; this explains why so many genotoxic agents can interact with liver DNA and yet so few are liver carcinogens. Indeed, with the possible exception of primary mitogens, unless a carcinogen is a cytotoxic agent and can induce cell death and reparative cell proliferation, such an agent cannot initiate liver carcinogenesis in the adult animal. Thus, cell damage with cell death is a major rate-limiting step in the initiation of cancer development in some organs as it allows fixation of some chemical changes in DNA during replication. This may have broad implications for humans in the genesis of cancer in organs that are quiescent, such as liver, pancreas, kidney, urinary bladder, brain, salivary glands, thyroid, etc. (2) the promoting agents stimulate the replication of dormant initiated cells, which, in the absence of the promoter, would not proliferate. Indeed, the phenotype of the initiated cell does not include any autonomous or spontaneous cell proliferation. If the initiated cells are to be expanded by cell proliferation (clonal expansion), they somehow must be stimulated or encouraged to proliferate. How does the promoter stimulate clonal expansion? This can be accomplished by generating a hostile environment for normal hepatocytes; under these conditions, "resistant" initiated cells – that due to the original mutations introduced by the carcinogen possess the capacity to elude this environment – will be selected. Indeed, in the PB model, while normal surrounding hepatocytes become refractory to the mitogenic stimulus exerted by PB and undergo apoptosis, hepatocytes within the early preneoplastic lesions are resistant to PB-induced cell death and slowly expand [129]. Similarly, in the CD model, hepatocytes of the early lesions are much more resistant to fat accumulation than those in the surrounding parenchyma. A selection of hepatocytes refractory to fat accumulation might occur in this model of hepatocarcinogenesis, a possibility that could have relevant biological consequences. Indeed, excessive intracellular accumulation of triacylglycerols appears to be one cause of hepatocyte death in rats fed a CD diet [130], in which the life span of normal hepatocytes is reduced to a few weeks. Thus, the hepatocytes which do not accumulate fat will be refractory to cell death, and, in the same time, they will take advantage of the proliferative stimuli generated to compensate the extensive cell death occurring in the surrounding liver. This situation has close similarities with that observed in individuals with alcoholic and nonalcoholic steatohepatitis. Perhaps the most striking evidence of the "resistance" property of initiated cells stems from the studies of Farber's group [131]. In the R-H model, nodules develop rapidly within few days after beginning their selection, consisting in a proliferative stimulus provided by 2/3 PH in the presence of a cytostatic dose of 2-acetylaminofluorene (2-AAF). Under these conditions, only DENA-initiated cells are able to proliferate, while the normal hepatocytes are inhibited, due to the formation of adducts to DNA. Thus, it appears that the first relevant modification observed in initiated cells is their inability to activate the cytostatic metabolite that will bind to and inhibit DNA synthesis. Notably, initiated cells do not show, at this stage, any proliferative advantage over normal cells, as indicated by the lack of clonal expansion when subjected to proliferative stimuli (i.e., PH), but in the absence of a cytostatic milieu. (3) the lack of involvement of proto-oncogenes or tumor suppressor genes during the initial clonal expansion of mutated cells. It should be stressed that the resistance property of initiated cells as well as the apparent lack of alterations in proto-oncogenes and tumor suppressor genes, are often neglected in transgenic mouse models, where hepatocytes overexpress, or lack genes involved in cell cycle/cell death, and where little consideration is given to the relationship between mutated cells and the environment (surrounding liver). (4) during the carcinogenic process, a slow but continuous regression of most of the putative preneoplastic lesions occurs, characterized by a progressive loss of the less-differentiated phenotype and a return to a normal one (remodeling) [132]; this remodeling phenomenon is not unique to the R-H model, as it is also observed in the PB and CD models, upon withdrawal of PB or the CD diet [133, 134]; this suggests that the remodeling process is a genetically programmed phenomenon. However, the exact nature of this process remains elusive. Although some studies have suggested that apoptosis selectively occurring in preneoplastic hepatocytes increases during regression of the lesions [135, 136], more recent studies have shown that a rapid regression of preneoplastic lesions accompanied by a loss of several preneoplastic markers occurs in the absence of cell death, following short term treatment with mitogenic doses of T3 and the TRβ-agonist GC-1 [137, 138]. The finding that the inhibitory effect on the progression of liver lesions occurs despite the strong mitogenic activity of T3 and GC-1 suggests

that enhanced cell proliferation per se is not necessarily critical in the progression of carcinogen-altered cells to HCC, and that the nature of the proliferative stimuli is the important determinant in the process of HCC development. Notably, the inhibitory effect exerted by thyroid hormone receptor-ligands appears to be unique and not shared by PPARα ligands [139]. The latter results, together with previous findings [137] and those of others reporting that short-term treatment with KAT-681 (a liver selective thyromimetic) also inhibits the carcinogenic process in the liver [140] suggest that TRs may represent a meaningful target for liver cancer therapy. In this context it is important to note that somatic mutations in both TR-α and TR-β are detected in human HCC [141, 142]. The precise contributions of these mutant TRs to oncogenesis remain incompletely understood; however, their high frequency in these neoplasias (65% of HCC samples display TR-α1 mutations; 76% display TR-β1 mutations, with both loci mutated in some samples) is highly suggestive of their critical role. Understanding of the molecular events involved in re-differentiation of preneoplastic hepatocytes to a fully differentiated phenotype may represent an important clue for selectively working on carcinogen-altered cells. Notably, support to the differentiating effect of T3 comes from a recent report showing that T3 administration accelerates the differentiation of hepatic progenitor cells into hepatocytes in vivo [143].

Thus, we must realize that in spite of the several efforts, we still do not quite understand how exactly cell proliferation and the interaction between genetically damaged cells and the microenvironment play a role in HCC development. That cell proliferation is directly associated with liver tumor development is evident from both epidemiological and experimental evidences suggesting its involvement in the several steps of the carcinogenic process: initiation, promotion, and progression. However, while it is generally accepted that the mechanism by which cell proliferation plays a role in initiation is to allow fixation of a miscoding lesion in the newly made DNA, the exact role of cell proliferation during promotion is still unclear. Indeed, it is still an open question whether cell proliferation favors the generation of more aggressive cells simply by increasing the chances of mutational events by repeated rounds of DNA synthesis, or whether other events (generation of reactive oxygen species, inflammatory cytokines, or, more generally, the presence of an environment to which initiated cells, but not normal hepatocytes, can respond) are needed. In rodent liver, an increased incidence of preneoplastic lesions and tumors has been observed when carcinogen treatment was followed by compensatory regeneration induced by multiple treatment with necrogenic agents, such as carbon tetrachloride (CCl_4), an agent that induces fatty liver and severe necrosis [144]; on the other hand, hyperplasia caused by the mitogen LN (in the absence of tissue damage) did not exert any promoting effect on DENA-initiated cells, even when it was given once every 2 weeks to ensure a wave of hepatocyte proliferation following each injection, for up to 1 year [145]. Similarly, repeated treatment with T3 did not promote the growth of carcinogen-induced initiated cells to preneoplastic lesions [146]. These findings suggest that DNA synthesis per se is not sufficient to amplify the population of initiated cell; most probably, the lack of promoting effect is due to the inability of these mitogens to provide an appropriate environment (normal hepatocytes proliferate as efficiently as the genetically damaged cells). The same findings also support the notion that, whatever is the nature of the environment that favors the expansion of mutated cells, these cells do not seem to have any particular molecular alteration (proto-oncogenes and/or tumor suppressor genes) that enables them to grow more than normal cells following a proliferative stimulus. These results obviously differ from those obtained in transgenic mouse models such as the *c-Myc*-TGF-α model, where the earliest and primary event is the capacity of hepatocytes to continuously proliferate, giving rise to the emergence of preneoplastic lesions [147]. In addition, from the LN experiment it is also evident that, since apoptosis is responsible for the regression of hyperplastic liver observed following every dose of LN, the fact that no preneoplastic lesions are observed even after various cycles of treatment allows one to conclude that initiated cells do not possess alterations in genes involved in the regulation of apoptosis that could make them more resistant to cell death.

PPs are non-genotoxic rodent liver carcinogens and also powerful hepatomitogens [12]. For quite a while, it was suggested that the proliferative activity of PPs could explain their carcinogenic effect. However, this hypothesis has been ruled out by the finding that hepatocyte proliferation occurs only for a very short period of time (1–2 weeks) while tumors arise 16–20 months thereafter [148, 149]. Thus, paradoxically, the only conditions where primary mitogens do promote cancer development is when normal hepatocytes no longer respond to the mitogenic stimulus. Again, this supports the view that initiation with one of many different carcinogens is at no time followed by spontaneous or autonomous proliferation of any cells in the liver, and that initiated cells can proliferate and develop preneoplastic lesions and HCC because they have acquired some property that makes them resistant to the growth inhibitory mechanism operating in normal hepatocytes, inhibiting a further expansion of the liver mass. Indeed, if a differential environment is generated, primary mitogens do promote tumorigenesis. This is also the case of TCPOBOP. A single dose of this agent induces proliferation of 50–60% of the hepatocytes and doubling of the liver mass in 4–5 days. However, less than 2% of hepatocytes enter DNA synthesis after the second dose, and probably due to its exceptionally large size, the liver becomes refractory to a further increase. It is likely, that under these conditions

only DENA-initiated cells respond to the mitogenic stimulus exerted by TCPOBOP, and following repeated treatment will develop HCC.

Genetically Engineered Models

To improve the effectiveness of animals models as tools for identifying and characterizing critical modifications relevant to human disease, mouse models in which tumor suppressor genes were knocked out, or oncogenes were overexpressed have been developed at an astonishing rate [150]. Notably, most of the used mouse models utilized in cancer research over-express or lack genes which induce or inhibit cell proliferation. However, a critical limit of these models is that they underestimate the role played by the microenvironment surrounding the hepatocytes in the development of HCC. The relevance of tissue environment on HCC development stems from the long standing observation that chronic inflammation can result in cancer [151]. Data collected both from experimental systems and clinical observations have, indeed, strengthened the idea that the tumor microenvironment and neoplastic cells act in concert and contribute as a functional whole to growth and progression of the tumor mass. In particular, a key role has been assigned to inflammation as it is now widely accepted that chronic inflammation promotes cancer development. Chronic inflammation is generally associated with regenerative growth of the organ, and the liver is not an exception. Indeed, the principal causes of human liver cancer are infection with hepatitis B or C virus, chronic alcoholism, aflatoxin exposure, haemochromatosis, nonalcoholic steatohepatitis, or other circumstances that predispose to cirrhosis. These causes are believed to produce liver cancer by inducing repeated rounds of hepatocyte death and proliferation, creating a permissive environment in which genetic or epigenetic changes could occur that confer gain of function to protooncogenes or loss of function to tumor suppressor genes. Many such changes have been reported, but the combinations of these changes that operate to produce HCC remain largely uncharacterized. Thus, it is not surprising that many mouse models have investigated the relevance of genes involved in liver-cell proliferation in the genesis and development of HCC. A brief summary of experimental studies focused on the role of some oncogenes and tumor suppressor genes is reported below.

C-Met

Overexpression and amplification of the protooncogene *c-met,* which encodes the tyrosine kinase receptor for HGF frequently occurs in human HCC, and studies undertaken to elucidate the role of *c-met* in liver cancer development showed overexpression of *c-met* in a large percentage of human HCCs [152–155]. To learn more about this pathway, please see Chap. 20. Unfortunately, the correlation between its expression and clinical features are contradictory [154, 155]. Studies with transgenic mouse models have also reported quite contrasting results: Bishop's group reported that overexpression of wild-type *c-met,* can initiate HCC genesis in mice [156]. In a subsequent study [157], the same group initiated tumorigenesis by transgenically expressing the protooncogene *c-met* or by hydrodynamically transfecting *c-met al*one or in combination with other genes into the livers of adult animals. In both instances HCC arose from cooperation between *c-met* and constitutively active versions of β-catenin. In contrast, adenomas were produced by cooperation between *c-met* and defective signaling through the transcription factor HNF1α. Notably, a coincidence between activation of the protein tyrosine kinase encoded by *c-met* and activating mutations of β-catenin was observed in a subset of human HCC, suggesting that approximately 20% of human HCC may arise through the cooperation of *c-met* and β-catenin; these tumors that may correspond to the subset of HCC have been recently described as those expressing a *c-met* signature [158]. From the same study, it was also apparent that inactivation of *c-met* transgenes led to regression of HCCs, despite the persistence of activated β-catenin; interestingly, the tumors eventually recurred in the absence of *c-met* expression, presumably after the occurrence of one or more events that cooperated with activated β-catenin in replacing *c-met.* From these results it appears that *c-met* plays a very important role in HCC and that its cooperation with β-catenin is essential to select initiated cells to malignancy. However, this conclusion is hampered by the finding that, when mice were subjected to hydrodynamic transfection of *c-met,* over the course of 1 year, none of them showed any evidence of liver tumors. Given that a *c-met* transgene can initiate tumorigenesis, it is difficult to explain the absence of tumors in mice transfected with *c-met.* A possible explanation was that the discrepancy may be due either to differences in the levels of expression of *c-met* or in the relative number of cells expressing the gene (which is much greater in the transgenic animals). Interpretation of the role of *c-met* in hepatocarcinogenesis is further complicated by other findings on HGF or *c-met* conditional KO mice. Indeed, it was shown that the loss of *c-met* signaling in hepatocytes enhanced rather than suppressing the early stages of chemical hepatocarcinogenesis. *c-met* conditional knockout mice (metfl/fl, A AlbCre$^{+/-}$ C-metLivKO) treated with DENA developed significantly more and bigger tumors and with a shorter latency compared with control mice [159]. Accelerated tumor development was associated with increased rate of cell proliferation and prolonged activation of epidermal growth factor receptor

(EGFR) signaling. C-metLivKO livers treated with DENA also displayed increased oxidative stress, and the negative effects of *c-met* deficiency were reversed by administration of antioxidant N–acetyl-cysteine, which blocked EGFR activation and reduced the DENA-initiated hepatocarcinogenesis. These results argue that intact HGF/*c-met* signaling is essential for maintaining normal redox homeostasis in the liver and has tumor suppressor effect(s) during the early stages of hepatocarcinogenesis. Essentially similar results were obtained by other investigators who made use of *c-met* conditional knockout model, in which the floxed exon 15 of the c-met gene, which codes for the ATP binding site, was deleted by activating the Mx-promoter controlled Cre recombinase. In these mice, no autophosphorylation and therefore no activation of the *c-met* receptor occurs. In these animals, *c-met* defect in hepatocytes significantly enhances DENA dependent tumor initiation in liver as shown by a significant increase in the number of GS-positive lesions in liver of c-met knockout mice [160].To further complicate the picture it should be mentioned that (1) while a study showed that HGF overexpression delays HCC development in double transgenic *c-myc*-HGF mice [161], other studies demonstrated that HGF-overespression led to hepatomegaly and a more rapid HCC development [162], and that, (2) the content of HGF and the expression of HGF mRNA were significantly decreased in the rat liver tumors induced by PPs [163].

WNT-β/Catenin

Please see Chap. 20 for a detailed account on Wnt signaling cascade. Activation of the Wnt signaling pathway is one of the important aberrant pathways identified in HCC, both in animals and in patients [164, 165]. Approximately 30% of human HCC have activating mutations in β-catenin [166], which prevent the phosphorylation of β-catenin by the Axin/APC/CK1/GSK3 complex, and thereby the subsequent degradation of β-catenin by the proteosome [167]. As a consequence of such mutations, the increase of de-phospho β-catenin and its enhanced translocation to the nucleus result in the aberrant activation of LEF/TCF target genes. Moreover, in HCC, loss of function mutations have been observed in the degradation complex of the Wnt pathway components, such as Axin2 and APC [168, 169]. Anomalous β-catenin expression as well as mutations in the *CTNNB1* are observed in around 25% of all HCC cases and up to 50% of all hepatic tumors in transgenic lines such as c-myc or H-ras [170]. However, what is the overall impact of aberrant β-catenin activation on the biology of HCC still remains elusive. There is a lack of consensus as to whether β-catenin activation imparts a poor or better tumor behavior; indeed, β-catenin activation has been associated with poorer prognosis [171, 172], but also with non-invasive forms of tumor and better prognosis [173, 174]. Additional reports have correlated overexpression of non-nuclear type β-catenin with poor cell differentiation, larger tumor size and significantly shorter disease-free survival lengths [175], while another study found a significant relationship between nuclear β-catenin, low Ki-67 positivity and favorable prognosis and 2-year survival [176]. Wnt/ β-catenin pathway in HCC in animal models is also unsettling. The studies involving β-catenin transgenic mice that overexpress truncated β-catenin in liver do not show any evidence of spontaneous carcinogenesis [177]. Another study overexpressing nontruncated human β-catenin gene under transcriptional control of albumin promoter/enhancer also yielded no tumors in the liver [178]. Similar lack of tumorigenesis was also observed following adenoviral-mediated overexpression of a dominant stable β-catenin mutant in liver [179]. However, interestingly, APC-null mice showed increased HCC development associated with activation of β-catenin [180]. In a classical initiation-promotion protocol, β-catenin mutation seems to confer a selective advantage to "initiated" hepatocytes under PB promotion. Indeed, the vast majority of mouse liver tumors arising from treatment with DENA alone shows H-Ras mutation but no β-catenin mutation; on the other hand, only β-catenin mutation was found in tumors treated with the same dose of DENA, but then exposed to PB promotion [181]. Overall, it might be that β-catenin mutations might be insufficient on their own and require cooperation with other pathways for hepatocarcinogenesis.

C-Myc

The *c-myc* oncogene encodes a transcription factor with multiple effects on diverse cellular activities [182, 183]. A central paradox of *c-myc* is that it acts as a dual-function protein, capable of inducing both cell proliferation and apoptosis, processes that have opposite biological outcomes [184, 185]. Given the known relationships between apoptosis and tumor development, it has been proposed that the apoptotic activity of *c-myc* eliminates cells that over-express the gene, thereby reducing its tumorigenic activity [186]. In human HCCs, there is a significant positive correlation between *c-myc* expression and the degree of apoptosis [187]. Animal models have confirmed that overexpression of *c-myc* can induce HCC [188–190], whereas inhibition of *c-myc* expression results in a loss of the carcinoma's neoplastic properties [191]. The acceleration of tumorigenesis by *c-myc* activation is also illustrated by studies of transgenic mice that express both *c-myc* and TGF-α in hepatocytes. These mice develop HCCs more rapidly and at a higher frequency than mice that express either of these transgenes individually [189]. Tumors

from TGF-α/TGF-α mice show high proliferation and reduced apoptosis, as compared with surrounding tissues and tumors produced in *c-myc* single transgenic animals [192]. These observations suggest that TGF-α may have an anti-apoptotic function in *c-myc*/TGF-α double transgenic mice [193–195]. An elegant study addressed to explore the possibility that *c-myc* inactivation may be effective in treating liver cancer, was performed by developing a conditional transgenic model whereby it was possible to regulate the expression of human *c-myc* in murine liver cells. [196]. In this study, inactivation of the *c-myc* oncogene is sufficient to induce sustained regression of invasive liver cancers. *c-myc* inactivation resulted in differentiation of tumor cells into hepatocytes and biliary cells forming bile duct structures, and this was associated with rapid loss of expression of the tumor marker α-fetoprotein, and with an increase in expression of liver cell markers. In vivo bioluminescence imaging revealed that many of the tumor cells remained dormant as long as *c-myc* remain inactivated; however, *c-myc* reactivation immediately restored their neoplastic features, suggesting that oncogene inactivation may reverse tumorigenesis in the most clinically difficult cancers. From most of the studies performed on *c-myc,* it seems evident that this oncogene plays an important role in HCC development. However, it is still unclear whether *c-myc* is important for sustaining proliferation of tumor cells, preventing their apoptosis, or inhibiting their differentiation to a normal phenotype.

C-Jun

The transcription factor *c-Jun* has been implicated in several cellular processes including proliferation, survival, and cell transformation [197, 198]. *c-Jun* is activated in chemically induced murine liver cancer and in human HCCs [199], suggesting an important oncogenic function for this gene. The oncogenicity of *c-Jun* is probably due to several mechanisms: *c-Jun* cooperates with Ras in promoting tumor cell proliferation [200] and it can affect the anti-proliferative activity of p53 by direct repression of p53 transcription [201]. In addition, *c-Jun* modulates TNF-α and TGF-β signaling pathways, both of which are implicated in tumor development [202, 203].

Besides several reports showing increased *c-Jun* expression in experimentally induced or human HCC, one of the few studies performed to investigate the role of liver-specific inactivation of c-Jun at different stages of tumor development in chemically induced HCCs in mice showed that the requirement for *c-Jun* was restricted to early stages of tumor development [204]. The number and size of hepatic tumors was dramatically reduced when *c-Jun* was inactivated shortly after initiation. The impaired tumor development correlated with increased levels of p53 and its target gene *noxa,* resulting in the induction of apoptosis, without affecting cell proliferation. Primary hepatocytes lacking *c-Jun* showed increased sensitivity to TNF-α-induced apoptosis, which was abrogated in the absence of p53. These data were interpreted to suggest that *c-Jun* prevents apoptosis of preneoplastic hepatocytes initiated by DENA, by antagonizing p53 activity; this mechanism might contribute to the early stages of human HCC development. However, considering that we still lack the evidence that p53 in mouse liver plays a role similar to that of human p53, the interpretation of these results needs a further analysis.

P53

The transcription factor p53 is a well-known tumor suppressor protein that induces cell-growth arrest, apoptosis, and senescence in response to various types of stress, and it is probably the most studied gene in oncology [205]. Although its role in carcinogenesis is unquestionable and more than 30 studies have shown p53 mutation, a remarkable differential mutation rate according to the geographic area has been found (from 0% in Spain to 67% in Senegal) [206], suggesting that mutation of p53 is directly correlated with the level of Aflatoxin exposure; its involvement in HCCs caused by other etiological agents is currently unclear. Moreover, although almost all data suggest that in western countries loss of p53 is a late event in hepatocarcinogenesis, whether p53 mutation may also contribute to cancer initiation is unknown and remains an area of active investigation. Unfortunately, convincing experimental models that could elucidate the role of p53 and the exact stage of the process at which this oncosuppressor acts, are lacking. Heterozygous p53 null mice show an overall increase in tumor burden when compared with wild type mice [207]; however, hepatocarcinogenesis is not enhanced on treatment of heterozygous p53 null mice with a chemical carcinogen [208–212]. This finding suggests that, quite in contrast to human liver where the frequent inactivation of p53 in HCCs points toward an important control function of the suppressor protein during malignant transformation, p53 does not play a major role in mouse liver carcinogenesis. Notably, human p53 knock-in mice do not differ in liver tumor response from their counterparts with murine p53 [213], ruling out the hypothesis that p53 harboring the human polyproline and DNA binding domains has a growth control function that is lacking in normal murine p53. Since mutations of p53 are extremely rare also in rat liver (with the exception of Aflatoxin-induced tumors), better experimental models for the study of the role of p53 in the several steps of the hepatocarcinogenic process are needed. One of the few studies on mouse models suggesting

a role of p53 in liver tumor development [214] showed that reactivation of endogenous p53 in p53-deficient Ras-overexpressing tumors can produce complete tumor regressions. However, the primary response to p53 was not apoptosis, but instead involved the induction of a cellular senescence program that was associated with differentiation and upregulation of inflammatory cytokines. This report highlights the critical role of wt p53 in regulating cellular senescence during tumor regression, suggesting p53 restoration in tumor cells as a potential therapeutic target. However, although interesting, this study relies on a quite artificial model and future studies are essential to establish if wt p53 expression would really lead to senescence of tumor cells in mouse HCC, and if the same conclusions can be applied to human HCC.

Why Experimental Carcinogenesis is Still Important

The finding that several mutations identified in human HCC do not correspond with those in rodents has generated the idea that experimental carcinogenesis is not helpful in the understanding of the molecular events underlying HCC development. However, it should be kept in mind that the molecular alterations observed in human HCC do not provide great therapeutic opportunities since it is impossible to identify which lesions are really involved in the genesis of the process and which are only a consequence of the completely deranged genome typical of tumors. It should also be stressed that the inability to observe in rodent HCCs the same mutations seen in human HCCs does not exclude the possibility that the same gene is activated/inactivated as a consequence of epigenetic changes (hypo or hypermethylation), or more simply that the function of a given gene in humans may be taken over by another gene in rodents. In any case, as only animal models provide the opportunity to dissect the carcinogenic process and to analyze in a step by step fashion the molecular alterations taking place in the process, we are compelled by the necessity to develop better animal models in the hope of recapitulating the human situation. In particular, the scientific community still awaits the results of critical control experiments that should aid in the decision as to whether these mutations are passive accompaniments of exposures to carcinogens, or whether they become, on activation (at a still unknown stage), involved mechanistically in the development of cancer. Advances in genetic engineering have lead to the development of many mouse models of cancer that simulate human HCC. Most of these models explore the interaction of different oncogenes and tumor suppressor genes in HCC development. The genetic manipulation of transgenic mice permits the design of mouse models to examine the role of multiple genetic and environmental factors in cancer. Unfortunately, these mouse models show severe limitation: (a) if we assume that in non-hereditary tumors, mutations are rare events, it is not conceivable that we can understand how a few cells clonally expand if we are introducing a mutation in all the cells of a given organ; (b) as already discussed, with the exception of hereditary tumors, there is no evidence that alterations in cell-cycle genes are the basis for the growth of initiated cells to focal lesions; nevertheless, most, if not all the genes overexpressed or deleted in mouse models, are genes related to proliferation and/or apoptosis; (c) the knockout models do not take in the right account the role of the environment of the organ, that is the complex of causes that can favor the growth of initiated cells. Thus also transgenic mouse models must be pursued and improved, and, at present, they can only complement but cannot substitute for the classical initiation-promotion models.

Conclusions and Perspectives

Even if many studies have been performed to understand the mechanisms controlling hepatocyte proliferation, many questions remain unanswered. This is mainly due to the fact that each of the experimental systems available has intrinsic limits, as none of them purely involves cell proliferation, but also adaptive and metabolic changes that render the analysis less clear and conclusive. Moreover, the data collected with the different models suggest the possible existence of several pathways leading to hepatocyte DNA synthesis, depending on the nature of the mitogenic stimulus. It is likely that the use of microarray technology will help to identify the genes critically involved in hepatocyte proliferation, by comparing genes that are up or downregulated in different experimental systems, such as liver regeneration and mitogen-induced hyperplasia.

Liver hyperplasia may also represent a promising tool in clinical conditions characterized by reduced regenerative capacity – such as in the elderly – or when a rapid increase of liver mass is required, as in living-related transplantation. Notably, at least three advantages can be envisaged from the use of primary mitogens (a) no surgical manipulation is required; (b) proliferative bursts of hepatocytes can be induced as many consecutive times as required; and, (c) cell proliferation occurs in the absence of any significant liver (or other organ) damage. Moreover, in view of the widely accepted notion that enhanced cell proliferation plays a major role in the genesis of cancer, the strong proliferative capacity coupled with the lack of toxicity of primary mitogens, may represent a unique model to discriminate between the

promoting effect possibly due to the increased mutational events associated with DNA replication, from that generated by the micro-environment surrounding initiated cells.

Finally, another important area to be further explored is the relationship between the inhibitory effect on hepatocarcinogenesis of some mitogens (i.e., T3), in spite of their mitogenic activity on both normal and preneoplastic hepatocyte proliferation. The finding that T3 transiently accelerates the proliferation of the oval cells, which is followed by rapid differentiation into small hepatocytes, and that preneoplastic hepatocytes also shift to a fully differentiated phenotype following T3-induced proliferation may help to identify the genes involved in this process and, consequently, to develop new targeted therapies to induce re-differentiation of preneoplastic hepatocytes. Moreover, to understand why only a minority of preneoplastic lesions do not regress after T3 treatment and give raise to HCC is critical to identify a subset of altered cells committed to cancer progression. Finally, because T3-based therapies result in undesired side effects, particularly cardiac dysfunction, i.e. tachycardia, arrhythmias, and precipitation of ischemic episodes or heart failure [215], the availability of new thyroid hormone analogs devoid of the cardiac effects of thyroid hormones, such as GC-1, will hopefully make possible to design new therapies against liver cancer. Targeting TR may reveal a promising area, especially in view of the recent case-control study showing the association between hypothyroidism and human HCC [216].

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Chapter 55
Stem Cells and Liver Cancer

Stewart Sell

Liver Cancer Stem Cells

The properties of the cells that make up a cancer are shown in Fig. 55.1. Three widely accepted functional properties of cancer stem cells are: (1) immortality, demonstrated experimentally by the ability to culture the tumor in vitro continuously without senescence; (2) transplantability; and (3) resistance to radiation and chemotherapy.

Immortality

Growth of cancer cells in vitro was first described for HeLa cells in 1955 [1] and later for cells from primary human tumors [2]. Salmon [3] found that 1 in 1,000 to 1 in 100,000 cells from a primary human adenocarcinoma would form colonies in soft agar (called "tumor colony forming units" or CFU). He proposed to use these cultured cells to study the effects of various drug treatments. This number of CFUs was similar to those found at that time for the number of cells in a solid tumor that were able to transplant the tumor, about 1 in 1,000 cells [4, 5]. The cells that formed CFU were referred to as clonogenic and not called tumor stem cells at the time. Thus, tumors contained three functional populations of cells: (1) cells that could be cultured in vitro without senescence; (2) cells that could be cultured for a limited number of passages; and (3) cells that could not be cultured. The populations correspond to stem cells, transit-amplifying cells, and terminally differentiated cells in normal tissues.

S. Sell (✉)
Department of Molecular Medicine, Wadsworth Center, Ordway Research Institute and University at Albany, Albany, NY, USA
e-mail: ssell@wadsworth.org

Rat Hepatomas

In vitro culture of chemically induced rat hepatocellular carcinoma (HCC) was documented over 60 years ago. Cell lines of rat HCC were established by Morris and Wagner [6], Novikoff [7], Yoskida [8], Zajdela [9], Ruber [10], and others. There was marked variation in the growth kinetics of these cell lines [11–13]; some were very slow growing lines that maintained metabolic properties of normal hepatocytes (minimal deviation hepatomas), while some grew rapidly and behaved like poorly differentiated HCC [6]. Although not so interpreted at the time, the results of the in vitro culture of hepatomas clearly indicate not only heterogeneity of cells within a primary tumor, but also a mixed population of cells within culture lines [14–19]. This is consistent with the existence of a lineage of cells including immature "transformed" cells that are immortal in vitro (stem cells), more differentiated cells that grow for limited times and then become senescent (transit-amplifying cells), and mature, post-mitotic cells that do not grow (terminally differentiated cells), both in the original tumor and in the derived cell lines. In any case, those studies documented the first defining characteristic of liver cancer stem cells (LCSC), namely the ability to grow continuously in vitro and produce differentiated progeny.

Cell lines derived from primary cell cultures of normal liver cells can also give rise to cells that produce cancers. Epithelial cell lines from the normal liver of 10-day-old rat were first established by subculture of cells under non-selective conditions from mass cell cultures [20]. These lines were maintained for up to 9 months of continuous culture, and were found to express enzymes of mature hepatocytes when the cells in culture became confluent [21]. Using a similar approach, Grisham et al. [22] derived a long-term cell line from a normal Fischer-344 rat that was made up of cells that resembled those in the terminal biliary ducts [23]. This cell line, known as WB-344, has been subcloned and maintained for over 30 years [24]. It has been extensively studied and appears to have properties most consistent with those of a liver stem cell [24]. Other investigators have obtained similar lines of cells that produce tumors upon injection into nude

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_55, © Springer Science+Business Media, LLC 2011

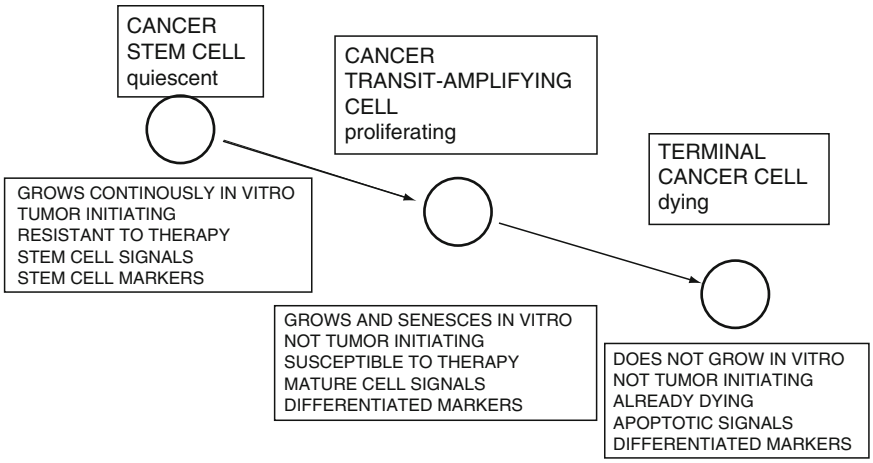

Fig. 55.1 Properties of the cells in a cancer. Cancers are made up of the same functional cells as normal tissues: stem cells, transit-amplifying cells, and terminally differentiated or dying cells. Cancer stem cells proliferate rarely, grow continuously in vitro (are immortal), are able to grow after transplantation into syngeneic or immune-suppressed recipients, are resistant to therapy, express stem cell signaling molecules, and exhibit stem-cell surface makers. Cancer transit-amplifying cells are actively proliferating, may grow in vitro but then die out, do not grow after transplantation, do express signaling molecules of mature cells, and do exhibit differentiated cell markers. Terminal cancer cells are dying. They cannot grow in vitro, cannot be used to transplant the tumor, do express apoptotic signals, and do exhibit differentiated cell markers

mice [25, 26]. A subclone of WB-344, designated WB-5–11, was mutagenized by exposure in vitro to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and 18 clonal subpopulations of WB-5–11 were derived. These transformed clones give rise to a variety of tumors with differing histologies when injected dorsally into irradiated 1-day-old isogeneic Fischer-344 rats [27]. Diploid non-transformed WB-F344 cells integrate into hepatic plates and acquire the phenotype of hepatocytes, after transplantation into the liver of the normal rat [28, 29]. In fact, the normal liver microenvironment can modify the potential of the transformed cell lines; some lines, which form tumors when injected into the back of newborn mice, will instead give rise to normal hepatocytes, when injected into the liver of normal adult rats [30]. The livers of young rats are less capable of directing such normal integration of the transformed cells than do the livers of older rats [31]. The parent cell line WB-F344 will undergo spontaneous transformation in vitro when maintained in a confluent state, producing multiple independent stable lineages of transformed cells [32]. Thus, it appears that there are stem cells in normal liver that can be converted to cancers when cultured in vitro and exposed to carcinogens, but that these cells are very rare.

Human HCC

Cultures derived from human HCC reveal stem-cell properties similar to those of the rat, but they have mainly been used to demonstrate the production and secretion of hepatitis B surface antigen (HBsAg). The first extensively studied human cell line (PLC/PRF/5) was derived from tumor that was cultured after transplantation and growth in an athymic (*nu/nu*) mouse [33]. This human HCC cell line was subsequently shown to produce high levels of HBsAg [34]. It also grows more rapidly than other lines and expresses higher levels of epidermal growth factor receptor (EGFR) than do other human HCC cell lines, considered possibly a manifestation of tumor progression [35]. The widely used human cell lines, HepG2 and Hep3B, were cultured from liver biopsies of two children with primary hepatoblastoma and HCC [36]. Hep3B secretes HBsAg, but HepG2 does not [37]. A number of other human cell lines have been reported. Some of these produce HBsAg [36–42], and some do not [37, 43, 44]. Difficulties in obtaining human HCC lines have been reduced with publication of a reproducible method for culturing human HCC [45]. A cell line (KYN-1) from a human HCC transformed to an adenocarcinoma with passage in vitro, but retained the proteome phenotype of liver cells (expression of AFP, CEA, ferritin, etc.) [43]. Such studies also show tumor heterogeneity similar to that seen in studies on rat HCC. For example, two human HCC cell lines derived from a single tumor nodule have different properties [44]. These lines produced cultures with different morphology (one less differentiated than the other) and different doubling times. A cell line with characteristics consistent with those of transit-amplifying cells (HACL-1) was derived from a human HCC [46]. This line had a finite life span, proliferating for 2 months, and then showing contact inhibition and inability to grow in soft agar (senescence). Although not as in-depth as studies on rat liver cancer cultures, the studies on human HCC cell lines gave results consistent

with the existence of a stem cell/transit amplifying cell/differentiated cell hierarchical lineage in human HCC cell lines.

Tumor Transplantation

The second property of a cancer stem cell is that it can initiate tumor growth when transplanted to syngeneic or immune-compromised recipients ([4, 5, 47–53], also recent review [54]). The earliest transplantation study found by the author was of an experimental mouse leukemia transplanted from an adult leukemic mouse to a young mouse of the same family, in 1932 [47]. A subsequent study in 1937 showed that transplantation of mouse leukemia could be accomplished with a single cell [48], presumably the leukemic stem cell. In more recent studies of transplantation of human leukemias to immune-compromised mice [51, 52], only one cell in 250,000 could transfer the leukemia [52]. Subsequently, the frequency of tumor-initiating cells for solid tumors was in general found to be of the same order of magnitude as the frequency of cells that survive after chemo- or radio-therapy and growth in soft agar [4, 5, 54]. Tumor cells that grow after transplantation are called tumor-initiating cells [53]. However, the frequency of tumor-initiating cells is dependent on how they are detected. For example, serious questions have been raised regarding the nature of the recipient in determining the ability of cancer stem cells to grow [55, 56]. For human cancer cells, the recipient is an immune-deficient mouse (xenogeneic transplantation). Transplantation studies using mouse tumors and syngeneic mice as recipients are more likely to show tumor growth than are studies using xenogeneic transplantation of human cancers to immune-deficient mice [55]. For example, the frequency of tumor initiating cells for a human melanoma depends on the nature of the immune-deficient mouse: fewer than 1 in 1,000 cells are able to transfer a tumor to NOD/SCID mice, but as few as one in two can transfer the tumor to NOS/SCID/Il2rg−/− mice [57].

Some insight into the presence of tumor initiating hepatoma cells is provided by studies on transplantation of Morris hepatomas using histocompatible rats as recipients [6, 58]. Transplantation of the Morris hepatomas reveals marked differences among the many tumors examined. Some can be transplanted by means of just a few cells and grow very quickly, whereas others can only be transplanted by means of large numbers of cells and grow very slowly. While these observations again suggest cellular heterogeneity in hepatomas neither systematic dilution nor fluctuation analysis experiments have yet been performed. Consequently, clear identification of the percentage of cells with tumor-initiating properties, tumor stem cells, in this model system is lacking.

The library of Morris hepatomas [6] provided a pre-clinical model to determine the relationship of the serum concentrations of alpha-fetoprotein (AFP) to growth rate, histologic grade, and chromosome composition of transplantable hepatomas in tumor-bearing rats [59]. Higher serum AFP levels were found in rats bearing faster growing tumors, tumors with poor differentiation, and tumors with aneuploidy as compared to the AFP levels in the serum of rats with slower growing, well-differentiated diploid tumors. The serum levels of AFP in recipient rats correlate closely with the growth rate (percentage of dividing cells) and size of the tumor. Later, clinical studies revealed similar correlations in human patients with HCC. Serum AFP levels could be used to follow the growth of HCC and response to therapy. For a preclinical model, we used a fast-growing transplantable rat HCC that produced high levels of AFP (Morris hepatoma 7777). Following transplantation of hepatoma 7777 cells into the thigh of histocompatible Buffalo rats, the serum concentration of AFP rose exponentially as the tumors enlarged, until about day 40, when the tumor size required that the animals be euthanized [60]. At this time the serum concentration ranged between 7,000 and 10,000 μg/mL. Thus, the kinetics of the elevation of serum AFP paralleled the growth of the tumor. When the transplanted tumors were removed surgically at 17 days, the serum AFP levels fell with a half-life of about 24 h. However, rapid elevations occurred with growth of metastatic lesions, mainly in the lung. Irradiation of the lung at the time of amputation of the tumor in the leg resulted in prevention of metastasis in a significant number of recipient rats [61]. Thus, this preclinical model proved to be a good one for determining how to use AFP in the diagnosis and clinical management of patients with AFP-producing tumors.

Resistance to Therapy

Normal quiescent cells are resistant to therapeutic measures that kill proliferating cells. Stem cells in normal tissues, as well as in cancer, are normally not dividing; i.e., in a quiescent G_0-state. Thus, cancer stem cells also exhibit predominant quiescence (i.e., only rare cell division), such that they are infrequently transiting the cell cycle during the course of administration of chemo- or radio-therapy. Therefore, even if all of the proliferating transit-amplifying tumor cells are killed therapeutically, cancerous tissues re-grow from therapy-resistant cancer stem cells [54, 62]. In support of this conclusion, highly tumorigenic subpopulations of cancer initiating stem cells derived from human glioblastomas have been found to display resistance to radiation because of protection against DNA damage [63]. Studies in the 1950s on radiation therapy showed that long-term regrowth of a tumor

after treatment is related to the histologic appearance of the tumor [64] and depends on the rate at which radio-resistant clonogenic cells are added to the tumor [65, 66]. In the recent cancer stem cell literature, the term cancer stem cell has been used to mean what clonogenic meant in the older literature. Studies on the clonogenic cells in experimental tumors show that if the clonogenic population is not eradicated, such tumors will regrow [67–69].

Radiation Therapy of Morris Hepatomas

William Looney and his collaborators extensively studied cell-proliferation kinetics and the response of transplantable Morris hepatomas to radio- and chemo-therapy [11, 70–73]. They showed that after high-dose radiation or chemotherapy [70], the transplantable Morris hepatoma were able to re-grow, indicating the presence of a therapy-resistant clonogenic cancer cell (stem cell) that is not killed by the treatment. They proposed that the term "radio-resistant" actually means that tumors regrow from the clonogenic population that is not proliferating at the time of treatment. The data clearly support the concept that tumors are made up of non-proliferating stem cells (resistant to therapy), proliferating transit-amplifying cells (susceptible to therapy), and cells that are dying. After treatment with effective doses of either 5-FU or radiation, there is an accelerated rate of proliferation of the tumor cells 12 days after treatment with a two to threefold increase in the number of cells in S phase of the cell cycle [72]. This may be explained by activation of cancer stem cells that were not proliferating at the time the treatment was given. The treatments most likely kill off the proliferating transit amplifying cells, and loss of these cells stimulates proliferation of the cancer stem cells. Looney proposes that by careful timing of the second dose of therapy to match the time of this accelerated proliferation, it may be possible to catch the now-proliferating cancer stem cells and effect complete destruction of the tumor [73]. This is, in fact, what we think happens with repeated doses of therapy for leukemias [74].

Nature of Therapy-Resistant Cells

Do the transplantable tumor cells that are resistant to therapy differ from other cells in the tumor (cancer stem cells as distinct from the cancer transit-amplifying cells), or are the transplantable cells merely quiescent or senescent cells that respond poorly to therapy, and survive the challenges of transplantability? This is not a newly recognized conundrum. For example, in 1999 Trott [75] and Danenkamp [76] provided opposing views. Trott suggested that 0.1–100% of all cells from transplantable mouse tumors meet the criteria of a tumor stem cell; i.e., "re-growth of the tumor preceded by clonal expansion from a single cell with unlimited proliferative potential." He concluded that tumors contain the same populations of cells as do normal tissues, namely, stem cells, transit-amplifying cells, and dying cells [75]. On the other hand, Denekamp [76], considering the same evidence, interpreted it to mean that therapy-resistant cancer cells are the least differentiated cells of a cancer cell population, functionally and kinetically different from the remaining mass of tumor cells. Thus, resistance to therapy could be due to the presence of cancer stem cells, to cellular heterogeneity of the tumors, or stochastically to the presence of non-dividing tumor cells at the time when therapy is administered.

Markers of LCSC

The problem of identifying LCSC rests with the ability to isolate such cells from liver cancers and to then characterize them as the stem cells that give rise to liver cancers after transplantation [77, 78]. However, authentic LCSC have not yet been isolated. In fact, there is no compelling reason to believe that true LCSC actually exists, except that it is the most plausible explanation for the properties of transformation, transplantability, and resistance to therapy. The genomics and signaling pathways in HCC have been adumbrated [79]. Not surprisingly, most of the HCC-associated gene products found were non-specific, i.e., they were proteins expressed in both normal and abnormal proliferating hepatocytes. Thus it is difficult to identify gene products specifically associated with HCC let alone with putative LCSC.

Stem Cell Transcription Factors

One way to identify putative stem cells is to determine whether the cells in question express the same levels of transcription factors as known stem cells. c-Myc, along with the three other transcription factors Oct3/4, Sox2, and Klf4, are able to reprogram adult skin fibroblasts into cells that behave like pluripotent human embryonic stem (ES) cells [80]. Although there is no evidence linking Oct3/4 and Sox2 with LCSC, these factors are known to augment stem cell function and to suppress differentiation in mouse and human ES cells. Signaling factors involved in stem cell functioning (such as c-kit [81]), in embryogenesis (such as Bmi1, Wnt, and β-catenin [82–85]), and in membrane transport (such as ABCG2 [78]), as well as cell surface molecules (such as CD133 that is used to isolate oval and other stem cells [86, 87]) have been implicated in oval cell expression and HCC formation in experimental and human tissue samples. Gene expression profiling of HCCs reveals a hierarchy of expression

similar to hepatoblasts (class A), immature hepatocytes (class B), and mature hepatocytes (class C) that correlates with the range of clinical outcome [88]. This finding is consistent with the concept of stem-like cells in liver cancer. It is likely that LCSC gene expression of transcription factors will share pathways with normal liver stem cell expression; the challenge is to find expression patterns specific to LCSC and direct therapy specifically to them [89].

Cell Surface Markers

A major conceptual jump in the cancer stem cell field was the conclusion that cancer stem cells responsible for tumor initiation can be identified by cell surface markers [53, 54]. In application of the surface-marker approach to HCC stem cells, several cell surface makers, specifically CD 133 [90, 91], CD90 [92], and epithelial cell adhesion molecule or EpCAM [93], have been reported to identify LCSC. Expression of CD133 and CD90 is associated with fetal liver-cell marker expression, tumor initiation, growth in vitro, and chemo-resistance [90–92], properties attributed to cancer stem cells. Unfortunately, as discussed above under transplantation, there is only a weak correlation between cancer cell marker expression and the ability of the cancer cells to initiate tumor growth. Further study is warranted, to establish whether LCSC can be identified by such markers.

Side Population Cells

Side populations (SP) in liver cancer cell lines are isolated by flow cytometry on the basis of their ability to exclude Hoechst 33342 dye, and are considered to have the property of resistance to chemotoxic drugs [94]; a property SP cells share with cancer stem cells. SP cells have adenosine triphosphate binding cassette (ABC) membrane transporters that export intracellular Hoechst 33342, whereas non-SP cells do not. SP cells from HCC [94], glioma, and breast cancer [95] initiate tumor growth after transplantation with as few as 100 cells, whereas the same or greater numbers of non-SP populations are unable to produce any tumors [95]. Thus, it was concluded that the cancer stem cells are contained within the SP.

Hoechst 33342 is cytotoxic; consequently, SP cells are protected by their membrane transport properties, whereas unprotected non-SP cells suffer toxicity and are unable to grow [55]. Thus, the differing tumor-initiation abilities of SP and non-SP cells are more likely due to the resistance of the SP cells to Hoechst 33342 toxicity, than due to intrinsic stem cell properties of the SP cells. This interpretation is consistent with observations that in the absence of exposure to Hoechst 33342, essentially all glioma C6 cells are transplantable [96]. Thus, identification of cancer stem cells by a criterion such as Hoechst 33342 exclusion does not correlate with the functional identification of a cancer stem cell, as determined by the ability to transfer the tumor. On the other hand, ABCG2 cell surface expression may have relevance, as a marker for liver cancer cells that have the property of resistance to chemotherapy [97].

Summary

Analysis of the cell populations in HCC indicates that these cancers contain the same cell populations as do normal tissues: stem cells, transit-amplifying cells, and terminally differentiated or dying cells [98]. LCSC have the properties of immortality (grow continuously in vitro), transplantability (tumor initiating capability), and ability to resist therapy. Isolation of tumor cells on the basis of expression of cell surface markers results in enrichment for LCSC, but a definitive marker for LCSC has not yet been identified. It is important that we learn more about the properties of LCSC so that we may direct therapy against these cells [89, 99, 100]

The Stem Cell Origin of Liver Cancer

From the end of the nineteenth century until the early 1980s, cancers were believed to arise exclusively through de-differentiation of mature cells [98–102], i.e., mature cells reacquired properties of less differentiated cells and continued to grow like fetal tissue. During that period, one of the observations that was used to support the de-differentiation theory of cancer was the appearance of altered foci of hepatocytes and nodules in the liver; this appearance preceded the development of liver cancer in rats and mice after exposure to chemical hepatocarcinogens. These foci and nodules were interpreted to represent sequential cellular alterations in mature hepatocytes that would eventually become malignant, in other words, de-differentiation. However, revision of the de-differentiation concept was stimulated by a series of detailed studies, which included morphologic and physiologic analyses of the production of AFP during experimental chemical hepatocarcinogenesis, and the expression of AFP in the eventual tumors, but not in premalignant foci and nodules [103–105].

Experimental Chemical Hepatocarcinogenesis

The cellular response of the liver preceding development of HCC induced by exposure to chemical hepatocarcinogens has been subject to different interpretations with respect to

the roles of various cell types in the histogenesis of liver cancer (for an extensive review with multiple references see [106]). Beginning in the mid 1930s, the development of cancer in rats and mice exposed to chemicals produced by the dye industry became a yardstick for testing carcinogenicity that persists to the present day [107–110].

Dedifferentiation

The most obvious cellular responses preceding the appearance of HCC were the appearance of foci of altered hepatocytes and enlarging hyperplastic nodules. From these observations it was concluded that HCC developed from transformation or de-differentiation of "pre-neoplastic" liver cells [111, 112]. However, the studies revealed that the cellular response to chemical hepatocarcinogens also involves various other cell types, including large numbers of small round cells, and sometimes massive ductal cell proliferation preceding development of preneoplastic nodules (Fig. 55.2). The extent of the small round cell and ductular response varied, depending on the carcinogen used, the elapsed time after carcinogen exposure, and the species and age of the animals tested.

Oval Cells

In 1956, Farber coined the term oval cells for the small round cells [111], but he did not consider these cells to be an essential part of the carcinogenic process ([112], see pages [441–447]). He did consider that more than one cell can serve as the cancer precursor, but he concluded that the cells in areas of nodular hyperplasia were the precursors for HCC [112]. The succession from foci to nodules to HCC became the ruling dogma, and was supported by numerous studies into the 1990s [113]. Thus, throughout most of this period, the cellular changes in the liver preceding HCC were interpreted to indicate that HCC arose from transformation or de-differentiation of mature hepatocytes. However, at least two laboratories had independently arrived at a different conclusion. In 1953, Gilman et al. [114] concluded that there were liver progenitor cells in the ducts that can undergo malignant neoplastic transformation. A few years later, Wilson and Leduc [115] also noted the presence of liver progenitor cells in or closely associated with the bile ducts.

In contrast to the views of Gilman et al. and Wilson and Leduc, the conclusions of two other detailed studies on bile duct proliferation, published in 1964, were directly contradictory [116, 117]. These later studies found no evidence for a common precursor of ductal cells and hepatocytes. It was then generally concluded that the oval cells and duct-like cells were not involved in the process of development of HCC during chemical hepatocarcinogenesis, and that HCC arose from de-differentiation or reprogramming of hepatocytes.

Blocked Ontogeny

However, the de-differentiation hypothesis was challenged by Potter [118–120], who deduced that liver cancers instead

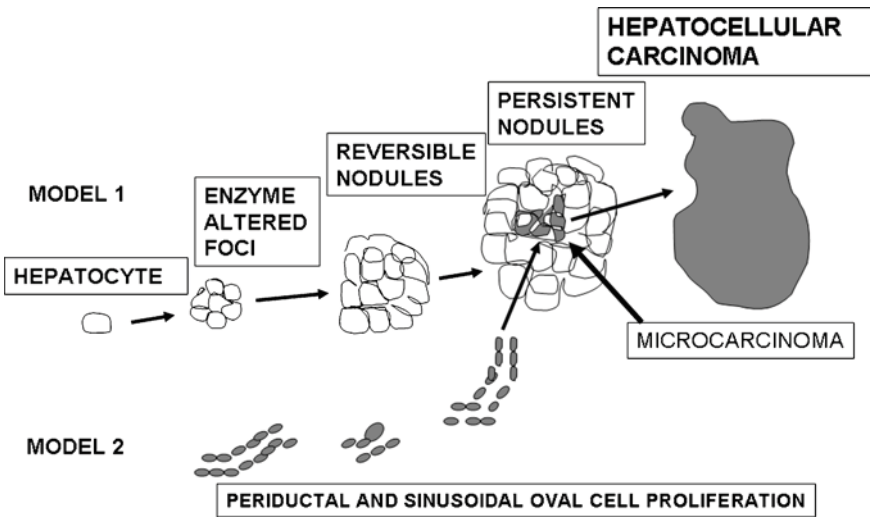

Fig. 55.2 Two models of chemical hepatocarcinogenesis: (1) de-differentiation [111, 112], and (2) maturation arrest of tissue stem cells (oval cells, [98]). Model 1, de-differentiaton is supported by the histologic sequence of changes in the liver preceding development of liver cancer. This sequence is (1) basophilic staining of hepatocytes; (2) small basophilic foci of hepatocytes; (3) small nodules, (4) larger nodules which distort the liver and (5) hepatocellular carcinoma (HCC). Model 2, proliferation of small oval cells beginning in the portal triad then extending between the sinusoidal lining cells and hepatocytes, eventually producing cells which enter the enlarging persistent nodules and give rise to HCC

arose by arrested differentiation of liver lineage cells. He called this process "blocked ontogeny." Potter originally developed his hypothesis after consideration of the enzymatic [121–124] and protein [59] changes in transplantable Morris hepatomas [58]. He theorized that the expression of fetal liver cell markers in such hepatomas was due to a "block" in the differentiation of those liver cells that gave rise to the cancer [125, 126]. Subsequently, fetal enzymes (isozymes) were found not only in HCC but also in pre-neoplastic nodules [126–130]. Although this process was attributed to de-differentiation by most workers [126–131], Potter [119, 120] interpreted that it reflected selection of progenitor cells during the carcinogenic process. This concept later received critical support with identification of the cells responsible for expression of AFP during the process of chemical hepatocarcinogenesis [77].

Alpha-Fetoprotein: A Marker for Early Cellular Events in Chemical Hepatocarcinogenesis

In the early 1980s, a serum protein was discovered in high concentrations in fetal blood and in the blood of animals with HCC by Abelev [132, 133], and in the blood of humans with HCC by Tatarinov [134]. This serum protein, designated alpha-fetoprotein [135], had properties similar to those of albumin: it was shown to be produced by fetal liver and yolk sac cells, and it was the major serum protein during fetal life [135–138]. A number of investigators, looking at the cellular changes during hepatocarcinogenesis, found AFP to be localized to the oval cells [103, 139–142] and not the foci or nodules [143–145]. Since AFP was also produced by most of the HCC that developed later in the carcinogenic process, the conclusion was that these oval cells, rather than the foci and nodules, are the true cellular precursors to the HCC, [103, 104, 139]. On the basis of the production of this fetal developmental marker by oval cells, and the indistinct immature morphology of the oval cells, it was concluded that these oval cells are liver stem cells [139, 146–148].

In an attempt to reconcile the two possible cellular origins of HCC, i.e., arising by de-differentiation of hepatocytes, or by maturation arrest of putative liver stem cells (Fig. 55.1), our laboratory carried out a series of studies, employing various models of experimental hepatocarcinogenesis. We used AFP and other markers to attempt to identify the precursor cells for HCC (for review see [77]). Because the cellular response preceding the development of HCC varies markedly, depending on the experimental hepatocarcinogenic

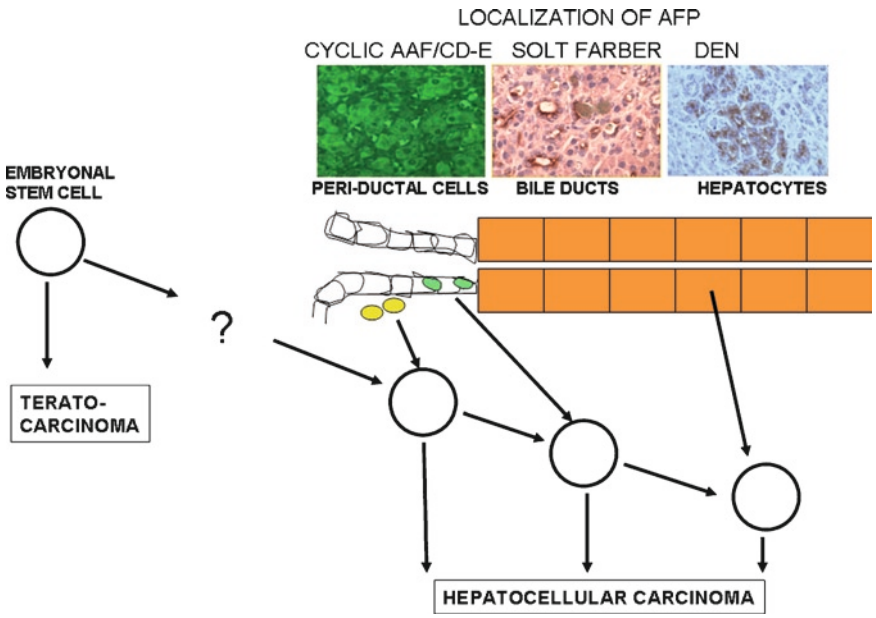

Fig. 55.3 Hierarchal model of liver cancer. Localization of AFP in three models of chemical hepatocarcinogenesis (Cyclic AAF, Solt Farber, and DEN) is related to the stage of maturation arrest in the hepatocyte lineage (The CD-E regimen gives results similar to the cyclic-AAF model). The maturation arrest model postulates that cancers arise from a block in differentiation of a cell lineage so that the cells no longer terminally differentiate and continue to accumulate. The earliest such block occurs in embryonic stem (ES) cells (teratocarcinomas). In the liver-cell lineage, a block may occur at the level of the stem cell (peri-sinusoidal oval cell), the duct cell, or the hepatocyte. Blocks at each of these stages results in HCC. The ? refers to the "missing link" in the stage of maturation between teratocarcinoma and HCC. Modified from: Sell [105]. Used with permission

regimen used, it is instructive to summarize here the results of four selected regimens which showed different cellular changes in the liver preceding liver cancer (Fig. 55.3).

Choline Deficiency (CD)

We began our studies in the late 1970s by feeding rats with ethionine or *N*-2-acetylaminofluorene (AFF) in a choline-deficient diet (CD-E, [149–154]). In this model, the first cells to proliferate are small, AFP-positive periductular cells in the portal triad, which extend into the lobule on the sinusoidal side of the liver cord as a single row of cells with little or no ductal structure. Proliferation of ductules and nodules ensues, and eventually there is occurrence of HCC. From this model it was concluded that HCC arises either from small periductular liver stem cells or from stem cells in the ducts, as well as from foci and nodules [103, 104, 139]. The sequence of cellular changes was actually very similar to the foci to nodule to HCC as observed by Farber [111, 112], but the striking difference was the early appearance of AFP positive periductular oval cells. Proliferating oval cells were seen next to ducts within 1–3 days after the CD-AAF diet was started. These oval cells expanded within 3 weeks from the portal triad across the entire liver lobule [152, 153]. Proliferating ductules appeared at 3–4 days, and foci and nodules appeared at 2–3 weeks, so that oval cell and ductule proliferation was prominent before foci or nodules appeared. AFP was not found in the foci or nodules.

Cyclic *N*-2 Acetylaminofluorene

The next regimen was selected because it is a model designed for optimal production of foci and nodules preceding HCC. We reasoned that this would be one of the best regimens to show whether or not AFP was produced by foci or nodules. As described by Teebor and Becker [155], the cyclic feeding of AAF for four rounds of a 2 week on, 1 week off regimen produces prominent foci and nodule formation, before development of HCC. At the end of the first round (2 weeks on, 1 week off) there is extensive development of small foci of about four to eight altered hepatocytes, with each cell larger than normal; and the cells have a more basophilic staining than normal. If AAF feeding is discontinued, these foci disappear with time. After the second round, 2 weeks on, 1 week off cycle, small nodules of 30–50 cells are seen. Again, if AAF feeding is discontinued, these nodules disappear. After the third round, large nodules of megahepatocytes distort the liver, but still, if AAF feeding is stopped, these nodules will disappear (so-called reversible nodules). However, after four rounds, much larger nodules that greatly distort the liver appear, and can number in the hundreds in a single rat liver. If AAF feeding is discontinued at this stage, HCC will appear, but with a delay of about 20 weeks. Thus, the cyclic AAF-feeding regimen provides a systematic model that elucidates the characteristics of foci, nodules, and cancer.

We took advantage of this regimen to study and attempt to correlate the production of AFP with the appearance of foci and nodules. Using a highly sensitive radioimmunoassay [156] for AFP, we were able to detect the low concentrations of AFP present in normal serum [157, 158]. Combining this assay with immunolabeling of cells producing AFP in the liver [104, 143], we followed the kinetics of AFP production in the serum, and performed the cellular localization of AFP during the carcinogenic process induced by cyclic AAF feeding [143]. After the first round, as described above, a marked increase in small oval cells was seen, as was a rapid elevation of serum AFP. After the second cycle, the number of oval cells as well as the serum level of AFP actually decreased, at the same time as the number and size of foci and nodules were greatly increasing; thus, the serum level of AFP correlated with the numbers of oval cells, not with the development of nodules. Immunolabeling clearly demonstrated AFP in the small oval cells, but not in foci or nodules. In fact, after four cycles, small AFP-positive oval cells were seen within the larger persistent nodules [104, 159]. It had previously been reported that HCC actually arises within persistent nodules and accordingly, the cells in the persistent nodules were believed to be the precursors of HCC. The localization of AFP-positive cells in the persistent nodules suggests that the cells in the nodules that give rise to HCC are not the persistent nodular cells derived from hepatocytes, but are instead oval cells that migrate into the nodules [104, 159]; this conclusion was independently reached by Evarts et al. [160]. We further postulated that the nodules, being resistant to the toxic effects of carcinogen treatment, actually provide a protective niche for the further development of oval cells [159]. Thus, the results of the cyclic AAF protocol supported the idea that the AFP-containing non-ductal oval cells were the precursor cells to HCC.

Solt-Farber Model

The possible role of ductal liver progenitor cells was examined using the Solt-Farber regimen for induction of HCC. The Solt-Farber model [161, 162] was designed to provide an initiation-promotion type of regimen for experimental hepatocarcinogenesis in the rat. A single dose of diethylnitrosamine (DEN) is the initiator. Two weeks after initiation, AAF is added to the diet, to inhibit the proliferation of hepatocytes. A week later (3 weeks after initiation), a partial hepatectomy (PH) is done to stimulate liver-cell proliferation. Because hepatocyte proliferation is inhibited by AAF, the PH instead stimulates the proliferation of small oval cells

(putative liver stem cells), many of which can be seen in small proliferating ducts. In fact, when the major bile duct was injected with gelatin-based medium, gelatin was found in both the preexisting ducts and the newly proliferating ducts [163]. This model, which has been extensively used by others (see review [106]), led to the conclusion that the liver stem (progenitor) cells are located in the duct of canal of Hering. These changes are similar to those seen in human atypical ductular reactions [164]. However, in our experiments, the first proliferating oval cells are seen in the periductular tissue, followed by extensive arborization of ducts, which migrate into the persistent nodules that develop later. The newly formed ducts are labeled by AFP and by monoclonal antibody, OV-6 [165, 166]. Again, elevation of serum AFP occurred during the early proliferative stage of oval cells and decreased with increasing appearance of more numerous and larger nodules. Clearly, the pattern of oval cell proliferation seen here (ductal) is distinct from that seen with the CD models (non-ductal). For example, Novikoff et al. [167] using CD-E, found proliferation of oval cells extending across the liver in single file in the sinusoids; however, when they used the Solt-Farber model, they found extensive proliferation of ductal oval cells [168]. Both periductular and ductular liver stem cells proliferate in one or the other of these models, and both cell types are candidates for the precursor cells for HCC.

Diethylnitrosamine

Continuous exposure to DEN was the regimen that we used, to determine whether hepatocytes could give rise to HCC. Analysis of AFP serum concentration and localization in cells in the liver at various times after 10–12 weeks continuous exposure to DEN revealed a pattern unlike that seen with the other models [104, 165]. There was no early rise in AFP levels in the serum, but instead a slow and gradual rise until about 8 weeks; after that, a rapid rise associated with the development of HCC. The cellular AFP localization showed a temporal sequence: initially, AFP-positive hepatocytes were seen in the mid-zone of the liver lobule, followed by foci of AFP-positive hepatocytes, AFP-positive micro-adenocarcinomas, and finally AFP-positive HCC, within 16–20 weeks of initiation of treatment. The successive cellular changes were examined with the use of monoclonal antibodies H-4 (to hepatocytes), T-6 (to tumor cells) and OV-6 (to oval cells) [165, 166]. Positive foci, micro carcinomas and HCC were seen to be labeled with T-6, but very few oval cells were present. Thus, continuous DEN treatment is a hepatocarcinogenic protocol that favors development of HCC from hepatocytes. Using repeated doses of DEN, Williams et al. [169] also found foci, nodules, and HCC with very few oval cells. However, one of the discrepancies was that, in our studies, AFP-positive micro carcinomas were seen to be surrounded by a few AFP-negative oval cells [104], leaving open the possibility that the AFP-positive micro carcinomas can arise from oval cells. Overall, the results using the DEN model were most consistent with the idea that HCC can arise from hepatocytes [104, 106, 165].

Liver-Cell Lineage and HCC

Taken together, the results of the above four experimental regimens for induction of liver cancer demonstrate that the liver contains the same lineage of cells as do other epithelial organs [106, 170, 171]. Depending on the nature of the injury, lost liver cells can be replaced by hepatocytes, ductal liver progenitor cells, or periportal liver stem cells. Similarly, depending on the carcinogenic regimen, liver cancer can arise from maturation arrest of stem cells (oval cells), bipolar ductal progenitor cells, or hepatocytes (Fig. 55.3). Thus, support for the ruling dogma (foci to nodules to HCC implying de-differentiation) in accounting for the origin of HCC began to flag in the 1980s; that paradigm was replaced by a maturation arrest concept for the origin of liver cancer [106, 170]. Since mature hepatocytes can divide, there is no reason why these mature hepatocytes cannot give rise to HCC. However, this process is not dedifferentiation; rather it is a block in the final step of maturation of hepatocytes, which can respond to liver injury by proliferation. Tumors that arise from each of the three types of cell in the mature liver (oval cells, duct cells, and hepatocytes), give rise to hepatocellular or ductal carcinomas, but there is no clear association between the histologic type of HCC that results and the particular carcinogenic protocol. The lack of correlation may be because most of the experimental studies are done in adult rodents. Do the liver stem cells of adult animals have less potential to produce undifferentiated HCC than do those of younger animals? With aging do the progeny of liver stem cells lose potential? The "blastomas" seen in young animals provides the missing link in the liver-cell lineage that answers these questions (Fig. 55.4).

Mouse Hepatoblastomas

Mouse hepatoblastoma (MHB) is a poorly differentiated small round cell liver tumor, which frequently appears associated with "ordinary" hepatocellular tumors (adenomas, carcinomas, or nodules). MHB was first described in the late 1960s as a cholangioma, cholangiocarcinoma, or unusual tumor with rosette formation. Later, the term hepatoblastoma was suggested [172], and the tumor was characterized in detail [173–176]. An efficient method for the induction of MHB is the two-stage treatment of 5-week-old male mice with

N-nitrosodiethylamine (initiator), followed by phenobarbital (promoter) [175]. Although infiltrative growth is not pronounced (the tumor may even be surrounded by a fibrous capsule), MHB is much more malignant than ordinary mouse HCC. Lung metastases are common, the tumor is readily transplantable, and the features are not common in HCC produced in older animals. MHB is made up of poorly differentiated cells with dark nuclei (morphologically similar to oval cells), and includes many cells in mitosis; the cells are often arranged in rows or rosettes, or may form peculiar organoid structures resembling embryonic or fetal liver. Osteoid formation, hematopoietic elements, and foci of keratinization are sometimes present [174, 176]. The tumors do not express the cytokeratins that are characteristic of duct cells or hepatocytes; they do not form bile duct-like structures and they usually do not express AFP (properties similar to those of the oval cells that appear early during chemical hepatcarcinogensis in rats, [152–154]). These negative traits indicate origin from a very early cell in the hepatocyte lineage, before the above noted markers are expressed. An age-related difference in the potential of liver-determined tissue stem cells to give rise to hepatoblastomas or HCC is also supported by the characteristics of childhood hepatoblastomas in humans.

Human Hepatoblastoma

Human hepatoblastoma (HHB) has features very similar to those described above for MHB [177, 178]. The most primitive small round-cell variant of HHB is essentially the same in appearance as the most prominent cells of the MHB. HHB is a childhood tumor; approximately two-thirds of them occur within the first 2 years of life, and 90% within the first 5 years of life [179]. The tumor is made up of cells at various stages of differentiation, within a variable mesenchymal matrix. The epithelial component consists of embryonal cells (small, fusiform, and darkly staining with hyperchromatic nuclei), and/or fetal-like liver (larger cells with formation of primitive hepatic cords) [180, 181]. Anaplastic small cells grow in sheets, and are similar to neuroblastoma and other primitive tumors of childhood. A variant called teratoid HHB contains osteoid, melanin, cartilage, and muscle [182, 183], suggesting pluripotency of the HHB cells. These tumors grow rapidly and respond poorly to therapy [179–182]. HHBs occurring in older children may show more differentiated cells and more adult-like liver cords. From the standpoint of the cellular origin of HCC, these observations suggest that there is a loss of potential with aging in the tissue-determined liver-stem cell; tumors arising in the livers of adults are the more highly differentiated HCC, whereas those arising in the livers of young children reflect the less differentiated stage of the liver-determined stem cell, i.e., HHB. In contrast to MHB, some small epithelial cells in HHB express CK-7, albumin, and oval cell associated markers OV-1 and OV-6 [184] suggesting some differentiation to bipotential liver stem cells in HHB.

Human Hepatocellular Carcinoma

The cellular responses to injury of the mature liver that are associated with development of human HCC have been extensively described; they support an origin of HCC in

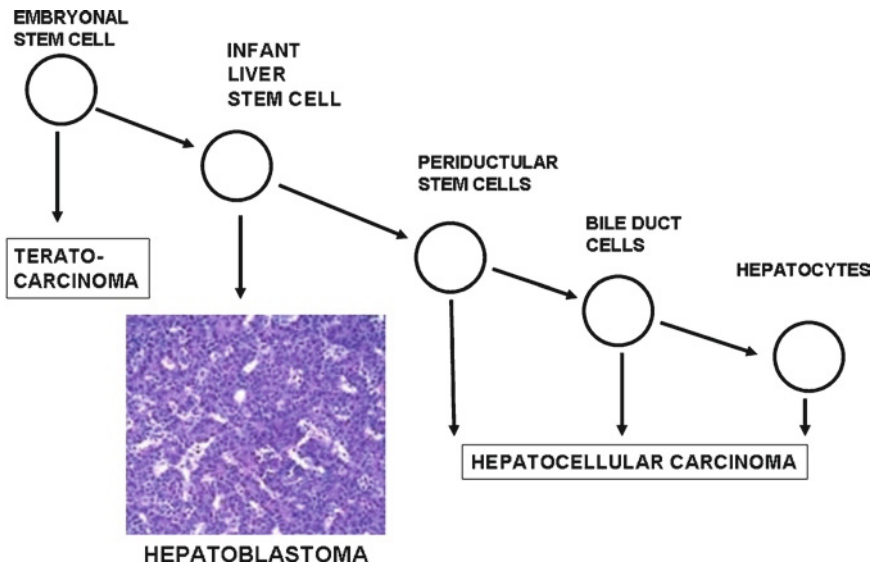

Fig. 55.4 Hepatoblastoma – the "missing link" in the hierarchical model of liver cancer. The occurrence of liver cancers of embryonic liver cell type (hepatoblasts) in children under the age of 6 years and in young animals exposed to chemical hepatocarcinogens implies that there are stem cells in the liver at these ages that are "less mature" than those in adults. These infant liver stem cells provide the missing link in the maturation arrest model of cancer between pluripotent ES cells (teratocarcinomas) and oligopotent liver stem cells (hepatoblastomas)

humans from a more differentiated cell than that giving rise to HHB [164]. Ductular reactions, which feature prominent increases in small ducts [185–188], were classified by the renowned liver pathologist Hans Popper into three types: I, II, and III [186]. Type I contains well-formed duct structures, type II has less well-organized biliary structures, and type III consists of duct structures containing both biliary epithelium and hepatocytes. Type II is associated with chronic active hepatitis and type III with submassive hepatic necrosis. Together, the latter two are also called "atypical ductular reactions." The atypical ductular reactions have many similarities to the ductular and oval-cell proliferation described above in the Solt-Farber rat model of liver injury and carcinogenesis. Typical ductular reactions occur after biliary obstruction and their appearance resembles the ductal proliferation that is seen in rats after bile duct ligation or treatment with chemicals such as 4,4′-diaminophenylmethane (DAPM) [189, 190]. Atypical reactions are associated with biliary cirrhosis, sclerosing cholangitis, alcoholic liver disease, and liver necrosis, as well as graft rejection and focal nodular hyperplasia [191]. Atypical ductular reactions feature increased numbers of bile ducts arranged in anastamosing cords with poorly defined lumina, and small cells located between the periportal zone and the adjacent parenchyma. On close inspection, the atypical ducts can often be seen joining with hepatic cords with an apparent transition of biliary-type cells to hepatocytes. In acute atypical reactions, the atypical ducts may disappear, whereas in chronic injury they persist and are associated with nodular hyperplasia. The cellular changes in human liver associated with chronic liver disease are similar, if not identical to those seen in the Solt-Farber and DEN models of experimental liver injury and hepatocarcinogenesis in the rat. Thus, it appears likely that the cell types that give rise to HCC in the rat also do so in humans [164].

Summary and Conclusions

Analysis of the cell types present in liver cancers indicates that the cell populations are the same as in normal tissue: stem cells, transit-amplifying cells, and terminally differentiated cells. The population of LCSC may itself be heterogeneous, but its cells are responsible for the defining three cancer-stem cell properties: (1) growth in vitro (immortality), (2) transplantability ability to transfer tumor, and (3) resistance to therapy. LCSC can be enriched in vitro by selection for cells with markers of immature cells, but a verified marker for LCSC has not yet been found. When the results from various experimental regimens of chemical hepatocarcinogenesis and histological observations on the lesions preceding liver cancer in humans are considered together, maturation arrest of cells at any of several stages of differentiation in a hierarchical cell lineage best explains the various types of liver cancer seen. The mature liver contains tissue-determined stem cells and transit-amplifying cells, but they are very few in number in as compared to the complement in the skin and gastrointestinal tract. In contrast to mature cells in most other tissues, mature hepatocytes are not terminally differentiated, and they respond to injury or loss by rapid proliferation. Thus, cancers of the adult liver may arise from periductal stem cells, bipolar ductal progenitor cells or mature hepatocytes. Which cell population is the origin of experimental HCC depends on age of the animal and the experimental regimen used to induce the injury. The cell population that gives rise to human HCC depends on the age, and nature of the injury that precedes the cancer.

Note: The concepts presented in the second part of this chapter are derived from a previously published extensive review of cancer stem cells in the liver (ref [105]).

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Chapter 56
Primary Hepatocellular Carcinoma

Jean-François Dufour and Caroline Hora

Introduction

Epidemiology

Hepatocellular carcinoma (HCC) is the main primary liver tumor, accounting for 85–90% of primary liver cancers diagnosed [1]. The prevalence and incidence vary greatly among the different regions of the world. High prevalence areas are Asia and Africa. Eastern Asia and sub-Saharan Africa account for over 80% of worldwide HCC cases, and more than half of the world's cases occur in China [1, 2]. In these regions, the incidence ranges from 18.2 to 39.7/100,000 in men and 5.7 to 14.2/100,000 in women. North and South America, Europe, and Australia are considered as low-prevalence regions, with incidence rates ranging from 3.4 to 11.6/100,000 in men and from 1.7 to 4.0 in women; the highest incidence being recorded in countries of Southern Europe [1, 2].

The male: female ratio for liver cancer averages from 2:1 to 4:1 [1]. This is also seen in animal models of HCC, an observation that favors the hypothesis that endogenous factors modulate male risk, rather than sex-specific differences in exposure to risk factors.

The age distribution of HCC is influenced by a variety of factors, in particular by the geographic region. As a general trend, HCC tends to concern younger age groups in developing countries, whereas in developed countries the highest age-specific rates are seen among older age groups (i.e., 75 years and older in the United States) [1, 3]; female rates tend to peak 5 years later than the corresponding male rate [1].

The geographic variability of HCC epidemiology largely reflects the distribution and frequency of underlying liver diseases, such as hepatitis B and C, as well as differences in the age of exposure to hepatitis virus. In high-prevalence regions, it is mostly hepatitis B virus that is in cause, with vertical transmission responsible for infection at a very young age. In some of these countries, a positive effect of wide-scale vaccination programs of new-born children could already be noted with a regression of HCC incidence [4]. The situation is very different in North America, Western Europe, and Australia where the incidence of HCC is on the rise [5, 6]. In the US, mortality due to HCC has nearly doubled in the last 20 years [7, 8]. This rising trend is mainly due to an increase in the incidence of HCV-related HCC [6, 8, 9]. As the HCV infection rates increased until the early 1990s, when blood conserves started to be screened for the virus, a further augmentation of HCV-related HCC is expected for these countries [3, 10, 11].

Etiology and Risk Factors

Seventy to ninety percent of HCC arise on a background of chronic liver disease characterized by inflammation and fibrosis that eventually progress to cirrhosis [1, 12, 13]. Cirrhosis is the largest single risk factor for HCC.

Viral hepatitis is a highly prevalent etiologic risk factor for HCC and a leading cause of cirrhosis development. Globally, chronic hepatitis B is the most frequent cause of HCC. It is estimated that 300 million people are infected with HBV worldwide (see Chap. 37). The majority of HBV-related HCC occurs in cirrhotic livers; however, HBV infection also evolves to HCC in the absence of cirrhosis. HBV carriers co-infected with hepatitis D virus (HDV) or hepatitis C virus (HCV) increase their risk of developing HCC. In general, a potentialization exists for most of the known risk factors cited below.

Chronic hepatitis C is another major cause of HCC, intravenous drug abuse and contaminated blood conserves propagated the virus in the second half of the twentieth century. A chronic course is found in about 80% of cases, cirrhosis develops in 15% of infected patients and it is estimated that 1–3% of infected individuals progress to HCC after 30 years [9, 14].

J.-F. Dufour (✉)
Department of Visceral Medicine, University of Berne, Berne, Switzerland
e-mail: jf.dufour@ilep.unibe.ch

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_56, © Springer Science+Business Media, LLC 2011

Toxic agents also contribute to hepatocarcinogenesis. Heavy alcohol intake (>50–70 g/day for prolonged periods) leading to alcoholic cirrhosis is a well-established risk factor for HCC [1, 15, 16]. A dietary toxic agent is aflatoxin B1 (AFB1), a mycotoxin of the *Aspergillus* fungus, contaminates food like corn and peanuts when stored in damp and warm conditions. High numbers of AFB1-induced HCC are found in Africa and Asia, often in synergy with HBV. Tobacco smoking and occupational exposure to vinyl chloride have also been linked to HCC, but the data are more controversial [1].

Metabolic etiologies include obesity, diabetes mellitus, and non-alcoholic steatohepatitis (NASH). The most compelling evidence for association of NASH and HCC comes from two conditions strongly implicated in NASH development: obesity and diabetes. Diabetes is a significant risk factor for HCC [17–19]. Obesity is a risk factor for cancer mortality in general and increasingly recognized as a risk factor of HCC [20]. Ninety percent of people with a BMI > 30 have fatty liver disease, ranging from steatosis to NASH and cirrhosis [21]. Men with a BMI > 35 have a risk of death by liver cancer increased by a factor of 4.5 [20].

Genetic disorders, namely hemochromatosis, primary biliary cirrhosis, and α1-antitrypsin deficiency represent an increased risk of HCC in the setting of established cirrhosis [22–25]. Autoimmune hepatitis may also lead to a cirrhotic liver, but yields a much lower risk for HCC [26]. Wilson's disease is another situation where the risk of HCC is low even in case of cirrhosis [27].

Genomics and Carcinogenesis

Mutations and Chromosomal Aberrations in HCC

The genetic information of the cells may be compromised at different levels, resulting in profound alterations of the cell's initial phenotype and function and setting the stage for malignant transformation. Such changes may imply:

- Epigenetic mechanisms
- DNA coding sequence alterations, referred to as qualitative alterations
- Changes in the regulation of gene expression (quantitative alterations)
- Perturbations at the posttranscriptional (microRNA)
- Posttranslational level

Quantitative changes and the latter two will be discussed below.

Genomic Changes in HCC

Epigenetic changes are predominantly characterized by modifications of the methylation status of the DNA double strand. DNA methyltransferases (DNMT) are enzymes involved in the methylation and demethylation of CpG islands; DNMT1 and DNMT3 are both upregulated in HCC [28]. The S-adenosylmethionine synthase and glycine N-methyltransferase contribute to the pool of available methyl groups; they are upregulated in a number HCCs. Another potential mechanism is changes in chromatin acetylation status.

Alterations of the DNA coding sequence consist of structural changes such as mutations, deletions, amplifications, and transpositions. Allelic deletions are present in almost all HCCs [29–31]. Impaired DNA mismatch repair leads to frameshift mutations and deletions [32]. Defective DNA-repair mechanisms are reflected by microsatellite instability, which may be found in chronic liver diseases and HCC [29–31, 33].

There may be a causal effect linking epigenetic methylation status to amplifications, deletions, and translocations at the DNA level [29, 34].

Loss of heterozygosity (LOH) is a feature recurrently encountered in HCC. Allelic losses on the following chromosomes are linked to hepatocarcinogenesis: 1p, 4q, 5q, 6q, 8p, 9p, 13q, 16p, 16q, and 17p [35–39]. LOH on chromosomes 17p is linked to p53 inactivation, 13q to RB1, 9p to p16, 6q to IGFR2, and 16p to Axin1 inactivation. Regional gains on chromosome arms are also recognized for 1q, 6p, 8q, 17q, and 20q [37].

Frequent Mutations in HCC

The most frequently mutated genes in HCC are p53 and CTNNB1, followed by Axin1 mutations [40–43]. The latter two account for the frequent activation of the Wnt-pathway in HCC. Mutations of CTNNB1 are found in 13–43% of HCC, most are exon 3 mutations modifying the phosphorylation site by GSK3β and thereby impairing the degradation of β-catenin [36, 41, 42, 44]. The axin1 gene is located on chromosome 16p13 and allelic losses at this site are found in ~30% of HCC [36]. Biallelic inactivation is found in 5–7% of HCCs, caused by homozygous deletions or a combination of LOH and inactivating gene mutations [36, 43]. Axin1 is a scaffold protein in the inhibitory complex formed by GSK3β, CK1, APC, and Axin. This complex mediates phosphorylation and inactivation of β-catenin. Deletion of Axin1 therefore further stabilizes β-catenin [43]. AXIN2 mutations have also been described in HCC, but not APC mutations [45, 46].

Also observed in HCC but less frequent are inactivating mutation of RB1, methylation suppression of CDKNA2 (p16ink4A), cyclin D1, and gankyrin overexpression [47, 48], the latter resulting in increased RB1 degradation [49, 50].

Even more seldom, PI3KA and K-ras mutations, inactivating gene mutation of IGFR2, SMAD2, and SMAD4 modify the TGF-β pathway [51–53].

Genetic Alterations Specific to Etiological Factors

HBV

HBV infection can induce hepatocarcinogenesis by:

- Chromosomal instability caused by HBV DNA integration in the host genome.
- Insertional mutagenesis, accounting for 20–40% of HBV-related HCC. Random integration, but also site-specific integration sites of HBV for the hTERT and IPR (inositol 1,4,5-triphosphate receptor) loci [54–56].
- Viral protein HBx mediated changes in cell survival and proliferation, of note, HBx binds to p53 and inactivates its downstream effects in vitro [57].

Aflatoxin B1

Aflatoxin is metabolized in the liver to exo-8,9-epoxide. This metabolite forms DNA-adducts and is responsible for mutational DNA damage [58, 59]. In particular, it reacts with guanine; the G-T transversion in codon 249 of TP53 leads to an amino-acid substitution (R249S) and this mutation typically occurs in regions with high AFB1 exposure levels [60, 61]. The individual susceptibility is influenced by the host's capacity to metabolize the AFB1-epoxide. Different enzymes have been recognized to have a role in this process, amongst them the epoxide hydrolase (EPHX) and glutathione transferases GSTM1 and GSTT1. Polymorphisms in these proteins influence their enzymatic capacity and have been associated with increased hepatocarcinogenic susceptibility, and the same holds for patients with allelic deletions; the carcinogenic effect of AFB1 in this setting was even more pronounced in HBV infected individuals [62, 63].

Vinyl Chloride

Next to its implication in the development of angiosarcoma in the liver, exposure to vinyl chloride has also been associated with the onset of HCC. HRAS and KRAS mutations have been observed in exposed patients [64].

MicroRNAs

MicroRNAs (miRNAs) are small (~22 nucleotides), endogenous RNA molecules characterized by a hairpin loop-structure, acting at the posttranscriptional level. They modulate mRNA expression by inhibition of translation and/or degradation of complementary mRNA strands [65]. Accordingly, depending on the gene product, they target, they may act as tumor suppressors or have oncogenic properties (*oncomirs*) [66].

A variety of miRNA have been reported to be deregulated in HCC [67]. Amongst them are miR-21, miR-122, and miR-221/222. MiR-21 and miR-221/222 are overexpressed in HCC and in a variety of other neoplasms; miR-221/222 promote cell-cycle progression by inhibition of the cyclin-dependent kinase inhibitors (CDKIs) p27 and p57 [68, 69]; additionally, miR-221 was shown to inhibit apoptosis in HCC cell lines [70]. MiR-21 targets the tumor suppressor PTEN and may thereby increase signaling via PI3K-AKT to promote cell survival [71]. Interestingly, hypoxia, present in the center of growing tumors, may be a trigger for miR-21 expression. MiR-122 is, in contrast, a hepatospecific miRNA, regulating cholesterol and fatty-acid metabolism. It was reported to be downregulated in >70% of HCCs [72] and may have a role in carcinogenesis via indirect inhibition of p53 [72–74].

MiRNA represent yet another level of gene regulation, but concomitantly they also represent new targets for molecular therapeutic intervention.

Telomeres and Telomerase

Telomeres are short tandem nucleotide repeats located at the end of chromosomes. They form a cap at the chromosomal ends, thereby preventing fusion with other chromosomes and ensuing chromosomal instability as well as activation of the DNA damage response (DDR) [75–77]. Each cell-division cycle implies telomere shortening due to the inability of the DNA polymerase to replicate the far end of chromosomes. This is counterbalanced by the enzyme telomerase, a ribonucleoprotein that elongates telomeres. Telomerase is composed of a template RNA component (TERC) and the reverse transcriptase (TERT), which catalyzes the addition of nucleotides [78–81].

The limiting factor of telomerase activity is the expression of TERT; whereas TERC is ubiquitously expressed, expression of TERT is downregulated after birth in the majority of human organs, comprising the liver [82].

Telomerase reactivation is a common feature in a wide variety of human cancers. Studies of telomerase activity in

the setting of liver diseases showed an upregulation in >90% of human HCC [83, 84] and some reported a reactivation occurring already in precancerous and dysplastic nodules [85, 86]. In parallel, telomere erosion is a common feature of hepatocytes with progressive shortening of telomeres from chronic hyperproliferative disease to cirrhosis and HCC [83, 87–89]. Further, telomere shortening is associated with chromosomal instability and hepatoma development [90].

Telomerase deficiency and thereby the impact of short telomeres has been studied in animal models. Mice deficient for the TERC component of telomerase displayed enhanced tumor initiation, but reduced progression of the cancerous lesions [91]. In the setting of chronic liver injury and absence of p53, these mice proved to have a reduced progression rate of HCC, with telomere dysfunction resulting in higher aneuploidy, DNA damage and apoptosis, pointing to a p53-independent suppression of tumor progression in this model [92]. Another approach analyzed tumorigenesis in mice lacking the TERT component of telomerase. Interestingly, in the context of liver damage and p53 deletion, these mice showed not only reduced progression, but also reduced initiation of HCC, and this was reversed in the presence of a functional p53 allele [93]. Moreover, TERT has been implicated in DDR and may have a dual role in carcinogenesis by limiting genomic damage in cancer cells and providing further survival advantage [94, 95].

Cell Signaling in HCC

p53 Pathway

Defects in the p53 response pathway are found in a wide variety of human cancers, including HCC. p53 is activated upon DNA damage and cellular stresses. It is a transcription factor acting to maintain genomic stability and prevent proliferation of genetically disrupted cells. Next to this tumor suppressor function it also mediates regulation of genes involved in development, differentiation, cellular senescence and aging [96–98].

The p53 protein structure bears three different domains. The TA (trans-activation) domain, located at the N-terminal end, is responsible for mediation of cell-cycle arrest and apoptosis. It contains several residues that are phosphorylated by up-stream regulators of p53, e.g., the PI3K-family kinases ATM, ATR, and DNA-PK [99]. The DNA binding domain (DBD) is a mutational "hot spot" with 80% of tumor-related TP53 mutations occurring in this region and preventing the binding of p53 to DNA [100]. This is also the site of aflatoxin-induced R249S mutations. Finally, the C-terminus holds the TD/Reg (tetramer/regulatory) domain, crucial for the tetramer formation of p53 [101, 102]. It further contains motifs that regulate its subcellular localization [103].

Upon DNA damaging insults (ultraviolet and ionizing radiation, oxidative and nitrosative stress) as well as in hypoxic stress conditions, p53 is activated. ATM, ATR, and Chk2 are kinases activated in the DDR [104]. They stabilize p53, which then translocates to the nucleus to induce and/or repress expression of target genes. Target genes mediate cell-cycle arrest, which allows the cell to repair its DNA damage, or apoptosis. The determinants leading to preferred apoptosis or cell cycle arrest are complex and are being investigated [105, 106]. One mechanism seems to involve binding affinities of p53 to different partners, implying a dose-dependent effect of nuclear p53 [107, 108]. Upregulation of the CDKI p21 promotes cell-cycle arrest in the G1 phase by inhibiting cyclin/CDK complexes mediating G1/S transition [109]. It is aided by Gadd45, another target gene of p53 [110, 111]. The G2/M transition is also affected preventing mitosis [112]. Next to cell-cycle surveillance, p53 is directly implicated in DNA repair, interacting with DNA repair proteins and regulating their expression [113, 114]. Also, it modulates the expression of anti- and pro-oxidant genes [115].

Another key function of p53 is activation of apoptosis, presumably following irreversible cellular damage. p53 impacts on both the intrinsic and extrinsic pathways of apoptosis by modulating gene expression of key players; direct mitochondrial interaction may also occur [116, 117].

Sustained p53 activity would be deleterious and is prevented by accurate regulation. One of the major mechanisms is the p53-Mdm2 autoregulatory feed-back loop [118]. p53 upregulates Mdm2, which in turn, inhibits the transcriptional activity of the tumor suppressor and also mediates its proteosomal degradation [119, 120]. Mdm2 itself is inhibited by binding of ARF ($p14^{ARF}$), another tumor suppressor.

In cancer, overexpression of Mdm2 as well as repression of ARF are next to p53 alterations, the most frequent causes of a dysfunctional p53 pathway [109]. p21 levels downstream of p53 may also be altered.

In HCC, p53 mutations are a late event, usually found in advanced disease. TP53 gene mutations may be a consequence of oxidative and nitrosative stress, but also result from genomic instability; LOH of chromosome 17p is frequently found in HCC. However, according to the etiological context, specific mutations or disruption of the p53 response may be causative factors in hepatocarcinogenesis. AflatoxinB1 is metabolized in the liver to exo-8,9-epoxide. This metabolite forms DNA-adducts and causes mutational DNA damage [58, 59]. It preferentially reacts with guanine; the G-T transversion in codon 249 of TP53 leads to an amino-acid substitution (R249S) and this mutation typically occurs in regions with high AFB1 exposure levels [60, 61]. In HBV-related HCC, the prevalent mechanism of p53 pathway disruption involves the HBx protein of the HBV. A variety of

interactions have been recognized [115]. HBx binds to p53 and compromises its downstream activities, including inhibition of p53-mediated transcriptional activation and blocking of apoptosis [57, 121–123]. HBx further represses transcription of TP53 [124] and interferes with p53-associated DNA-repair mechanisms [121, 125, 126].

WNT/β-Catenin Pathway

The Wnt-signaling cascade plays a crucial role in normal development by controlling a variety of cellular events comprising cell proliferation, differentiation, epithelial-mesenchymal transition, and cell death. Parallel to its physiological function, it is recognized as an oncogenic pathway, with overactivation being described in several malignancies including HCC [127–129].

In HCC, overactivation of the Wnt-pathway is characterized by aberrant cytoplasmic/nuclear β-catenin immunostaining. Recently, a more sensitive marker was proposed using anti-glutamine synthetase (GS) antibody [130]. Overall, 33–67% of tumors show an overactive pathway [131].

Extracellular binding of the glycoprotein Wnt to its transmembrane receptor Frizzled (Fz) induces a complex formation with LRP5/6, which mediates the cytoplasmic stabilization and nuclear translocation of monomeric β-catenin [132–134]. In the nucleus, β-catenin associates with the transcription factor TCF/LEF to initiate target gene expression [135, 136]. Prominent target genes in the liver encode proteins involved in proliferation and survival, adhesion, signal transduction, as well as glutamine and detoxification metabolism. Deregulation therefore confers growth and survival advantages, but also facilitated dissemination to tumor cells.

The pathway is regulated at several levels; of particular interest is the negative regulation of β-catenin by a cytoplasmic complex consisting of GSK3β, CK1, Axin, and APC. This complex promotes phosphorylation of β-catenin; phosphorylated β-catenin is ubiquitinated by βTrCP followed by its proteosomal degradation [137]. As described in the previous section, activating β-catenin mutations modify N-terminal phosphorylation sites, preventing its degradation. Axin1 and APC are tumor suppressors. Interestingly, somatic APC mutations are not described in HCC whereas they are the most prevalent mutation in human colon cancer. Moreover, loss of Axin1 expression is found in <10% of HCC; however, absence of Axin1 does not modify Wnt/β-catenin target gene expression [138]. Inactivating Axin1 mutations do not cluster with the HCC gene expression signature profile characterized by an active Wnt/β-catenin pathway (and CTNNB1 mutations). Therefore, Axin1 is likely to exert its tumor suppressor function via other pathways, possibly by its positive regulation function in p53-, JNK-, and TGF β-signal transduction [139].

The Wnt receptor Frizzled7 is another actor of the pathway, which was shown to be upregulated in HCC and results in stabilization and nuclear accumulation of β-catenin, independently activating β-catenin mutations [140, 141]. Secreted frizzled-related protein (SFRP) binds and sequestrates Wnt, acting as an antagonist [142]. Methylation and silencing of SFRP family genes is another event described in HCC and was also found in viral chronic hepatitis and cirrhosis, pointing to a possibly early event in hepatocarcinogenesis [143].

Sonic Hedgehog Signaling

The Hedgehog pathway (Hh) is a prime regulator of processes involved in embryogenesis, development, and tissue remodeling [144–146] and is evolutionarily highly conserved. Hh signaling activity increases growth and viability of target cells, whereas withdrawal of stimulation usually triggers apoptosis [144, 147]. The activation of the pathway is mediated in a para- or autocrine fashion, via secretion of the Hh ligands, Sonic (Shh), Indian (Ihh), and Desert (Dhh); the specificity of Hh signaling is accomplished by differences in Hh-responsivity, with some cell types equipped to respond to Hh ligands and others that do not express Hh receptors [144–147].

Signal transduction is initiated by binding of an Hh ligand to the receptor Patched (Ptc); ligand-binding terminates the Ptc-mediated repression of the transmembrane protein Smoothened (Smo). Smo then activates an intracellular cascade, which results in the stabilization and nuclear translocation of the Gli (glioma-associated oncogene) family of zinc finger transcription factors. Gli1, Gli2, and Gli3 regulate the expression of target genes that include proliferative and anti-apoptotic genes, as well as Hh pathway components [148–150].

Adult hepatocytes lack Hh pathway activity and are not responsive to Hh [151, 152]. Despite this finding, the Hh pathway has been implicated in HCC in recent years, as demonstrated by various studies analyzing both hepatoma cell lines and HCC tumor tissue of patients [151, 153–156]. The Hh pathway was found to be active in a significant fraction of HCC samples, with upregulation of pathway components such as Shh, Smo, Gli1, Gli2, and downregulation of Hhip, when compared to the surrounding non-tumorous tissue [151, 153–156].

Hhip is a negative regulator of the pathway; it competes for Hh ligand binding at the cell surface [157, 158]. Hhip downregulation in HCC may result from promoter hypermethylation as well as from LOH at the corresponding gene locus 4q [154].

Following injury, mediated by various insults such as oxidative stress and inflammation, the Hh pathway becomes

upregulated in Hh responsive cells (e.g., hepatic stellate cells [HSC], endothelial cells, immature cholangiocytes, and hepatic progenitors). In the normal liver, this is fine-tuned by auto-regulatory mechanisms which involve positive and negative feed-back circuits. However, in the setting of chronic liver injury, Hh signaling persists [159]. This deregulation has been implicated in tissue remodeling and could represent a carcinogenic trigger, with abnormal proliferation of hepatic progenitor cells (which, on the contrary to hepatocytes, are Hh-responsive in the adult liver), evolving to self-sufficiency and uncontrolled growth.

Inflammation and HCC

HCC, in most cases, arises on a background of chronic liver inflammation. As mentioned before, a variety of etiologies promote continuous inflammatory stimuli in the liver parenchyma leading to fibrosis, the regenerative nodules characteristic of cirrhosis, and eventually culminating in HCC.

Although observed for many years, the molecular mechanisms linking inflammation and cancer have only recently begun to be unveiled [160].

So far, our discussion concerned mainly the malignant cells, their mutations, and deregulations. However, a fundamental aspect of all tumors is the tumor microenvironment (TM), consisting of the surrounding vessels, extracellular matrix (ECM), and resident cells. Without intense interaction with their surroundings, malignant cells would be unable to proliferate, expand, and metastasize (Fig. 56.1). This implies molecular crosstalk between the transformed cells and supporting cells such as endothelial cells, fibroblasts, and tissue macrophages, also called tumor associated macrophages (TAM).

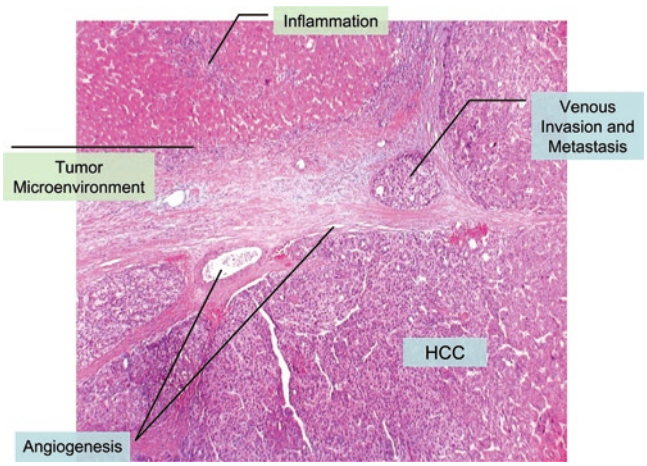

Fig. 56.1 Microscopic view of hepatocellular carcinoma (HCC) and its microenvironment. Features of the tumoral stroma and surrounding liver tissue. Inflammation is a hallmark of chronically damaged liver and the inflammatory microenvironment is implicated in hepatocarcinogenesis. Angiogenesis at the tumor periphery supplies the growing tumor with nutrients. It further provides access to the systemic vasculature, enabling the dissemination of metastatic cells

Different inflammation-associated intracellular signaling pathways have shown to be implicated in the genesis, promotion, and progression of HCC. Some of the best characterized will now be discussed.

Inflammatory Pathways in HCC

NFκB

NFκB may be activated by the pro-inflammatory cytokines TNFα and IL-1, via the TNFR-1 and IL-1R receptors, respectively, or by TLR receptors which signal through the adaptor protein MyD88 [161]. IL-1R is a member of the TLR receptor family. TLR receptors are activated via pathogen-associated molecular patterns (PAMPs) released by viruses and bacteria [162]. Interestingly, they may be further activated by DAMPs (damage-associated molecular patterns), endogenous ligands released by stressed, necrotic, or apoptotic cells to elicit what has been called a "sterile inflammatory response" [163, 164].

Upon ligand-receptor binding, the IKK complex (consisting of IKKα, IKKβ, and NEMO) is activated and phosphorylates the inhibitory protein IκB, which is subsequently ubiquitinated and degraded. This mediates the release of the NFκB dimer, which can now translocate to the nucleus and induce transcription of pro-inflammatory and anti-apoptotic genes [165].

NFκB was found to be constitutively active in some HCC and animal models have underscored its role in hepatocarcinogenesis [166–169]. At the level of hepatocytes and early in the course of tumor initiation, NFκB has a tumorsuppressive role, whereas in later stages of tumor promotion it has an oncogenic function, protecting malignant cells from TNF-mediated apoptosis [167–169]. To add to the complexity, NFκ activation in Kupffer cells has a tumor-promoting function [168]. Sustained activation in Kupffer cells leads to continuous secretion of hepatotropic/mitogenic cytokines such as IL-6, stimulating hepatocyte proliferation, which in a setting of oxidative stress and hepatocyte injury eventually culminates in hepatocarcinogenesis. This scenario of perpetual immune cell/macrophage stimulation occurs in the setting of chronic liver injury and is a plausible explanation for the elevated IL-6 levels found in patients with chronic liver diseases.

IL-6 and JAK-STAT3 Signaling

The inflammatory cytokine IL-6 is a downstream target of NFκB signaling. In the liver, it is produced by Kupffer cells.

IL-6 signals through a hexameric cytokine receptor complex composed of two molecules each of IL-6, IL-6R (gp80), and of the co-receptor gp130 [170, 171]. Complex-bound gp130 activates the JAK tyrosine kinases, which in turn phosphorylates and activates STAT3 (signal transducer and activation of transcription). Activated STAT3 dimerizes and translocates to the nucleus to activate transcription of target genes. Amongst them are members of the SOCS (suppressor of cytokine signaling) family of proteins, regulating JAK-STAT signaling in a negative feed-back loop.

STAT3 may also be activated by COX2, receptor tyrosine kinases (RTKs) such as EGFR and VEGFR, and receptor-associated tyrosine kinases such as SRC [172]. In the setting of inflammation and cancer, STAT3 signaling has differential but congruent effects in immune and tumor cells. It is immunosuppressive in immune cells, impairing host-mediated tumor rejection [172], and has pro-mitogenic and anti-apoptotic effects in tumor cells [173, 174]. SOCS play a fundamental role in controlling the physiologically transient activation of STATs. In HCC, the JAK-STAT pathway is often activated and associated with downregulation of negative regulators such as SOCS [175, 176]. SOCS3 has been shown to have lower expression levels in HCC tissue compared to surrounding non-HCC tissue. In animal models, deletion of SOCS3 in hepatocytes yielded enhanced and accelerated tumorigenesis following DEN-treatment [177, 178]. These findings account for an important role of IL-6 and STAT3 signaling in the fragile balance between inflammation and cancer.

As we already mentioned, one of the characteristics of HCC is its worldwide predominance in men. This male prevalence is also observed in experimental hepatocarcinogenesis. Recently, a research group has brought new insights in the mechanisms underlying these gender differences, involving both NFκB signaling and IL-6. They treated male and female mice with the carcinogen DEN and compared the IL-6 serum concentration and the incidence of HCC [179]. A particular feature of IL-6 is its regulation by sex hormones. Estrogens inhibit both NFκB and the CCAAT-enhancer-binding protein beta (C/EBPβ), thereby impeding IL-6 promoter activity and IL-6 transcription [180]. IL-6 levels were significantly higher in males compared to females, and correlated with markers of acute liver damage. These effects were abolished by administration of an estrogen receptor agonist as well as in male IL-6 knockout mice. Further, deletion of IL-6 in males reversed the gender differences in tumorigenesis. The DEN-induced rise in IL-6 came from Kupffer cells, dependent on DAMP-stimulated TLR and the adaptor protein MyD88. MyD88 ablation in males also conferred protection following DEN-treatment and resulted in reduced tumor burden. This example nicely illustrates the interplay between immune cells, cytokines, and carcinogenesis, with the modulation by sex hormones conferring an additional level of complexity (Fig. 56.2) [164, 179–181].

Liver Stem Cells

Hepatic progenitor cells (called oval cells in rodents) reside in the peripheral branches of the biliary tree, the ductules, and canal of Hering [182]. They form a progenitor cell compartment that can be activated in the setting of liver injury, when mature hepatocytes and/or cholangiocytes are unable to proliferate to restore liver mass (i.e., due to cell damage or inhibited replication) [183]. The activation of progenitor cells, called "ductular reaction" results in amplification of the progenitor-cell niche and differentiation of precursor cells in biliocytes or hepatocytes. These processes can be followed by immunostaining for a variety of markers, such as cytokeratin (CK)7, CK19, and oval cell markers like OV6.

HCC is a multistep process evolving from focal precursor lesions [184]; additionally, liver tumors appear to be monoclonal, deriving from a single cell. The earliest premalignant lesions are large-cell and small-cell dysplastic foci, <1 mm in size. These may evolve into dysplastic nodules and HCC. Fifty five percent of small dysplastic foci consist of progenitor cells and intermediate stage hepatocytes [185], and 28–50% of HCC express markers of progenitor cells [186–190]. Moreover, expression of different progenitor cell markers (e.g., CK19) correlates with patho-clinical features such as worse prognosis, shorter survival, post-operative recurrence, and metastasis [190, 191]. Stem cell features in HCC can be explained in two ways: either the cell of origin is a progenitor cell (differentiation arrest theory) or it is a mature hepatocyte that dedifferentiated in the course of hepatocarcinogenesis, presenting with progenitor-cell characteristics (dedifferentiation theory). The question remains to know whether both mechanisms (co)exist, and whether particular settings (i.e., underlying liver disease and risk factors) influence the cellular source of HCC.

What makes liver stem cells become malignant? Studies of normal tissue development have identified different signaling pathways regulating stem cell behavior. TGFβ/Smad and HGF/c-MET signaling control parallel pathways that promote liver development [192]. In the regenerating human liver, progenitor-cell niches are characterized by a variety of markers (Oct4, STAT3, and Nanog), including the TGFβ-pathway components TGFβ Receptor 2 (TBRII) and the intracellular adaptor protein ELF [193, 194]. Analysis of HCC tissue showed small cell clusters, lacking precisely TBRII and ELF. Given that $elf^{+/-}$ heterozygote mice develop HCC with an incidence of 40% [193], it can be speculated that a dysfunctional TGFβ-pathway disrupts normal differentiation of progenitor cells and thereby promotes hepatocarcinogenesis. Recently, ablation of the HGF/c-MET axis was found to enhance tumor initiation and accelerate early stage hepatocarcinogenesis in chemically induced HCC models [195, 196]. In contrast, overexpression of MET, as compared to surrounding tissue, is observed in 28–40% of

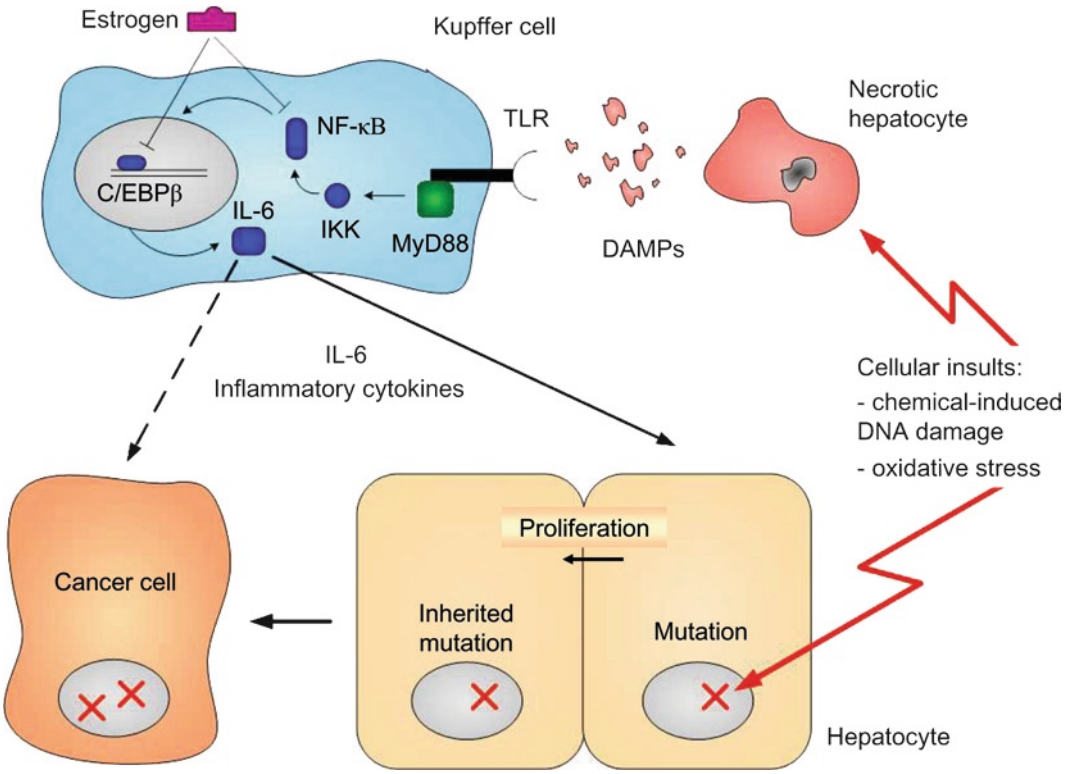

Fig. 56.2 IL-6 and hepatocarcinogenesis. A possible mechanism of inflammation-derived tumorigenesis. Cellular insults such as chemicals and oxidative stress promote DNA damage and cellular necrosis. IL-6 is secreted by Kupffer cells upon release of necrotic cell debris (DAMPs, damage-associated molecular patterns). The production of IL-6 is dependent on signaling via Toll-like receptor and the adaptor protein MyD88. Estrogens interfere with this process by inhibiting the transcription factors NF-κB and C/EBPβ. IL-6 then stimulates hepatocytes to proliferate; proliferation of hepatocytes bearing genomic damage sets the stage for malignant transformation and development of HCC [164, 179–181]

HCC [197–201]. Possibly, in these experimental models, interruption of HGF/c-MET signaling in progenitor cells contributes to tumorigenesis.

Angiogenesis in HCC

HCC is a highly vascularized tumor. Angiogenesis is defined as the formation of new blood vessels from existing vasculature. An HCC focus is not able to grow more than 1–2 mm^3 if it is not accompanied by tumor-driven angiogenesis. The new blood vessels supply the tumor with oxygen and nutrients, they also permit the recruitment of supporting cells such as TAMs (tumor-associated macrophages) to the site of neoplasia; moreover, they are an essential feature for malignant cells to enter the circulation and metastasize to distant sites.

The potential therapeutic implication of tumoral dependence on angiogenesis was first postulated nearly 40 years ago by Folkman [202]. Since then, a broad field of research focused on angiogenesis-modulating agents and some have been approved for the treatment of HCC.

Cellular Actors of Angiogenesis

In the normal liver, endothelial cells are quiescent and have a slow turnover rate with only ≤0.01% of cells dividing at any given time point [203]. Endothelial proliferation and vessel stability are tightly regulated by the opposing effects of pro- and anti-angiogenic factors. In the course of tumorigenesis, tumor cells gain the ability to shift this balance toward stimulatory factors of angiogenesis, an event called angiogenic switch [204] and a rate-limiting step in tumor progression. Upon activation, the turnover time of endothelial cells is greatly increased and a change in expression of surface markers is induced (i.e., CD31, CD34, VEGFR2), leading to a distinct phenotype [203, 205]. The endothelial cells in HCC further lose their fenestration and deposit a basement membrane, a process known as sinusoidal capillarization. These differences permit to differentiate tumor-associated from normal blood vessels (e.g., by immunohistochemistry); moreover, they are the basis for anti-angiogenic therapies targeting tumor blood vessels.

Circulating bone marrow-derived cells are also involved in tumoral neo-angiogenesis. Hematopoietic (CD45+) cell types are recruited to the perivascular regions, amplifying

angiogenesis, whereas the endothelial progenitor cells (EPC), a nonhematopoietic (CD45−) bone marrow cell population, are recruited to merge with the growing blood vessel where they differentiate into mature endothelial cells [206]. The contribution of EPCs to tumor vessel growth is usually low, also in HCC; however, EPCs can be markedly mobilized under vessel disrupting circumstances (i.e., treatment with vascular disrupting agents), contributing to the rapid restoration of tumoral blood supply [207, 208]. In HCC, the levels of EPC are increased and may correlate with advanced tumor stage [209, 210]. EPCs express distinct surface molecules, amongst them VEGFR2, allowing targeting of their regenerative action by VEGFR-blocking compounds [207].

Another functionally important cell type in tumor vasculature is pericytes. These are vascular smooth muscle cells in direct contact with endothelial cells, supporting their function, maturation, and survival. In the liver, HSC function as liver-specific pericytes. Pericytes are characterized by expression of PDGF B-receptors, members of the superfamily of RTK. Cancer cells and ECs release PDGFB thereby stimulating and recruiting pericytes to sites of tumoral vasculogenesis [211]. Pericytes in turn secrete vascular endothelial growth factor (VEGF), acting back on endothelial cells. The newly-formed immature blood vessels, as opposed to quiescent mature vessels, strongly depend on stabilization by pericytes and PDGF signaling [212–214]. For example, disruption of pericyte function with tyrosine kinases inhibitors (TKI) targeting PDGFR, takes advantage of this feature and has a selective effect, reducing vascularity in tumoral tissue [215].

Thrombocytes are also involved in angiogenesis; they contain high concentrations of angiogenesis stimulators and inhibitors, which can be selectively released [216].

Modes of Tumor Vascularization

As pointed out before, tumors rely on neo-angiogenesis to prosper; it is therefore not surprising that they have developed several modes of angiogenesis.

Sprouting angiogenesis is the most important mode of angiogenesis. It is found in physiological conditions of angiogenesis, for example, liver regeneration and also in pathological HCC-associated angiogenesis. It consists of the growth of new capillaries from existing vessels, triggered by growth factors such as VEGF, placental growth factor (PlGF), and basic fibroblast growth factor (bFGF). These activate local endothelial cells to proliferate, migrate, and form sprouts which are subsequently canalized and annexed to the pre-existing microvasculature [217].

Intussusceptive angiogenesis is a form of microvascular remodeling leading to the formation of transcapillary pillars. Growing endothelial pillars successively fuse and partition the vessel lumen, leading to sinusoidal multiplication. This alternative mode of angiogenesis can contribute to neo-angiogenesis concomitantly with classical sprouting angiogenesis and is found in liver regeneration as well as in HCC [217, 218].

Vasculogenic mimicry designs the formation of vascular structures formed by tumor cells that have adopted an endothelial phenotype [217, 219]. These vessels are lined by tumor cells and may contain endothelial cells. Mosaic vessels, in turn, consist of blood vessels normally lined by ECs but containing interspersed tumor cells [220]. In HCC, vasculogenic mimicry is associated with an aggressive tumor profile.

Vascular remodeling, rather than forming new vessels, mediates an adaptive response of existing vasculature to physiologic and pathologic stimuli. It implies structural and functional changes in the vessels, e.g., loss of sinusoidal fenestration, changes in cell–cell contact between pericytes and ECs. In HCC, this was described as an adaptive response of the tumor vasculature to escape anti-angiogenic therapy [217, 218].

Vascular Characteristics of HCC

A particular feature of HCC microvasculature is a nearly exclusive arterial blood supply. This so-called arterialization evolves progressively from dysplastic nodules to HCC and its degree was shown to correlate with tumor differentiation [221, 222]. In liver imaging using contrast agents, it leads to the typical hypervascularized enhancement of HCC tissue during the arterial phase [217, 222]. Another characteristic finding that evolves with progressive HCC is unpaired arteries, not accompanied by bile ducts [223, 224].

When considering the architecture of HCC microvasculature, several properties delineate it from normal blood vessels in the liver (Fig. 56.3): it shows widened lumina, aneurismal dilatations leading to irregular blood flow, regions of stasis, incomplete coverage by pericytes, high permeability and leakiness, bleeding and expression of a different set of endothelial cell markers (e.g., CD31, CD34) [217, 222, 225, 226]. The fenestration of hepatic sinusoids is lost and transformed into capillaries with deposition of a continuous basement membrane and collagenization of the Disse space [227, 228]; this process, called sinusoidal capillarization, is also found in cirrhosis.

Angiogenic Pathways in HCC

VEGF Signaling

VEGF are the best studied mediators of angiogenesis. The VEGF family of glycoproteins comprises VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF [229, 230]. The main mediator

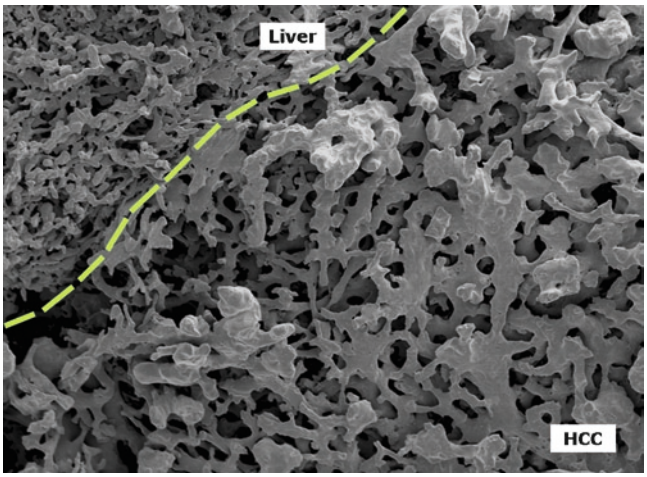

Fig. 56.3 Tumor vessels in HCC are abnormal and different from the hepatic microvasculature. Scanning electron microscopy of a polymer cast of normal sinusoids (upper left corner) and HCC tumor vasculature (center and right lower corner). Tumor vessels have irregular diameters and an atypical branching pattern (image kindly provided by V. Djonov, University of Fribourg, Switzerland)

of tumor angiogenesis is VEGF-A and it will be subsequently referred to as VEGF. The receptors that bind these growth factors are RTK. VEGFR-2 (KDR/Flk-1) is the principal receptor responsible for sprouting angiogenesis; the role of VEGFR-1 (Flt-1) in neo-angiogenesis is not quite elucidated and VEGFR-3 (Flt-4) binds VEGF-C and promotes lymphangiogenesis [231]. In the normal liver, VEGFR-1 is found on portal endothelial cells and macrophages and VEGFR-2 is expressed on sinusoidal endothelial cells [232]. In HCC, both receptors, but especially VEGFR-2, are expressed by endothelial cells of tumor vessels [232]. Further, HCC tissue has distinctly higher VEGFR-2 mRNA levels than the non-tumoral parenchyma [233].

Various environmental factors can induce or increase VEGF expression in tumors, such as, hypoxia, inflammatory cytokines such as IL-6, growth factors like bFGF, chemokines, and sex hormones (androgens and estrogens). VEGF upregulation can also occur as a consequence of tumoral mutations with gain of function of oncogenes (e.g., ras, c-myc, EGFR) or loss of tumor suppressors (e.g., p53, PTEN) [208]. Also, VEGFA gene amplification has been described as a cause for augmented VEGF levels in HCV-related HCC [234]. Hypoxia is an important trigger for VEGF expression. Hypoxic conditions, mainly occurring in the center of solid tumors, are sensed by the cells leading to intracellular stabilization of hypoxia-inducible factor HIF-1α. HIF-1α acts as a transcription factor promoting the expression of hypoxia-response genes, such as VEGF [235] and decreasing angiogenesis inhibitors like thrombospondin [236].

Binding of VEGF to its receptor on endothelial cells induces a cascade of signaling pathways, such as PLCγ-Raf-MAPK and PI3K-Akt-mTOR signaling. Downstream effectors mediate proliferation, migration, survival, and increasing vascular permeability of ECs [231] leading to the sprouting of new vessels, mainly in the tumor periphery. This increased blood supply promotes peripheral tumor growth, whereas unaltered hypoxic conditions in the tumor core sustain the vicious circle.

Angiopoietin

Angiopoietin 1 and 2 are further key elements of the angiogenic switch and are also specifically implicated in HCC. Both bind competitively to the same receptor Tie-2, a RKT principally expressed on vascular endothelium [237, 238]. Angiopoietin 1 has a vessel-stabilizing function, promoting the recruitment of pericytes; and angiopoietin-2 an antagonistic function, reducing vessel stability at sites of remodeling, and it also acts as a pro-angiogenic factor, namely in cooperation with VEGF. Angipoietin-2 expression is elevated in HCC and reflects high vascularity [239, 240].

Notch-Deltalike Ligand 4 (D114)

In recent years, growing attention has been given to the interaction of notch-deltalike ligand 4 (D114) and its downstream signaling, emerging as a new angiogenesis pathway [241–244]. Concerning HCC, the role of signaling via Notch, a family of transmembrane receptors comprising Notch 1–4 is currently being investigated. It appears that Notch receptors are aberrantly expressed in HCC tissue [245, 246] and Notch4-D114 signaling may be involved in angiotensinogen-mediated suppression of tumor growth [247].

(Tumor)Cell–Matrix Interactions

The ECM is a primordial regulator of angiogenesis as well as of many other cell processes. In its highly cross-linked network of fibrous proteins and proteoglycans, it holds a variety of cell-stimulatory molecules, which are released upon matrix degradation. This also concerns a variety of pro- (i.e., VEGF, bFGF) and anti-angiogenic factors (i.e., thrombospondin 1 and 2 or the collagen-derived endostatin, arrestin, canstatin, and tumstatin) [248]. Tumor growth and expansion as well as tumor neo-angiogenesis depend on the ability of tumor cells to break down the surrounding ECM. Multiple enzymes function as matrix-degrading factors, including the two families of matrix metalloproteases (MMP) and the serine proteases (e.g., urokinase, tPA). Their action is counterbalanced by specific protease inhibitors, namely tissue inhibitors of MMP (TIMPs) and serine protease inhibitors (serpins). Human HCC cell lines express high levels of

MMPs [249, 250], correlating with an invasive character [251]. On the other hand, TIMP overexpression reduces experimental hepatocarcinogenesis [252]. An elevated MMP/TIMP ratio is a predictor of aggressive, metastasizing HCC [253, 254].

HCC Classification

Edmondson and Steiner Grading

The histological classification of hepatocellular tumors is based on the tumor grading by Edmondson and Steiner [255]. According to the degree of cellular dysplasia, HCC is staged into grade I–IV. The scoring holds predictive value. However, it has no therapeutic implications and has not been linked to molecular pathomechanisms.

HCC Signature – Molecular Profiling of HCC

With the emergence of microarray gene technology in the past years, molecular profiling of neoplasms has opened a new field of possibilities regarding classification of tumors, prediction of survival, and the search for targeted anti-tumoral therapies.

Regarding HCC, different gene expression analyses of tumoral tissue have been undertaken. The progresses in diagnosis of HCC result in increasing detection of early stage HCC, a stage when there are little indices of clinical outcome and progression. Concerning intermediate stage and late stage HCC, the natural course and prognostic factors have been defined; however, these tumors are heterogenous and remain difficult to classify according to clinical parameters [256, 257].

In the recent literature, classes of tumors with distinct gene expression patterns and genetic profiles have been described. These studies integrate clinical features, cellular biological pathways, and genomic characteristics comprising epigenetic changes (promoter methylation), mutations and aberrations in chromosomes as well as gene copy numbers.

Among the different genetic profiles described, two emerge: one characterized by high proliferation, shorter survival rates, and chromosome instability. Tyrosine kinase activation is found in this class, with increased phosphorylation rates of IGFI-R, AKT, and other PI3K downstream targets [234, 258, 259].

The other class features mutations of CTNNB1, the gene encoding β-catenin. Mutation of exon 3 results in stabilization of beta-catenin and this group has an overactive Wnt signaling pathway [42]. It is associated with a better survival prognostic [234, 258, 259].

In parallel to these two main genetic profiles, different entities have been described, some of which may be considered as subgroups and will briefly be described.

Among the high proliferation class, different characteristics have been clustered in three subgroups: two subgroups share common properties comprising HBV infection, AKT activation, and Axin1 mutation [259]. An interesting characteristic of the first subgroup is an expression pattern reminiscent of fetal hepatocytes. While expression of progenitor cell-markers are likely to represent an HCC entity originating from hepatic progenitor cells, upregulation of the fetal growth factor IGF-II may reflect dedifferentiation of mature hepatocytes [259, 260]. TP53 mutations are present in the second and third subgroups. The latter distinguishes itself by a high incidence of CDKN2A (p16ink4) promoter hypermethylation and overexpression of a large number of genes involved in cell-cycle control [259].

In the CTNNB1 mutation class, differences are found in the prevalence of mutated CTNNB1 gene. One subgroup has a higher mutational rate and shows E-cadherin underexpression associated with satellite nodules. Another subgroup displays downregulation of genes involved in immune response and stress, including IFN-regulated genes [259]. Of note, despite an important association of HBV infection with two subgroups belonging to the high-proliferation-profile class, HBV, HCV, and alcohol abuse are found throughout the different clusters.

A third class of genetic profile was found to be related to the host-mediated immune response. It is distinguished by overexpression of IFN-stimulated genes associated with smaller tumors, possibly due to higher immune clearance reflected by lymphocyte infiltration and tumor apoptosis [234, 261]. This IFN-related class has been linked to HCV-mediated HCC, but not exclusively.

The classical methods to predict survival and recurrence in patients with HCC are based on histopathological findings (i.e., venous invasion, histological grade defined by Edmondson and Steiner). Owing to improved diagnostic tools and surveillance, HCC is increasingly recognized at an early stage. Tumor ablation is a potentially curative treatment at this point, nevertheless, most patients eventually present with recurrences [262]. Various studies sought to define gene expression profiles with prognostic value, able to predict outcome of early stage disease and gene expression patterns identifying patients at risk for recurrence and metastasis were described [263–265].

There is a debate concerning the origin of recurrent HCC. The recurrent tumor may originate from the primary malignancy, implying that the primary tumor features a pro-metastatic profile. On the other hand, the local TM may predispose to *de novo* tumorigenesis, implicating the host's genetic background [266–268], or, finally, a combination of both may facilitate metastatic processes. Genetic profiling of TM

confirmed that expression profiles influence survival and accurately predict recurrence [269, 270]. Further, highly discordant motifs of gene copy number alterations were detected when comparing the primary and recurrent tumors of HCC patients [270]. These findings led to the concept of "field effect," where late recurrences (>2 years after curative resection [271]) emerge as new tumors in the setting of a damaged organ [270].

Another possibility is to study quantitative changes in miRNA expression [272]. One such study identified alterations in miRNA expression associated with specific risk factors, namely upregulation of miR-96 in HBV-related HCCs and downregulation of miR-126* in HCC of alcoholic origin [273]. Also, low expression of miR-375 was found in tumors characterized by an activating β-catenin mutation [273]. A different approach found a 20 miRNA tumor-signature able to significantly predict venous metastases and survival in HCC patients [274]. MiRNA profiles of the tumor environment have not been identified thus far.

Next to classification purposes the main implication of molecular profiling lies in the development of new target drugs and adapted treatment of individual HCC, which will take in consideration distinct cellular pathobiology that allow to overcome survival advantages developed by cancer cells.

Therapeutic Considerations

Despite the large availability and use of conventional chemotherapies in the second half of the last century, the prognosis of HCC remains dismal; none of these treatments proved to be effective for primary liver cancer. For small tumors, surgery – either resection or transplantation – remains the best curative approach. In recent years, advances in our understanding of the molecular mechanisms involved in tumorigenesis opened the field for the discovery and development of new molecules targeting specific aspects of cancer cells. Regarding HCC, a multityrosine kinase inhibitor with antiangiogenic properties, sorafenib, targeting VEGFR1–3, PDGFR, c-Kit, FLT-3, and Raf/MEK/ERK signaling has proven effective in case of advanced disease, by prolonging survival in these patients [275]. Although the survival benefit is modest, it is the first systemic therapy able to modify the course of HCC and confirms that molecular therapies can be effective for its treatment. A variety of other small molecule inhibitors such as tyrosine kinase inhibitors (TKI) and antibodies against receptor ligands have been developed. Several of those are currently being tested in patients suffering from HCC, amongst them EGFR and PDGFR blockers (i.e., erlotinib and sunitinib) and inhibitors of mTOR targeting the PI3K/Akt pathway (i.e., rapamycin) [276]. Despite promising results, resistances to these agents do exist and the future of molecular therapies in HCC will likely comprise a combination of different treatment strategies.

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Chapter 57
Fibrolamellar Hepatocellular Carcinoma

Sanjay Kakar

Introduction

Fibrolamellar hepatocellular carcinoma (FLM) is a rare tumor that occurs in young adults. It occurs in the absence of chronic liver disease or cirrhosis. Serum alpha-fetoprotein is typically normal. FLM is characterized by a triad of morphological features: polygonal tumour cells with eosinophilic cytoplasm, prominent nucleoli and lamellar pattern of fibrosis. A central scar is seen in some cases. FLM has to be distinguished from conventional hepatocellular carcinoma (HCC). Scirrhous variant of HCC can closely resemble FLM, but lacks its typical cytological features. Acinar differentiation and focal mucin production can be seen in FLM and can be confused with adenocarcinoma. Focal neuroendocrine differentiation occurs in a minority of FLMs and can be mistaken for neuroendocrine tumours. Some of the molecular changes typically observed in conventional HCC like p53 and beta-catenin mutations are not observed in FLM. FLM is an aggressive neoplasm with 5-year survival of around 50%. Although the prognosis in FLM has been described as favorable compared to conventional HCC, this is likely to be related to absence of cirrhosis rather than unique morphological features of the tumor.

Fibrolamellar hepatocellular carcinoma (FLM) is a rare hepatocellular neoplasm that comprises less than 1% of primary liver tumors. This tumor was first described by Edmondson [1], but was firmly entrenched in literature by later reports by Craig and Berman [2, 3]. It has been referred to as eosinophilic hepatocellular carcinoma (HCC) with lamellar fibrosis and polygonal type HCC with fibrous stroma. Although both FLM and HCC show hepatocellular differentiation and share some morphological features, FLM differs from HCC in its clinical setting, morphological features, and genetic characteristics, hence justifying its designation as a distinct entity or a variant of HCC (Table 57.1).

S. Kakar (✉)
Department of Anatomic Pathology, University of California, San Francisco and San Francisco VA Medical Center, San Francisco, CA, USA
e-mail: sanjay.kakar@ucsf.edu

Epidemiology and Clinical Features

FLM occurs in noncirrhotic liver in young adults (mean age, 26 years), with only 6% of patients being above 50 years at diagnosis [2, 4, 5]. Even among young patients, conventional HCC is more common than FLM [6]. Rare cases have been reported in infants [7]. Its incidence has been variously estimated as 0.6–5.8% of all primary liver cancers. A slight female predominance has been described, but both genders are equally affected in most series [2, 3, 8]. FLM is more common in the USA and Europe, compared to Asia [9].

Clinical presentation is often nonspecific with abdominal pain, anorexia, nausea, and weight loss. Abdominal mass and jaundice can be present. Rare presentations include hemoperitoneum, fulminant hepatic failure, obstructive biliary disease-like symptoms, Budd-Chiari syndrome, deep vein thrombosis, metastatic bone disease, massive ascites, shoulder pain, nonbacterial thrombotic endocarditis, liver abscess-like symptoms, hypoglycemia, and gynecomastia [10]. The latter is a result of conversion of androgens to estrogens by aromatase produced by tumor cells, and not due to liver failure as in cirrhosis [11]. FLM has been described in the setting of pregnancy, ulcerative colitis with primary sclerosing cholangitis, autoimmune cholangitis, and Fanconi's anemia as well as with other liver tumors including conventional HCC and cholangiocarcinoma.

Unlike conventional HCC, serum levels of alpha-fetoprotein (AFP) are normal in the majority of FLM. Mild elevations may be seen in 10–15% of cases, but are usually below 200 ng/ml [2, 3, 5, 12, 13]. Serum levels of glypican-3 (GPC-3), another oncofetal antigen, are elevated in around half of conventional HCC; the data about GPC-3 in FLM is less clear [14]. Elevated serum levels of des-carboxy prothrombin, neurotensin, and transcobalamin I (vitamin B12 binding protein) is seen in many FLMs [15, 16]. The latter two have been observed in 70–80% of FLMs, but are not typical of conventional HCCs. It has been suggested that these markers can be useful for diagnosis and monitoring for recurrence after tumor resection. However, their diagnostic utility is limited due to low sensitivity and specificity.

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_57, © Springer Science+Business Media, LLC 2011

Table 57.1 Clinical features of fibrolamellar carcinoma and conventional HCC

	Fibrolamellar carcinoma	Conventional HCC
Clinical features		
Age at presentation (average) (years)	26	65
Gender	Males = females	Males > females
Serum AFP	Usually normal, mild elevation in 10–15%	Often elevated (>80%)
Serum neurotensin	Often elevated	Usually normal
Serum B12 binding protein	Often elevated	Usually normal
Serum glypican-3	Insufficient data	Elevated in ~50%
Vascular invasion	Rare <5%	Common
5-year survival (%)	40–60	10–20
Pathologic features		
Cirrhosis	Not present	Present (80–85%)
Central scar	Often present	Typically absent
Calcification	Present in 50%	Typically absent
Oncocytic cytoplasm	Characteristic	Rare
Lamellar fibrosis	Characteristic	Absent or focal
Pale bodies	Often present	Uncommon
Stainable copper	Often present	Typically absent
CK7 expression	Often present	Typically absent
AFP expression	Absent	Positive in 30–50%
Molecular features		
Chromosomal gains/losses (%)	55–70	>95
Chromosomal gains/losses (mean number)	3.2–3.6	5.4–12.8
β-catenin mutation	Absent	10–40%
Phosphorylated β-catenin	High levels	Usually low
P53 expression (%)	<10	30
Survivin overexpression	Absent	55%

Imaging Characteristics

Computed tomography (CT) is regarded as the most accurate imaging modality for diagnosis and staging [17]. Punctate, nodular, or stellate calcifications are seen in 33–55% of FLMs, usually in the central scar [9, 18]. The tumor usually appears as a lobulated, well-defined mass with mixed echogenecity on ultrasound and hypoattenuation on noncontrast CT scan. The non-scar portion of the tumor shows enhancement during the arterial phase in contrast-enhanced CT and magnetic resonance (MR) imaging, while the scar is best seen on the delayed phase. The absence of scar enhancement has been used to distinguish FLM from focal nodular hyperplasia (FNH) and cholangiocarcinoma, but scar enhancement can occur in up to 25% of FLM [9]. The hypointense appearance of the scar on all MR images is very helpful in distinction from FNH. Retraction of the capsule adjacent to the tumor is often present. This finding can be seen in other malignant neoplasms, but can be helpful in distinguishing FLM from benign processes like FNH. Positron emission tomography (PET)–CT performed with 2-fluoro-2-deoxyglucose (FDG) delineates anatomic and metabolic characteristics of neoplasms and has been used to demonstrate primary as well as metastatic FLM [19].

Etiology

There are no definite known risk factors for FLM. In contrast to conventional HCC, most FLM cases do not occur in the background of chronic liver disease and cirrhosis. The oncogenic role of hepatitis B has been proposed based on the presence of DNA sequences of the virus in tumor cells as well as antibodies directed against hepatitis B surface and core antigens in rare cases [20, 21]. Rare cases of FLM have been reported in patients with chronic hepatitis B [22]. These cases are anecdotal and there is no concrete evidence of involvement of hepatitis B in FLM.

FLM resembles FNH as both lesions often show a central scar. FNH-like nodules have occasionally been seen at the periphery of FLM [23, 24]. This has led to the speculation that FLM represents the malignant counterpart of FNH. The nodularity surrounding FLM is likely the result of local perfusion abnormalities [25] and there is no definite evidence that FNH is a benign precursor of FLM [26].

Pathologic Features

Gross Features

FLM is a firm, tan-white to brown, well-circumscribed but unencapsulated, lobulated mass that arises in a background of normal liver. FLMs are significantly larger than conventional HCC [8, 12, 27, 28] and can measure up to 17 cm. The larger tumors often show foci of hemorrhage and necrosis. Rarely, extensive necrosis can lead to a multicystic appearance [29]. A majority of the FLM present as a single mass, but multiple tumors, generally in the form of satellite lesions may be present. An unusually frequent involvement of the left lobe has been noted. A prominent central stellate scar similar to that of FNH is present in around half of the tumors and up to 75% in some series [2, 17, 18, 30]. The central scar has also been observed in lymph node metastases.

Microscopic Features

The tumor cells are typically arranged in cords or solid nests. Acinar structures can be occasionally present. The defining feature of fibrolamellar carcinoma is its triad of histologic characteristics [2, 5, 8, 12, 25, 31, 32]:

1. Large polygonal tumor cells with abundant eosinophilic granular cytoplasm (Fig. 57.1). The tumor cells are typically one and half times larger than cells in well-differentiated conventional HCC [33]. The cytoplasmic granularity is due to the presence of abundant mitochondria. Ground glass-like cytoplasm is seen in some tumors. Other cytoplasmic features include round to oval, lightly eosinophilic or clear inclusions ("pale bodies"), which may contain fibrinogen and/or albumin (Fig. 57.2). Periodic acid-Schiff stain with diastase (PASD)-positive globules, probably representing glycoproteins, can be present. Bile plugs are common, but fat is usually absent.
2. Prominent macronucleoli. In addition, the nuclei are often vesicular with intranuclear cytoplasmic invaginations and margination of chromatin (Fig. 57.1).
3. Lamellar fibrosis. This comprises of plate-like stacks of collagen lamellae of variable thickness (Fig. 57.3). The dominant collagen is type I, III, and V, and is produced by stromal fibroblasts. The lamellar pattern may not be present in all portions of tumor, but generally is present in at least half of the tumor.

Areas resembling FLM can be seen in conventional HCC. These tumors more closely resemble conventional HCC as they tend to occur in older patients, have high AFP and/or arise in the setting of viral hepatitis/cirrhosis [34, 35]. A case of FLM that recurred as conventional HCC has been described [36].

Other features that can be occasionally present are acinar structures, mucin secretion, multinucleated tumor cells, copper, epithelioid granulomas, peliosis hepatic, and widespread amyloid deposition. Degenerative atypical nuclei as a result of degenerative changes can be seen. A clear, cell-variant of FLM has been described [37]. Portal or hepatic vein invasion is uncommon, being present in <5% of FLM [9] compared to more than 40% in HCC [38].

Histochemistry

Stainable copper has been reported in 75% of FLM. It was proposed that the presence of copper and copper-binding protein is specific for FLM [39], but was later described in conventional HCC [40]. Mucin stains can be focally positive, especially in areas of acinar differentiation.

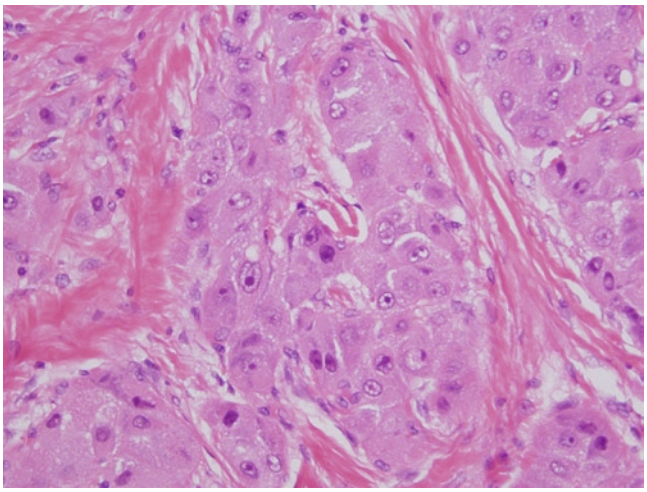

Fig. 57.1 Typical cytological features of fibrolamellar carcinoma: abundant granular cytoplasm, vesicular nuclei with margination of nuclear chromatin, prominent macronucleoli, 200×

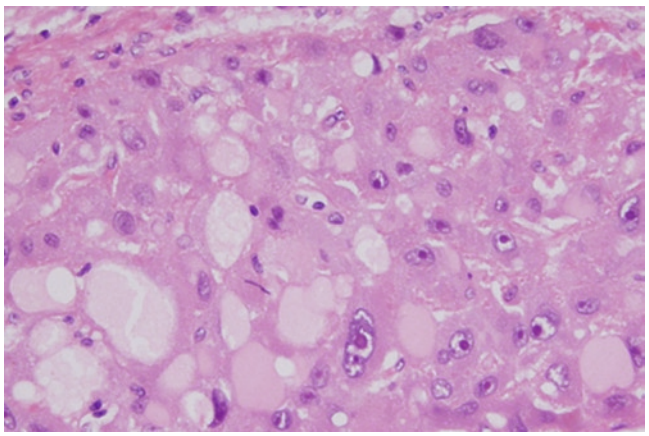

Fig. 57.2 Eosinophilic cytoplasmic inclusions ("pale bodies") are characteristic, but not diagnostic, of fibrolamellar carcinoma and usually composed of fibrinogen or albumin, 200×

Immunohistochemistry

FLM resemble conventional HCC and express Hep Par 1 and polyclonal CEA (canalicular pattern). AFP immunoreactivity is absent except in rare cases with focal immunoreactivity in a few tumor cells. Glypican-3 is expressed in around 60% of FLM compared to 80% in conventional HCC [41]. CK7 expression occurs in nearly 80% of FLM compared to 20–30% of conventional HCC [6]. Neuroendocrine differentiation can occur in FLM with expression of markers like nonspecific enolase and neurotensin [42, 43]. Synaptophysin and chromogranin are usually negative. The significance of "neuroendocrine differentiation" is unclear, and has been observed in up to one-fourth of conventional HCC as well [44].

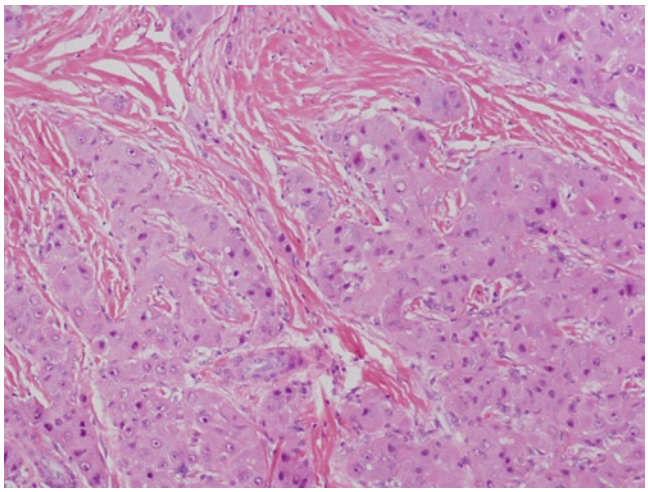

Fig. 57.3 Lamellar pattern of fibrosis in fibrolamellar carcinoma, 200×

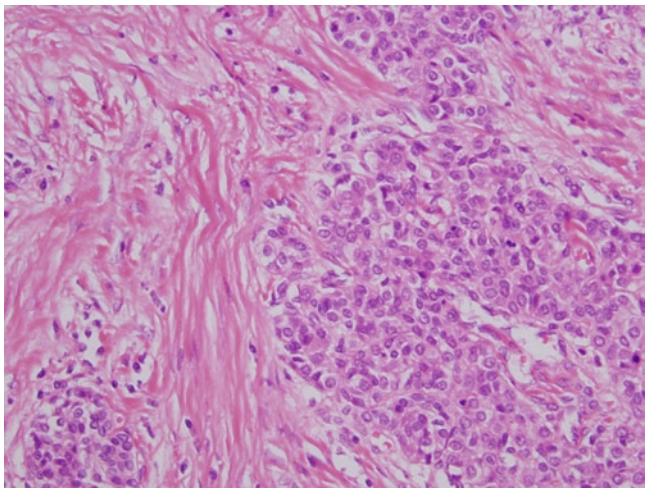

Fig. 57.4 Hepatocellular carcinoma. Lamellar pattern of fibrosis is present, but typical cytological features of fibrolamellar carcinoma are not seen, 200×. Such tumors are best classified as conventional HCC (scirrhous variant) rather than fibrolamellar carcinoma. This tumor occurred in a 50-year-old man

Ultrastructural Features

The most striking feature is the presence of mitochondria. Structures resembling neurosecretory granules have been noticed in some [42], but not all ultrastructural studies [2].

Differential Diagnosis

Conventional HCC

It is important to strictly use the triad of morphological features for the diagnosis of FLM [31]. In ambiguous cases, the presence of "pale bodies" favors FLM, although they have been described in conventional HCC, especially the scirrhous variant. The characteristic lamellar fibrosis may not be uniformly present throughout the tumor leading to sampling related errors in biopsy diagnosis. It is important to distinguish FLM from conventional HCC as the diagnosis of the former can lead to consideration of extended hepatic resection and lymph node dissection.

The scirrhous pattern contains focal or diffuse prominent areas of fibrosis that can be mistaken for FLM (Fig. 57.4). The lack of the lamellar pattern of fibrosis and absence of typical cytological features of FLM help to establish the correct diagnosis. Fibrotic changes can occur in HCC after radiation or chemotherapy and should not be labeled as scirrhous pattern. The term "sclerosing HCC" was used to describe a variant of HCC characterized by hypercalcemia and marked stromal fibrosis [45]. It may not represent a distinct entity and many of these tumors are likely to represent intrahepatic cholangiocarcinomas.

On rare occasions, HCCs show lamellar fibrosis without characteristic cytological features or show oncocytic morphology without the typical lamellar fibrosis of FLM. It is not clear whether these represent variants of FLM or conventional HCC. These tumors tend to occur in older individuals [46].

Adenocarcinoma (Cholangiocarcinoma or Metastatic Adenocarcinoma)

The presence of fibrosis, pseudoacinar pattern, occasional mucin production, and frequent CK7 expression in FLM may mimic cholangiocarcinoma or metastatic adenocarcinoma, especially in limited biopsy samples. Adenosquamous carcinomas of the gallbladder with liver invasion can have some overlapping features with FLM [10]. The lamellar pattern of fibrosis and the typical cytological features of FLM should enable the correct diagnosis. If required, hepatocellular differentiation can be confirmed with immunohistochemistry.

Neuroendocrine Tumors

Focal neuroendocrine differentiation has been noted in FLM. Since prominent fibrosis can be seen in neuroendocrine tumors, they can be mistaken for FLM. In most cases, FLMs express less specific neuroendocrine markers like neuron-specific enolase, protein gene product 9.5, vasoactive

intestinal peptide, and calcitonin. Diffuse staining with chromogranin or synaptophysin strongly support a neuroendocrine tumor.

Angiomyolipoma

The epithelioid variant of angiomyolipoma (AML) can be difficult to distinguish from HCC. It lacks the oncocytic features and lamellar fibrosis seen in FLM. Immunohistochemistry can confirm the diagnosis by demonstrating coexpression of smooth-muscle markers like smooth muscle actin and melanoma markers like HMB-45, melan A, and microphthalmia-associated factor. Hepatocellular markers like Hep Par 1 and polyclonal CEA are not expressed in AML.

Melanoma

Melanomas can have prominent nucleoli similar to FLM, but oncocytic features and lamellar fibrosis typical of FLM are not seen. Expression of S-100 and melanocytic markers like HMB-45, along with absence of Hep Par 1 and polyclonal CEA distinguish melanoma from FLM.

Other

FNH can be in the differential diagnosis based on radiological findings, while childhood tumors like mesenchymal hamartoma, hepatoblastoma, and embryonal sarcoma can be part of the clinical differential diagnosis. The distinction of FLM from these entities is straightforward based on histological features.

Genetic Features

Conventional HCC shows characteristic chromosomal gains or losses in >95% of cases [47, 48]. Similar genomic instability has been reported in 55–70% of FLM [49, 50]. The most commonly involved loci include chromosomes 1q,7p,7q, and 8q. Most changes are similar to conventional HCC, but some abnormalities like loss of 7p and 18q may be more common in FLM. The number of abnormalities in FLM is lower [49, 50] than conventional HCC (Table 57.1). Even early conventional HCCs show more aberrations (mean 6.5) than fibrolamellar carcinomas (mean 3.4) [48]. However, it is unlikely that FLM is similar to early HCC based on cytogenetic changes. Certain cytogenetic features like losses at 3q, 4q, and 13q, and gains at 20q are considered as intermediate or advanced steps in hepatocarcinogenesis [48]. These abnormalities are observed in 5–20% of FLM [49, 50] as well as in case reports [51, 52] indicating that at least some FLM are cytogenetically equivalent to advanced HCC. It has been suggested that these tumors may have a more aggressive course [50]. Ploidy studies have shown that all FLM are aneuploid or polyploid, which is in keeping with an aggressive tumor [53]. Primary FLM with minimal or no chromosomal aberrations and numerous aberrations in subsequent recurrence or metastatic FLM can occur [51, 52]. It has been speculated that FLM may not have major chromosomal aberrations in the initial stage of evolution and develop multiple chromosomal gains and losses with progression [50].

Since FLM is a rare tumor, there is limited information about the role of individual genes and pathways involved in tumorigenesis. Some of the genetic abnormalities commonly observed in conventional HCC like p53 mutations, survivin overexpression, and beta-catenin mutations are not seen in FLM [54–56]. Even though mutations of beta-catenin are absent in FLM, high levels of phosphorylated beta-catenin have been observed in FLM, but not in conventional HCC [56]. Phosphorylated beta-catenin can translocate to the nucleus and activate several target genes. Indeed cyclin D1, a known target of beta-catenin, is overexpressed in FLM [56]. Phosphorylation of beta-catenin is regulated by growth factors like epidermal growth factor as well as epidermal growth factor receptor (EGFR) [56]. Overexpression of EGFR as a result of gain of chromosome 7p has been observed in nearly all FLM cases [57].

Overexpression of genes involved in the RAS, MAPK, PIK3, and xenobiotic degradation pathways has also been described in FLM [58]. Activation of the total as well as phosphorylated mTOR has been observed in FLM [59]. Anterior gradient-2, a protein critical for normal embryonic development, is aberrantly expressed in 75% of FLM compared to <5% of conventional HCC [60]. The significance of this is not known, but may be related to abnormal MAPK signaling. Loss of fragile histidine triad, a putative tumor suppressor gene, has been described in 15–65% of HCC, but not in FLM [61, 62].

Transforming growth factor-beta (TGFβ) plays an important role in fibrosis in several pathologic conditions. TGFβ is aberrantly expressed in 80% of FLM compared to 21% of conventional HCC, and may play an important role in the development of lamellar fibrosis [63]. Higher levels of matrix metalloproteinase-2 have been observed in FLM compared to conventional HCC [64]. This enzyme is involved in extracellular matrix turnover and may contribute to the distinctive fibrosis seen in FLM.

Aberrant CpG island methylation of tumor-related genes is a frequent event in conventional HCC. The methylation process starts early and involves increasing number of loci with tumor progression [65]. In contrast, methylation is a less common event in FLM. E-cadherin and RASSF1 are frequently methylated in FLM, while methylation of other genes like p16 is seen in 20% of FLM compared to 65–75% of conventional HCC [66]. Methylation in FLM at most loci was similar to conventional HCC in noncirrhotic liver [66].

Natural History and Treatment

FLM presents with regional or metastatic disease in one-third of the cases at presentation [67]. Lymph node and peritoneal metastasis are more common than conventional HCC [2]. Lymph node involvement has been reported in up to 70% of cases [19, 68, 69] and distant metastasis occur in nearly half the cases [12].

Complete excision of the involved lobe is the therapy of choice and offers the only hope of cure. A cure rate of 50–75% has been achieved with successful resection [70]. Relapse rate for FLM is high (36–100%) with the median time to relapse being 10–33 months [19, 67, 68]. Hence, aggressive surgery with lymph node dissection is recommended. Invasion into adjacent organs, lymph node metastasis, or limited metastatic disease should not preclude curative tumor resection [71]. The median survival for unresected patients is 12 months with zero 5-year survival [69]. The large tumor size and high incidence of lymph node metastasis probably contribute to the high recurrence rate.

When the tumor is not amenable to resection due its location or extent, liver transplantation can be considered, but the outcome is less favorable compared to resection. This may be due to the large tumors size and unresectable status of tumors selected for transplantation. Recurrences have been observed in around 40% of FLM post-transplantation with a 5-year survival of 40–55% [72].

The limited experience with chemotherapy in FLM indicates that it does not prolong survival when used as adjuvant therapy with surgery [67, 71]. It is also ineffective for recurrent FLM [67]. In some cases, unresectable tumors may become amenable to resection after platinum-based chemotherapy [67, 73]. The combination of 5-fluorouracil combined with interferon-alpha has been successfully used in FLM to achieve complete remission in 60% of patients and its role in adjuvant therapy with surgery has been proposed [74]. Initial response has been reported with intra-arterial doxorubicin in unresectable FLM [75]. Arterial chemoembolization may be used to reduce tumor size in unresectable cases. Most of these reports involve a small number of patients, and no systematic study has examined the benefits of chemotherapy. The utility of newer agents like EGFR antagonists also remains to be assessed.

Prognosis and Survival

It has been widely regarded that FLM has a relatively favorable natural history and is associated with better outcome compared to conventional HCC. Numerous publications have reported that FLM is a less aggressive neoplasm compared to conventional HCC [2, 4, 8, 25, 27, 28, 32, 67, 70, 76, 77]. However, several well-designed studies have failed to confirm the better outcome in FLM [5, 12, 13, 78–80].

The discrepant results regarding behavior of FLM in various series may be attributable to a number of factors. One of the foremost reasons is the small size of most series since FLM is a rare tumor. It is also possible that uniform criteria have not been used in making the diagnosis of FLM. The use of a triad of histological features has been advocated for the accurate diagnosis of FLM. In many studies, the diagnostic criteria have not been outlined [76] or the slides were not reviewed before the case was included in the study [4]. Some studies have included cases that occurred in cirrhotic liver [2], while others have included tumors with FLM-like areas even though other areas showed features typical of conventional HCC [8]. It is likely that these tumors represent conventional HCC rather than FLM. The lack of adequate follow-up may also have led to the erroneous conclusion regarding prognosis in FLM. In one series, 86% FLM patients were alive after transplantation or resection [70]. However, 5-year follow-up was available in only 16% of patients.

Another major reason for the varying results in literature is that FLM has been compared to conventional HCC as a group. HCC is unlikely to be a homogenous entity in terms of prognosis and can be divided into several subgroups with distinct biological behavior. A vast majority of conventional HCC (>80%) arise in cirrhotic liver. Cirrhosis is a well known adverse prognostic feature in conventional HCC [81]. Hence, when survival in FLM is compared to conventional HCC, the poor outcome in the latter group as a result of cirrhosis is likely to affect the results. In a vast majority of studies including some large series [4], FLM has been compared to conventional HCC as a whole.

There are few studies in literature that have compared FLM to conventional HCC by separating the latter cases into those arising in noncirrhotic and cirrhotic liver. None of these studies were able to confirm the favorable outcome in FLM [5, 12, 13, 78]. The survival in resected FLM and conventional HCC in noncirrhotic liver is similar and is significantly better than conventional HCC arising in cirrhotic liver. The better survival in FLM is likely to be related to absence of cirrhosis rather than the unique clinicopathologic features of

the tumor. Young age and higher resectability may also contribute to better survival when compared to conventional HCC with cirrhosis [4, 78, 82, 83].

Irrespective of how FLM behaves compared to conventional HCC, it is important to realize that it is an aggressive malignant tumor with 5-year survival of around 50% in most series. In resected cases, the 5-year survival ranges from 25–75% and is even lower for cases with metastatic disease at presentation [12]. Though most of FLM occur in young patients, nearly 40% of patients die of the disease even when the tumor is localized to the liver at presentation [12]. Even in studies reporting better survival for FLM compared to conventional HCC, the 5-year survival was only 20–40% [2, 32, 76].

The most important prognostic factor in FLM is resectability of the tumor [25]. Solitary tumors, free margin status, normal liver enzymes, low stage, and absence of thrombosis or vascular invasion have been associated with increased survival [5, 13, 67, 70, 84–86]. Tumor size, gender, cellular proliferation, and atypia do not affect outcome [86]. Young age has been reported as a favorable prognostic factor in some studies, but not in others [84, 85].

Absence of genetic abnormalities typically observed in conventional HCC may also account for the favorable clinical outcome in some FLMs. These include absence of p53 or beta-catenin mutations, absence or lower number of aberrations, low frequency of allelic loss [87], and lack intratumor heterogeneity by DNA fingerprinting techniques [88]. It is possible that despite being morphologically similar, a subset of FLM may have genetic abnormalities like genomic instability and high risk cytogenetic aberrations like 3q loss that are associated with aggressive behavior [50].

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Chapter 58
Biology of Metastatic Liver Tumors

Alan Wells, Yvonne Chao, and Qian Wu

Introduction

The liver is the organ most frequently involved in metastases, to the point that cancer found in this organ is more likely to be a metastatic nodule than a hepatic neoplasia. Almost all cancers metastasize with some frequency to the liver, with this dissemination often being a harbinger of impending mortality. This includes not only carcinomas, with colorectal, breast, and lung cancers frequently disseminating to the liver, but also mesenchymally-derived tumors, with melanomas being prominent among liver metastases. This wide variety of involved tumors begs the question of the special attributes of this organ that cause it to be hospitable "soil" for so many different types of tumor "seeds" [1]. Speculation as to why the liver serves as such, in the absence of tested hypotheses in the literature, will be the topic of this chapter.

Liver involvement in cancer metastases often goes unnoticed or underappreciated as even extensive cancer infiltration may not alter the liver function or homeostasis. Unlike other liver pathologies, metastases to the liver, as a rule, do not cause cirrhosis or biliary blockage. Additionally, these lesions are painless, unlike some other sites of metastases. A case in point is prostate metastases. It is generally viewed that bone marrow is the predominant site of metastases as the osteoclastic lesions cause localized pain; however, thorough autopsy accounting reveals liver involvement to an equal, if not greater degree [2, 3]. In other cases, such as breast carcinomas, involvement does lead to tissue damage though not whole organ functional derangement, so that tumor metastasis or recurrence is sought by testing for hepatocyte damage and leakage of liver-specific enzymes into the circulation (such as alanine aminotransferase [ALT] and aspartate aminotransferase [AST]). While this immense functional reserve often masks metastatic involvement, it is exploited in the case of a limited number of colorectal metastases to the liver, in which a surgical lobectomy can be curative without compromising critical liver functions. However, most hepatic metastases present as multifocal lesions affecting multiple lobes of the liver, eliminating surgical resection as a therapeutic option.

The high frequency of liver metastases cannot be explained on the basis of access or mechanical entrapment within the sinusoids. Obviously, for colorectal and other gastrointestinal (GI) tract carcinomas, any cells that escape into the venous circulation would go directly to the liver. But, for other cancers, these disseminated cells would see the liver as the second or more likely the third capillary bed. Tumor cells that gain the venous circulation, whether directly or via lymphatic drainage, will need to pass through the lungs and most likely the GI tract, as 90% of the liver circulation is via the portal tract, prior to lodging in the liver capillary structure. Furthermore, many organs that have equal or greater fractional whole circulation, such as the kidneys, skin, GI tract, and skeletal musculature to name a few, do not evince this predilection for metastatic seeding. Thus, we will explore the local environment and architecture that may predispose to this lethal hospitality.

The Metastatic Cascade

The steps that a tumor cell must take to generate a metastatic focus need to be contemplated when considering the biophysical and molecular bases of liver metastases. In short, the cells need to escape from the primary mass, gain access to a vascular conduit for dissemination, survive this transit through shear stress and multiple capillary beds, escape into liver parenchyma, and then survive and proliferate to form a macroscopic nodule (Fig. 58.1).

The initial step of separation from the primary carcinoma mass requires loosening of the cell–cell bonds [4, 5]. This is characteristic of the dedifferentiation or epithelial-to-mesenchymal transition (EMT) as loosely defined [6, 7], noted in a variety of carcinoma cells [8]. The hallmark of EMT is a loss of the E-cadherin-mediated cell–cell junctions that provide

A. Wells (✉)
Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: wellsa@upmc.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_58, © Springer Science+Business Media, LLC 2011

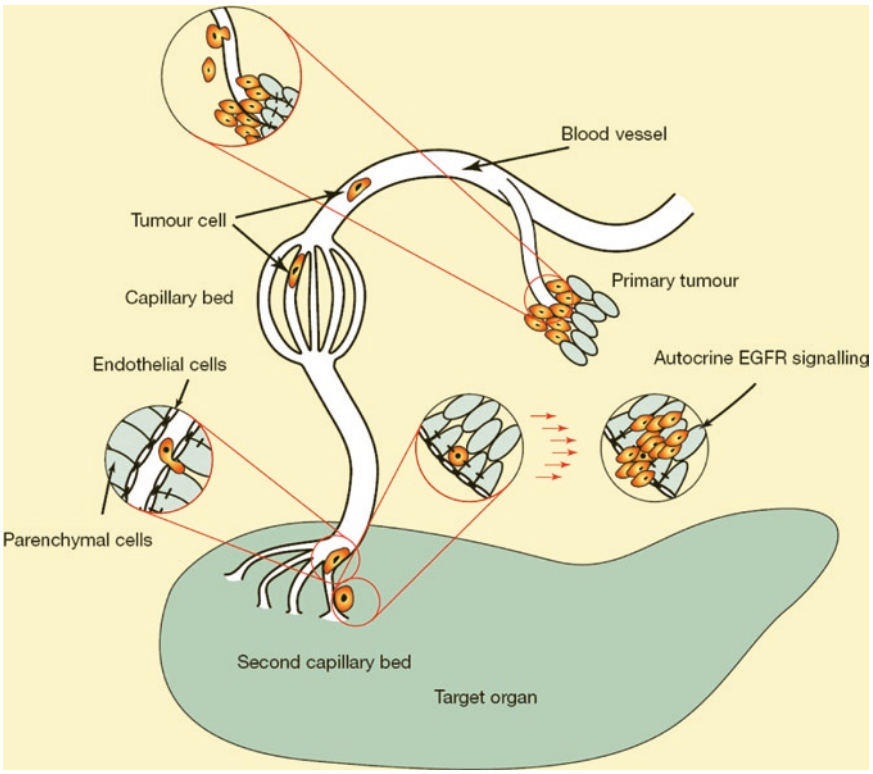

Fig. 58.1 Schematic of tumor dissemination with metastatic seeding in the liver. This proposed model represents the phenotypic plasticity of disseminating carcinoma cells with an initial epithelial-to-mesenchymal transition (EMT) promoting escape and dissemination, and with a liver-located mesenchymal-to-epithelial reverting transition (MErT) enabling metastatic seeding and survival. The final step to clinically evident metastatic nodule is still not understood. From Wells et al. [19]. Used with permission

for organ cellular architecture. This coincides with the slide down the histological scale toward a carcinoma being undifferentiated; with the widely-accepted inverse correlation between carcinoma differentiation status and likelihood of dissemination. Still, a tumor mass is generally heterogenous, with clusters of cells that span a range of differentiation levels, leaving open the question of which carcinoma cell escapes. Imaging of an experimental model of orthotopic breast cancers has found that individual tumor cells move toward the vasculature to disseminate [9], rather than the tumor moving as a syncytium as may occur during localization extension and invasion of adnexal sites [10, 11].

The mechanisms by which human tumor cells find and access hematological and lymphatic conduits is still ill-defined; even as to whether they intravasate mature vessels or enter among immature, open circulations. In the aforementioned experimental model, there is a clear paracrine signaling between blood-derived macrophages and breast carcinoma cells that leads to active intravasation [12], but the generalizability of such findings are open; and such a network would not explain escape via lymphatics. In the past, much was made of differences between hematogenous vs. lymphatic dissemination. More recently, these modes have converged in the consideration of distant metastases. This distinction likely maintains for tumors with regional-local spread along lymphatic drainage tracks such as carcinomas of the oral cavity, and does explain lymph node involvements, which are a harbinger of but not necessarily a precursor for distant metastases. To seed the liver, cancer cells must attain the portal, or to a much lesser extent, the hepatic circulation, implicating an end state of hematogenous dissemination.

Transit through the vasculatures (both hematogenous and lymphatic) is poorly understood, as to the track, length of time, and survival of the tumor cells. Recently, the ready finding of "circulating" tumor cells in peripheral venous blood [13], raised the possibility of tumor cells being able to pass at least two capillary beds during their time in circulation. The signals that allow these large cells to deform and survive this difficult passage are still unknown, and as such do not, at present, offer opportunities for targeted interventions.

Extravasation involves both tumor cell and endothelial adaptations. The initial step of arrest was captured as a physical mismatch between vessel caliber and tumor cell size. Unlike the rolling/attachment of hematological cells, tumor cells appear to flow to precapillary sites which have a lesser diameter, physically lodge, and then extravasate [14]. The molecular basis of tumor cell–endothelial cell interaction is via cell adhesion molecules (CAM) which lead to intracellular

signaling. In fact, in liver it may not be so much a cell–cell interaction and subsequent transit occurs between endothelial cells as rather a complete retraction of the endothelial-cell lining in the liver sinusoid [15]. In fact, it may be this unique fenestration of the sinusoidal endothelial cells that allows access of arrested cells to the underlying basement membrane/extracellular matrix components that promotes liver metastasis. A further unique aspect of the liver sinusoid is the "loose" attachment of the vasculature to the endothelial conduits; the potential "space of Disse" is the first place extravasated tumor cells reach when viewed in real time during experimental models of dissemination in which tumor cells were directly introduced into the portal vein and viewed as the tumor cells lodged in and moved from the sinusoids [14]. Extravasation through an endothelial lining requires recognition of the endothelial cells first; some tumor cells will express N-cadherin that can interact with endothelial cell N-cadherin, though this is not always the case [16, 17]. The ability to "sample" the underlying matrix allows for attachment by integrins and other adhesion molecules that actually provided the traction for the initial escape in the first place. This is a major advantage for metastatic seeding of the liver, in that recognition of location does not require altered gene expression beyond that which is provided for escape.

While this vascular access and transit is critical for dissemination, it appears not to be the major rate-limiting step under experimental challenge situations [14]. Even extravasation appears not to be rate-limiting, in that both normal and transformed fibroblasts leave the portal circulation to attain the liver parenchyma at an equal rate [18]. This is supported by the relative inefficiency of metastases in experimental models of dissemination, in which monoclonal cell lines are directly injected into the tail vein for lung seeding or heart for bone marrow and other sites; up to a million cells are introduced to obtain a few score of nodules. Still, if any of these steps are more efficient or preferential in the liver, the net number of established metastases would be increased.

The true rate-limiting event for the establishment of metastases is the ability of the tumor cells that reach the liver to first survive and then grow to establish a clinically relevant metastasis. While there is a structural advantage to the extravasation in the liver, namely the fenestrated sinusoidal endothelium and the "loose" underlying matrix, there also appears to be advantages to the "soil," the micro-environmental milieu that promotes tumor cell survival and subsequent proliferation. This will be discussed in the ensuing sections.

One caveat that needs mention, and will be highlighted in the next section, is that the cell behaviors and elements that promote escape and dissemination may be distinct from those that enable metastatic seeding and survival, and may even be detrimental to such [19]. These differences are often noted upon histopathology. Despite this visual evidence, the molecular basis have not been as well-studied as those denoting transcriptome and proteome profiles, which correlate with predilection to metastasize to the liver in human specimens. Even with some liver-preferential tumor lines developed, the analyses of early liver metastases are sparse. Still, what few studies that are available point to a different tumor phenotype in the liver metastasis than in the primary, from which the metastasis was derived. For instance, a set of genes highly correlated with metastatic ability in many primary carcinomas was found to be expressed at much lower levels in the paired liver nodules of gastric carcinomas [20]. Thus, the molecular basis of ability to disseminate or the correlations found in primary nodules of subsequent metastases, are not necessarily operant on the metastatic site.

Histology of Metastatic Carcinomas

Metastatic tumors usually present markers and architecture that betray their site of origin; tumors of unknown origin, while a diagnostic quandary with high fatality, represent only ~3% of diagnoses [21]. Still, the tissue assignment is often made based on prior knowledge of a primary neoplasia in situ. Furthermore, metastatic nodules are often only sampled to confirm it is a metastasis by needle biopsy or upon resection for palliation. In the former case, the nodule architecture is lost and in the latter, the metastatic nodule has now evolved extensively from the original mononuclear seeding. Thus, the histology of the developed metastasis may not reflect that of the original seeded micrometastasis.

Tumor dissemination is highly negatively correlated with differentiation status of the primary tumor. Thus, the vast majority of primary carcinomas contain undifferentiated clusters of tumor cells. However, it is now being more and more appreciated that many, though not all, or even most, metastases appear quite differentiated (Fig. 58.2). For instance, in many cases of breast carcinoma metastases to the liver, the breast carcinoma cells appear to recreate the hepatic cords rather than disrupt the parenchymal architecture [22]. Often, breast cancer metastases appear more differentiated from the primary tumor from which they presumably derived. This is not unique to breast carcinoma as it has been reported in liver metastases from prostate carcinomas [23] and colorectal carcinomas [24]; nor is it unique to the liver, but has been reported in metastases to the bone marrow [25]. However, as metastases are rare events, it is possible that these differentiated lesions arose from a well-differentiated cell that somehow escaped the primary mass during angiogenic invasion. That these well-differentiated metastases can derive from poorly or undifferentiated primary tumor cells has been supported experimentally by demonstrating phenotypic

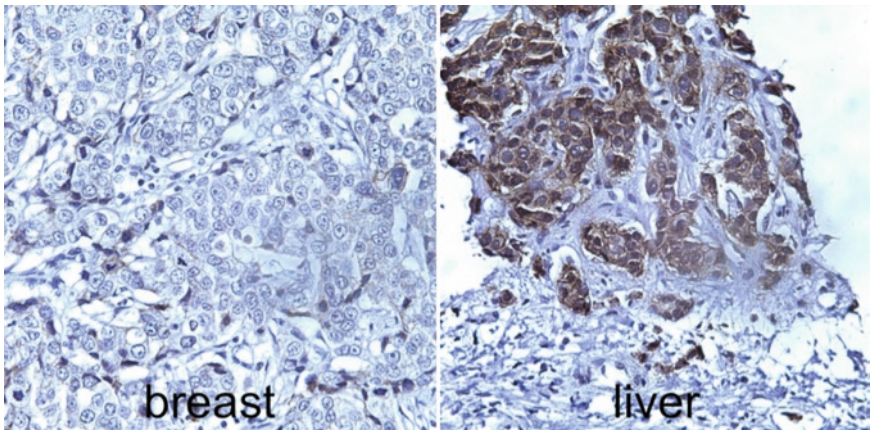

Fig. 58.2 Breast cancer metastasis to the liver demonstrating greater differentiation in acinus structure and E-cadherin staining (shown by IHC) than its cognate mammary gland primary

plasticity in breast cancer cells [19, 26, 27]. Thus, it appears that these early metastatic nidi coexist within the liver parenchyma rather than to wildly grow as it would be expected from their poorly-differentiated ancestry.

Interestingly, liver metastases tend not to demonstrate an inflammatory response. These nodules do not appear to elicit even the normal nonspecific inflammatory reaction that accompanies any foreign body that disrupts organ architecture. This absence of a reaction also argues for a coexistence within the existing liver structure.

Phenotypic Plasticity of Cancer Cells

The histological presentation of seemingly differentiated metastases in the liver argue either for metastases arising from dissemination of differentiated carcinoma cells or a plasticity of disseminating tumor cells. It is likely the latter as differentiated tumor cells do not form metastases in animal models and phenotypic dedifferentiation presents the strongest correlation with distant metastatic dissemination of carcinomas. This change in cell expression and proteome profile represents the carcinoma-associated EMT [6–8, 19, 28]. This carcinoma-related EMT is distinguished from true EMT that occurs during developmental processes in that the cancer cells do not fully attain the physiological role of stromal cells [4–7], but rather approach these cells in select behaviors. Such a partial phenotypic transition is not unique to cancer cells, occurring during normal wound repair processes; importantly, during repair, the EMT is reversed upon healing [29]. This suggests that carcinoma-related EMT may be similarly reverted.

The main signals that drive EMT and even dissemination are regulated via epigenetic mechanisms and through mutations [30]; this is in distinction to the genetic mutational events that are critical for carcinogenesis. The hallmark of EMT, regardless of the myriad of initiating signals [31–33], is downregulation of cell–cell adhesion structures based on E-cadherin [34]. This downregulation almost always occurs via epigenetic processes and not the irreversible and stochastic mechanisms of gene mutation or deletion [35]. Autocrine signals via receptors with intrinsic tyrosine kinase activities drive this loss of E-cadherin-based structures [19, 36]. Practically ubiquitous among disseminated carcinomas [37], autocrine signaling through the EGF receptor family and c-Met, direct adhesion disassembly secondary to catenin phosphorylation with subsequent E-cadherin degradation [23, 38–40]. Transcription repressors also conspire to suppress *de novo* transcription [31, 32, 41]. Lastly, promoter methylation maintains this EMT state [42, 43]. While the loss of E-cadherin is the common hallmark, different tumor types and even individual tumors, demonstrate a varied profile of suppressive strategies, with prostate carcinomas less likely to demonstrate extensive promoter hypermethylation than breast cancer cells [44, 45]. Regardless of the manner of E-cadherin downregulation, these mechanisms are reversible, even the promoter methylation appears to be dynamic rather than static [42]. This situation allows for adaptation to the host environment, as will be discussed.

Tumor Microenvironment

It has become increasingly evident that a tumor is actually an organ in which the cancer cells not only reside within a mix of non-neoplastic cells, stromal cells, and matrix, but are intimately influenced by the external milieu [46–48]. This includes traditional soluble signals and also cell- and matrix-associated interactions; even chemo-physical aspects of the environment impact the neoplastic cells [49]. While this has

been most studied in the original site of neoplastic development and dissemination, the same likely holds for the metastatic target organ. This is the crux of the "seed and soil" hypothesis in which the target organ, in this case the liver, provides key signals to allow for tumor cell survival, persistence and, eventually, proliferation [1].

The liver provides not only a unique architecture that likely enables more efficient arrest and extravasation of tumor cells, but also presents a rich signaling environment. The sinusoidal endothelial cells and loose but complex and bioactive base membrane provide the initial signals, but even once these are transmigrated, the signaling is particularly fertile. First, in addition to the usual stromal and parenchymal cells, the liver has a high density of resident monocyte-derived cells, Kupffer cells. Monocytes and their derivatives produce a large variety of soluble and matrix factors trophic for carcinoma cells [12, 50]. The liver is particularly rich in the CXCL12/SDF ligand for the CXCR4 chemokine receptor that mediates cell homing and is expressed on both carcinomas and melanomas [51]. Secondly, the parenchymal hepatocytes have an array of integrins, including the alpha-9beta1 tenascin-C receptor that bespeaks of a complex matrix composition that approaches that noted during development. This may underlie the high regenerative potential of the liver. Thus, upon liver injury, the response aims to support epithelial cell growth and survival with an outpouring of TGFalpha and active HGF along with production of tenascin-C. Thus, when a tumor cell disrupts the normal architecture, the normal wound response leads to a rich soil in which a wide variety of growth factors, pro-survival cytokines, and pro-survival matrix components are available as a fertile soil for the carcinoma seed.

Many micro-metastases and small nodules in the liver appear not as separate mini organs, but as integrated within the liver parenchyma. This "hepatization" of the tumor would support survival and escape from the nonspecific pro-death inflammatory response that any foreign body elicits. This inflammatory response would occur just from extravasation as infiltration of cancer cells into the liver often creates a pro-inflammatory response (from the unique environment of Kupffer, NK cells, and other immune cells that reside in the liver) that results in the upregulation of adhesion molecules such as selectins and CAMs on sinusoidal endothelial cells. A conundrum arises in that such integration would require E-cadherin-based adhesion to the parenchymal hepatocytes, and it is E-cadherin that is downregulated as part of the EMT-based dissemination. E-cadherin loss is epigenetic and even the promoter hypermethylation is unstable and reversible thus being potentially reversible [42, 52]. We found that coculturing of highly aggressive, E-cadherin-negative prostate and breast cancer cells with normal hepatocytes results in re-expression of E-cadherin with loss of promoter hypermethylation [23, 52]. Thus, these cadherins can form receptor homotypic, cell heterotypic interactions within the hepatocyte cords. These types of cell–cell close contacts have been noted in histological examination of metastases and upon transmission electron microscopy in bioreactors (Fig. 58.3).

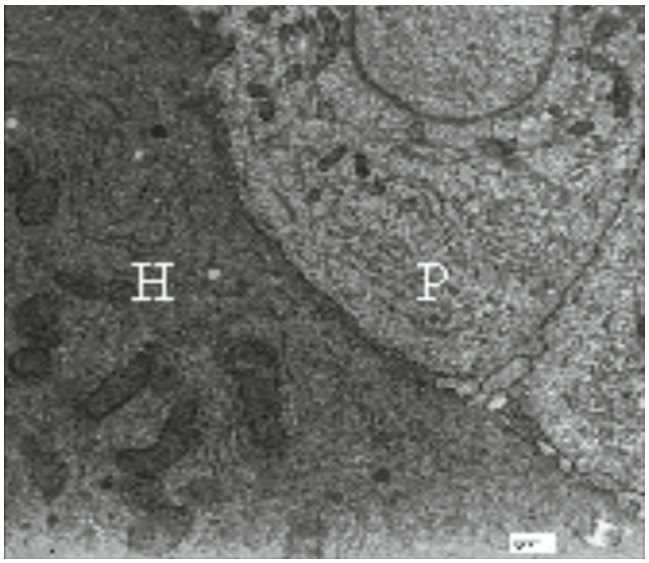

Fig. 58.3 Intercalated connections between prostate carcinoma cells (P) and parenchymal hepatocytes (H) in an organotypic model of metastasis to the liver as noted by transmission electron microscopy. Scale bar is 1 μm. From Yates et al. [52]. Used with permission

The above would speak toward carcinoma metastases, but the issue of non-epithelial metastases remains. Chief among the mesenchymal tumors that metastasize to the liver are melanomas. It may be merely coincidental or truly mechanistic that melanocytes normally reside singularly among keratinocytes, with which they form cell–cell communications. The integration of cancer cells with hepatocytes that may allow for survival does not necessarily have to be cadherin-mediated, as other adhesion or cell communication molecules such as connexins, claudins, etc. may also be involved.

Tumor Dormancy

One of the most vexing issues in tumor metastasis is the dormancy of these cells; in many cases dissemination only becomes evident a decade or more after the initial primary lesion is removed. This is coupled with the situation in which these micrometastases are not eliminated by adjuvant chemotherapy; as one example adjuvant chemotherapy reduces recurrence by only one third in small, node-negative breast carcinomas [53, 54]. This suggests that the micro-environment provides a survival advantage to these cells even in the face of chemotherapies that kill the primary tumor cells [46, 47]. It is likely that this drug resistance is part and parcel of

the ability of these micrometastatic cells to lie dormant and persist as singular cells for extended periods [55]. This non- or low-proliferative state would render cells generally drug resistant as clinical chemotherapeutic agents and regimens generally target cycling cells. In a manner of speaking, these cells would classify as a type of "cancer stem cell"; interestingly, stem cells are also generally resistant/less susceptible to chemotherapeutic killing.

The reasons for this dual tumor-cell quiescence/dormancy and drug resistance are not known. However, it is tempting to speculate that integration within the liver parenchyma would provide such signals. E-cadherin engagement sequesters the catenins to the membrane and out of their proliferative nuclear locale, while providing a low level, but tonic activation of survival signaling via Akt/PKB [56–58]. The importance of down-regulating the mitogenic signaling of nuclear catenins is supported by the absence of gamma-catenin in liver metastases of an experimental breast cancer model that preferentially seeds the liver [59]. Thus, the two aspects of cellular quiescence and survival signaling provide for long-term persistence of the carcinoma cells, and escape from adjuvant chemotherapy.

Key to cellular quiescence, and thus dormancy, is the suppression of the ubiquitous carcinoma-associated autocrine growth factor signaling. A significant part of carcinoma EMT is due to autocrine signaling via EGFR and c-MET receptors with intrinsic tyrosine kinase [60, 61]. However, co-incident with micrometastasis redifferentiation via a mesenchymal-to-epithelial reverting transition (MErT) [19] is suppression of EGFR autocrine activation [23, 24, 38]. The signals that drive this signaling suppression are ill-defined, but may actuate the negative transattenuation via PKC phosphorylation of EGFR. This finding has implications for bio-therapeutics that target receptors with intrinsic tyrosine kinase activities, in that inhibition of such signaling may limit EMT and initial escape, but at the same time perversely drive MErT in the liver metastatic niche and promote carcinoma seeding and survival.

Investigative Models of Liver Metastases

Exploration of the molecular cell biology of liver metastasis needs to be explored in organotypic models. Most studies utilize various animal models, overwhelmingly of rodents [62]. Immune compromised mice can host human xenografts of cell lines or tumor fragments. As few tumors metastasize from the subcutaneous site, other approaches are used to determine metastases. Orthotopic inoculations are often used for breast, prostate, and colorectal tumors, with a search for metastases after weeks to months of tumor growth. Unfortunately, in rodents, liver metastases appear less frequently than in humans. To increase the propensity to seed the liver, individual cells are introduced into the portal circulation area often via intrasplenic or cecal inoculation. In the study of just the seeding steps, the dissemination event is often bypassed with singular cells directly injected into the tail vein (with mainly lung seeding) or the heart. These models take advantage of being able to manipulate the tumor cells or challenge transgenic animals to probe the role of specific molecules. Additionally, the human/rodent difference allows for species-specific probing of tumor and liver microenvironment, thus enabling ease of discerning the origin of signal and matrix components. However, there are two major limitations, while these approaches do generate liver metastases with some frequency, the examination of the liver metastases represent snapshots in time similar to human biopsies; thus the natural course of metastases and the early events of seeding and establishment are difficult to capture. Second, the use of immunocompromised hosts limits the exploration of immunotherapies or deciphering the role of immune function cells in tumor metastasis.

Rodent tumors have been used to address the issue of immuno-modulation of tumor dissemination. One approach involves examining genetically-promoted "spontaneous" tumors. While a number of these do metastasize, the frequency of liver metastases are lower than in human situations and the underlying engineered molecular mutational event leaves open the question of relevance to the human situation. A second approach has been to utilize the small number of rodent lines derived from truly spontaneous tumors, such as B16 melanoma or MTLn3 mammary carcinoma cells. Introduction of these into syngeneic hosts has highlighted a surprising role for tumor-associated macrophages in promoting dissemination via a bidirectional paracrine signaling loop [9]. However, again, the examination of liver metastases presents a snapshot in time.

Intravital microscopy has enabled metastatic events to be examined in real-time. Rats have been maintained on a microscopic stage for hours so as to discern tumor cell escape from breast tumors [63] and initial seeding of the liver [64]. It is these studies that provided definitive documentation for the role of EGF-induced motility in tumor escape and the rate-limiting nature of cell survival on liver metastases, respectively. These approaches are excellent for capturing events on the timescale of minutes to a few hours, but are laborious, low-throughput, and limited in scope.

New approaches are needed to capture the metastatic seeding events over the span of days to weeks while this establishment occurs. Recently, the field of bioengineering and organotypic bioreactor development has been applied to the study of cancer. This is a partial perversion of a field that has focused on regeneration of organs [65, 66]. Still, the goal is to understand the cancer behavior so as to define targets for intervention. These bioreactors allow for many days to weeks of observing tumor cell behavior in an ex vivo organ context. In a few of these studies, the target has been liver metastases.

A simplified liver chip was used to determine that many of the cells reaching the sinusoids of a liver chip die secondary to cytotoxic ischemia/reperfusion injury, but that high IL-10 production can save these colorectal carcinoma cells [67]. A more elegant and longer-lived approach uses an engineered bioreactor that yields a functional liver lobule that is stable for over a month [68, 69]. Introduction of tumor cells into this liver has demonstrated phenotypic reversion to a more differentiated state and close cell–cell contacts between the tumor cells and the hepatocytes [23, 52]. The use of such models is still in its infancy, with the potential to follow cell fates and microenvironmental adaptations over many weeks.

Conclusions

The liver is the most common target organ for metastatic seeding after regional lymph nodes. However, liver metastases are more strongly correlated with poor outcome and impending demise. These metastases may occur early in the natural course of the tumor prior to clinical discovery of the tumor [70]; targeting treatments to prevent escape of tumor cells from the primary site may be too late to affect outcomes. Thus, we need to understand the molecular, cell, and organ biology of this critical metastatic niche. Such studies are in their infancy, but the findings that have arisen, speak of a critical communication between the tumor cell and liver parenchyma that alters the tumor cell behavior such that the cancer cell can survive in its new ectopic home. These suggest that the targeting of undetected micrometastases may be feasible, but will require agents that act upon novel targets, often different from those that promote initial escape.

Acknowledgments The authors thank Dr. Eric Lagasse (University of Pittsburgh) and other members of Wells (University of Pittsburgh) and Griffith (MIT) laboratory for discussions and feedback. Studies surrounding this topic have been supported by a VA Merit Award and DoD Predoctoral Fellowships from the CDMRP on Breast Cancer. The human specimen shown in Fig. 58.2 was obtained under Exemption 4e (de-identified excess pathological specimen) as determined by the University of Pittsburgh IRB, from the Cooperative Human Tissue Network. The authors have no conflicts of interest to disclose.

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Chapter 59
Cholangiocarcinoma

Gianfranco D. Alpini, Heather L. Francis, Marco Marzioni, Domenico Alvaro, Eugenio Gaudio, Ivano Lorenzini, Antonio Benedetti, and Giammarco Fava

Introduction

Cholangiocarcinoma (CC) is a rare and aggressive malignant neoplasm of the epithelial cells (cholangiocytes) lining the bile ducts. Histologically, more than 90% of CCs are adenocarcinomas [1, 2]. They can arise at any level of the intrahepatic or extrahepatic biliary tree and are anatomically classified as intrahepatic or extrahepatic [1, 2]. Fifty percent of CC develops in the liver hilum, 42% within extrahepatic biliary ducts and only 8% originate in the intrahepatic biliary ducts. Epidemiological data show that the incidence, prevalence and mortality of intrahepatic CC (ICC) are increasing worldwide [3]. The poor outcome of such disease is due to the lack of tools for early diagnosis and treatment. Indeed, the only curative treatment is surgery, but this therapeutic option is not often possible because the patient typically presents with an unresectable disease at the time of diagnosis since there is a lack of specific symptoms coupled with high invasiveness and easy involvement of critical anatomical structures [1, 2]. CC is characterized by a high rate of recurrence after surgery and is generally unresponsive or weakly responsive to chemotherapy or radiotherapy, which thus often have a palliative role. Recent therapeutic options include brachytherapy and photodynamic therapy (PDT), but their role efficacy is not well established as yet [1, 2].

Consistent with other major malignant tumors, CC develops as a multistep process, with the accumulation of genetic and epigenetic alterations in regulatory genes, leading to the activation of oncogenes and the dysregulation of tumor suppressor genes (TSG) [4–7]. A multitude of mutated genes and pathways have been described in malignant cholangiocytes.

The principal characteristics of malignant cholangiocytes are: (1) dysregulated growth, (2) high capacity of tissue invasiveness; and (3) capacity to metastasize [4, 7]. In this chapter, we have summarized what is known about the pathways and mechanisms implicated in every passage of the multi-step process of cholangiocarcinogenesis.

The Process of Cholangiocarcinogenesis

The mechanism leading to the malignant transformation of cholangiocytes, named cholangiocarcinogenesis, can be depicted as a multistep process (Fig. 59.1). Even if a small number of CCs arise in normal liver, it is largely described that CCs generally develop in a background of chronic inflammation of bile ducts and the consequent cholangiocyte injury is associated with the obstruction of bile secretion [1, 4]. Liver fluke infestation by *Opistorchis viverrini* and *Clonorchis sinensis*, primary sclerosing cholangitis (PSC), hepatolithiasis, Caroli's disease, congenital choledochal cysts, and anomalous pancreaticobiliary-junction malformations are well-known risk factors for CC [1]. Other factors predisposing CC development are: age greater than 65 years, bile duct adenoma, papillomatosis, liver cirrhosis, smoking, diabetes mellitus, thorotrast, dioxin and vinyl chloride intoxication, human immunodeficiency virus (HIV), hepatitis B (HBV), and hepatitis C (HCV) infections [8]. However, these specific conditions are often undetectable in patients affected by this neoplasia. In particular, independent of the existence of risk factors, malignant transformation of cholangiocytes arises in a milieu of chronic inflammation of the biliary tree [4, 7, 9]. The network of cytokines and molecules secreted in high concentration in the course of chronic inflammatory processes triggers and maintains the multistep process of cholangiocarcinogenesis [1, 4, 7] (Fig. 59.1). The mechanism of carcinogenesis is a stepwise process characterized by a progressive accumulation of chromosomal, genetic, and epigenetic alterations [5, 10, 11] (Fig. 59.1). The final result is a sustained overproduction of cytokines, stimulatory or inhibitory growth factors, and hormones that interact with the malignant cholangiocytes leading to irreversible changes in cell physiology (Fig. 59.1).

G. Fava (✉)
Division of Gastroenterology Azienda Ospedaliero-Universitaria "Ospedali Riuniti", Ancona, Italy
e-mail: favag@hotmail.it

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_59, © Springer Science+Business Media, LLC 2011

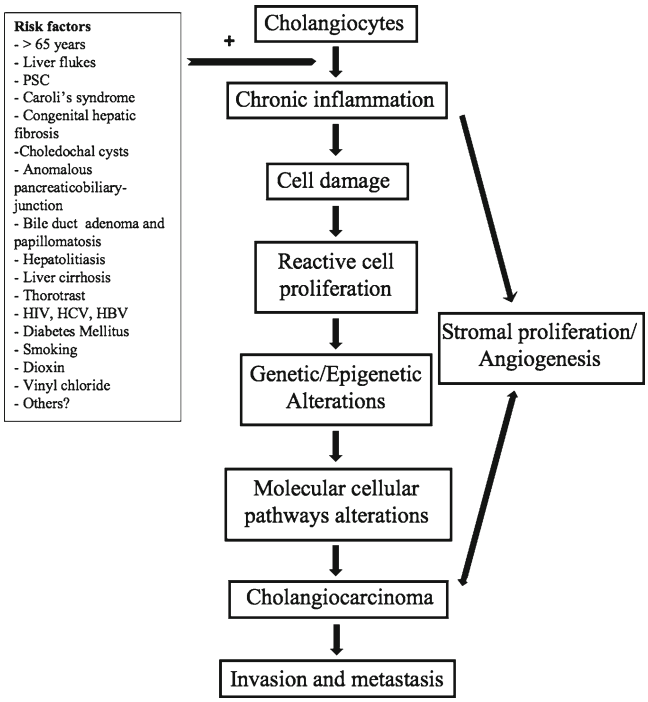

Fig. 59.1 The multistep process of cholangiocarcinogenesis. Proposed mechanisms leading to malignant transformation of cholangiocytes. Adapted from Sandhu et al. [5], with permission

Genetic Alterations

Genetic alterations induce specific changes in cell physiology such as [12]:

- Stimulation of growth induced by autocrine signals
- Insensitivity to growth inhibitory mechanisms
- Escape from cell apoptosis
- Dysregulated mechanisms of replication
- Neo-angiogenesis, tissue invasiveness, and metastasis

The final result of these altered processes is uncontrolled cell growth.

In this chapter we describe the principal genes altered in cholangiocytes during the development of CC. K-ras, p53, p14ARF, p16INK4a, and β-catenin are specific genes implicated in the development of many cancer types [5].

K-ras and p53

CC cells often present point mutations of K-ras and p53 oncogenes [5, 13–15]. Such mutations have been found in codon 12 that consists of changes from glycine (GGT) to aspartic acid (GAT) or less frequently to valine (TGT). Furthermore, mutations have been found less frequently at codon 13, involving GGT to GAT; and codon 61, involving CAA to CAC [16]. In addition, a recent study indicates that the expression of K-ras depends on the location of the neoplastic lesion in the liver [16]. Indeed, K-ras gene alterations are present in the periductal-spreading type tumor, but absent in the mass-forming type tumor, suggesting that such a mutation is important in CCs arising in the proximity of the hepatic hilus [16]. Moreover, K-ras mutations are expressed variably from 0–56% of ICC and from 0–100% of extrahepatic CC (ECC) cells [5]. The explanation for these differences in K-ras mutations could be due to the existence of different subtypes of cancers, racial and geographical differences of the study population, and use of different assay techniques [5].

p53 is a TSG with two important functions: the induction of cell cycle arrest and suppression of Bcl-2 protein expression with consequent blockage of apoptosis. Similarly to K-ras, the incidence of p53 gene mutation is variable, ranging from 20–80% [5]. This mutation appears more frequent in the mass-forming type tumors. This means that p53 mutation could be related to the development of ICCs of the peripheral small bile ducts [5].

p14ARF and p16INK4a are cell-cycle regulator genes implicated in the genesis of CCs. However, their mutation or deletion is not frequent in CCs [17, 18].

NKG2D

Natural killer (NK) cells play a critical role in tumor surveillance by cell-mediated cytotoxicity [19]. The NK group 2, member D cell receptor, also known as NKG2D, is expressed by NK cells and T-lymphocytes and is involved in their cytotoxic activity towards target cells [20]. In particular, some studies suggest that high levels of cytotoxicity protect PSC patients from the development of CC in a background of chronic inflammation of the biliary tract. E Melum et al. recently evaluated genetic polymorphisms of the NKG2D gene to address their influence on the risk of developing CCs in patients with PSC. They showed that two single nucleotide polymorphisms (SNPs) of the gene were associated with an increased risk of CC in PSC-affected patients [21]. In addition, homozygous condition for the non-risk alleles has an extremely low risk of CCs [21]. This finding could be helpful in identifying PSC patients with a low risk of CC. Development of cancer is a complex biological process, and other yet unknown polymorphisms are likely associated with CC risk. Combining *NKG2D* SNPs with other polymorphisms in a panel of markers may increase the specificity of a test for CC risk [22].

Activation-Induced Cytidine Deaminase

Activation-induced cytidine deaminase (AID) is a member of the DNA/RNA-editing cytidine deaminase, apolipoprotein B mRNA-editing enzyme catalytic-polypeptide (APOBEC) family. Recently, it was shown that AID production was significantly induced in cholangiocytes by proinflammatory cytokines, abundant in clinical conditions such as PSC and CC [23]. Specifically, the DNA mutator AID represents a target gene of the IKK-β–dependent NF-κB activation pathway [23] and it is aberrantly expressed during chronic inflammation [23]. Consequently, aberrant expression of AID in biliary cells resulted in the generation of somatic mutations in tumor-related genes, including *p53, c-myc,* and the promoter region of the *INK4A/p16* sequences. Komori et al. [23] speculated that proinflammatory cytokine stimulation is responsible for the aberrant *AID* gene expression in human cholangiocytes, thus providing a possible link between chronic biliary inflammation and the development of CC. However, further studies are necessary to clarify the significance and the role of AID production on leading precancerous cells to acquire a critical number of genetic changes.

Cellular Senescence

Cellular senescence is a physiologic process accompanied by growth arrest due to telomere shortening. Malignant cells, on the contrary, highly express the enzyme telomerase [4], which blocks telomere shortening, thus maintaining chromosomal length. This permits to preserve cell replication activity. The expression of human telomerase was homogeneously detected in ICC cells, whereas its expression was heterogeneous in the dysplastic biliary lesions [4, 6, 7]. Furthermore, this enzyme was not detected in nondysplastic biliary epithelia in hepatolithiasis and in normal livers, thus suggesting that malignant cholangiocytes acquire telomerase activities at the dysplastic condition thus triggering the process leading to malignant transformation [4, 6, 7]. Recent studies have shown that interleukin-6 (IL-6) is able to increase telomerase activity [4, 6, 7].

Epigenetic Dysregulation

Epigenetics refers to heritable changes in gene expression that occur without alteration in DNA sequence. Two primary and interconnected epigenetic mechanisms are known in CC: DNA methylation and covalent modification of histones [5, 6].

DNA Methylation

DNA methylation is a reversible chemical modification that affects cell function by altering gene expression and refers to the covalent addition of a methyl group, catalyzed by DNA methyltransferase (DNMT), to the 5-carbon of cytosine in a CpG dinucleotide [5, 6]. DNA methylation does not change the genetic information; it just alters the readability of the DNA and results in the inactivation of genes by subsequent transcript repression [6].

Epigenetic alterations in several candidate tumor-related genes have been evaluated in CCs. The functions of genes found to be methylated in CCs are shown and their location with reported CpG island methylation frequencies is reported in Table 59.1 [18, 24–37]. A description of the principal genes methylated in CC will be briefly provided.

p16INK4A

p16INK4A, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), is a TSG located at human chromosome 9p21 [5]. This gene regulates cell proliferation and the oncogenesis process. The specific function of p16INK4A is to codify for a protein binding to cyclin-dependent kinases 4 and 6 and inhibiting their interaction with cyclin D1 [38]. As well as for mutations of K-ras and p53 genes, different studies found a variable methylation frequency of p16INK4A in CCs, ranging from 17.7–83% [24, 29, 30, 33, 36, 39]. Interestingly, p16 gene promoter region hypermethylation is associated with a poor prognosis [29]. A recent study compared ICC and ECC and found a 77% methylation frequency of the p16INK4A gene in a high number of ECC [34]. In this group, promoter hypermethylation of the p16INK4A gene was more commonly noted in tumors with vascular invasion [34]. Moreover, Ahrendt et al. [18] demonstrated that the inactivation of the p16 TSG secondary to promoter hypermethylation is commonly observed in PSC-associated CC, and may play in the development of CC in such patients.

Ras Association Domain Family 1A

The Ras effector homologue gene (RASSF1A) is located at 3p21.3 and encodes a protein similar to the RAS effector proteins [5]. The tumor suppressor function of this gene is suggested from the fact that its loss or altered expression has been implicated in the pathogenesis of a variety of cancers [5]. RASSF1A interacts with the DNA repair protein XPA and its inactivation was found to be correlated with the

Table 59.1 Methylation frequency of candidate genes in different studies of cholangiocarcinoma

	Gene	Location	Function	[18]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]	[35]	[36]	[37]
1	FHIT	3p14.2	Purine metabolism (%)			42												
2	RASSF1A	3p21.3	Cell cycle arrest (%)		65	68	69							27				
3	hMLH1	3p21.3	DNA mismatch repair (%)		25			44.6	0				46	8.1				
4	14–3-3	1p36.11	Apoptosis (%)							59.5								
5	MINT1		Unknown (%)							40.5								
6	MINT2		Unknown (%)							0								
7	MINT12		Unknown (%)							50.6								
8	MINT25	22q11	Unknown (%)							15.4								
9	MINT31		Unknown (%)							1.3								
10	MINT32		Unknown (%)							35.4								
11	APC	5q21	Cell division and attachment (%)		46					26.6								
12	E-cadherin	16q22.1	Proliferation, invasion and metastasis (%)		43					21.5			41	27				
13	INK4a p16	9p21	Cell proliferation and oncogenesis (%)	25	50					17.7	83		70	24.3	77		53.5	
14	INK4b p15	9p21	Cell cycle arrest (%)		50													
15	TIMP3	22q12.1	Apoptosis (%)		9					8.9								
16	p14ARF	9p21	Cell cycle arrest (%)		38						25							
17	p73	1p36.3	Cell cycle regulation (%)		36													
18	MGMT	10q26	DNA mismatch repair (%)		33									49				
19	GSTP	1q43	Drug/xenobiotic metabolism (%)		14													
20	RAR-b	3p24	Cell growth and differentiation (%)		14													
21	DAPK	9q34.1	Apoptosis (%)		3								32	21.4				
22	TMS1/ASC	16p11.2	Apoptosis (%)									36.1						
23	COX-2	1q25.2	Prostaglandin metabolism (%)							5.1								
24	RUNX3	1p36	Apoptosis (%)											56.8				
25	CHFR	12q24.33	Apoptosis (%)											16.2				
26	BLU	3p21.3	Unknown (%)													20		
27	Sema-3b	3p21.3	Apoptosis (%)													100		
28	SOCS-3	17q25.3	Cytokine signaling suppression (%)															88
29	THBS1	15q15	Cell to cell interactions (%)							11.4						11		

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hypermethylation of its CpG island promoter region. RASSF1A also inhibits the accumulation of cyclin D1, thus leading to cell cycle arrest [5]. RASSF1A promoter methylation has been reported to occur in 27–69% of CCs [24–26, 33, 35]. However, methylation of the RASSF1A promoter was more common in ECC than ICC [24].

Human mutL Homologue 1

Human mutL homologue 1 (hMLH1) is a DNA mismatch repair gene located at 3p21.3. Mismatch repair represents an important mechanism used by cells to correct errors occurring in DNA during cell growth to maintain the integrity of the genome [40]. Genetic and epigenetic alterations of hMLH1 have been described in several tumor types [41, 42] as well as CCs [5].

In sporadic CC, a variable frequency of hMLH1 gene promoter methylation is described [33] and in a series of liver fluke-related CCs, hypermethylation of the hMLH1 promoter was described in about half of the cases [27]. In addition, an association between hypermethylation of hMLH1 and poorly differentiated subtype of CC with vascular invasion was described [5].

Other Hyper-Methylated Genes

A number of methylated genes were described in CC [5]. Table 59.1 reports the functions and the location of these methylated genes, accompanied by CpG island methylation frequencies and their corresponding references [24–37].

Histone Modifications

Histones are proteins with basic properties that complex with genomic DNA to form nucleosomes, the basic units of the compacted structure of chromatin [5]. Acetylation, methylation, and phosphorylation are the mechanisms by which histones are post-translationally modified. Among them, acetylation is more important. Histones are acetylated on lysine residues by histone acetyltransferases (HATs). On the contrary, acetylated histones are deacetylated by histone deacetylase (HDACs). The removal of acetyl groups from lysine residues on the amino termini of histones by HDAC leads to chromatin condensation and transcriptional inactivation [43, 44]. This process can lead to inhibition of TSG expression and consequently favor the mechanism of tumorigenesis. Conversely, HDAC inhibitors enhance chromatin decondensation and TSG expression, thus blocking cholangiocarcinoma growth. The synergistic effect of HDAC together with DNA methylation inhibitors could be a valid option to treat biliary malignancies [45, 46]. However, further studies on the role of hypermethylation of genes and histone modifications are warranted and needed.

Molecular Cellular Pathways

As a consequence of the activation of cellular oncogenes or the inactivation of TSGs, deregulation of several molecular mechanisms have been described in CCs such as ErbB-2, MUC-1, Met, β-catenin, IL-6, transforming growth factor-β (TGF-β), STAT-3, Bcl-2, DCP4/Smad4, hepatocyte growth factor (HGF), reduced glutathione (GSH), Notch-1, TRAIL, p16INK4a, Ras/Raf, and WISP1v [4, 7].

Initiation of the Malignant Process

The network of cytokines released in the biliary microenvironment in the course of inflammatory processes is responsible for the induction of malignant transformation of cholangiocytes. The trigger of the cholangiocarcinogenesis process is the activation of autonomous proliferative signaling in the biliary epithelium [4, 7]. Cytokines are abundant in the course of chronic inflammation and are secreted in the liver by a multitude of cell types such as hepatocytes, hepatic stellate cells, sinusoidal endothelial, and Kupffer cells. In addition, recent studies demonstrated that cholangiocytes themselves produce and release cytokines [8] such as IL-6, TGF-β, IL-8, tumor necrosis factor alpha (TNF-α), and platelet-derived growth factor (PDGF) B chain [9], which interact with biliary epithelium in an autocrine/paracrine manner, thus regulating biliary-cell pathophysiology. Several works demonstrated that these cytokines play a fundamental role in the development and growth of biliary-tract cancers [10]. This effect is favored by the fact that cholangiocyte intracellular signaling in response to specific cytokine stimuli is altered during malignant processes [5]. The constitutive activation of cellular receptors and the mitogenic factors abundant in proximity of the biliary epithelium stimulates the uncontrolled growth of malignant cholangiocytes [1, 5].

IL-6 and Mcl-1

IL-6 is a cytokine secreted in the liver by several cell types, including cholangiocytes, in the course of inflammatory pro-

cesses [47]. Moreover, biliary tract inflammation is associated with increased concentration of IL-6 in bile and serum [48] (Fig. 59.2). Such a cytokine has multiple functions. In particular, it mediates the immune responses and stimulates the growth of normal and tumoral cells, and several data demonstrate a fundamental role of IL-6 in the pathogenesis and growth of CC [47] (Fig. 59.2). The mitogenic effect of IL-6 is also suggested from the fact that the concentration of this molecule is increased during chronic inflammation of the biliary tract, a condition predisposing cholangiocarcinoma development. IL-6 acts both in autocrine and paracrine manner activating several intracellular pathways involved in survival and growth of malignant cholangiocytes [49]. Among them, p44/p42 and p38 mitogen activated protein kinases (MAPKs) have been largely studied [50]. Tadlok et al. [50] showed that activation of p38 MAPK by IL-6 decreases expression of p21(WAF1/CIP1), a cell cycle controller protein, and mediates growth independent of anchorage signals, whereas mitogen activation of p44/p42 MAPK mediates an anchorage signal-dependent growth pathway (Fig. 59.2).

IL-6 also influences the apoptotic process of malignant cholangiocytes. Several studies showed that IL-6 upregulates Mcl-1 or myeloid cell leukemia-1, a potent key antiapoptotic Bcl-2 family member protein. It has been recently shown that this effect of IL-6 is mediated by increased activation of STAT-3 (that is constitutively activated in malignant cholangiocytes), which regulates Mcl-1 transcription [51]. Moreover, Mcl-1 increases cancer-cell resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [52], and therefore, this molecule appears to have a fundamental role for CC development [37]. Inhibition of IL-6 induced expression of Mcl-1 restores sensitivity to TRAIL [53].

The activation of the JAK1/STAT-3 pathway [51] by IL-6 enhances SOCS-3 which, through recruiting Tyr759 of gp130, turns off IL-6 signaling by a negative feed-back to JAK1 (Fig. 59.2). The importance of this pathway in maintaining tumor growth is underlined by the fact that this inhibitory loop is inactivated in several human CC cell lines, and re-expression of SOCS-3 reduces STAT-3 activation with consequent inhibition of its target gene Mcl-1. This reduction in Mcl-1 sensitizes CC cells to TRAIL induced cytotoxicity [37]. In summary, SOCS-3 reexpression can sensitize CC cells to TRAIL cytotoxicity by inhibiting IL-6/STAT-3 pathways, with consequent downregulation of Mcl-1. These findings suggest that the upregulation of SOCS-3, for example, by demethylating agents, could have an important role in

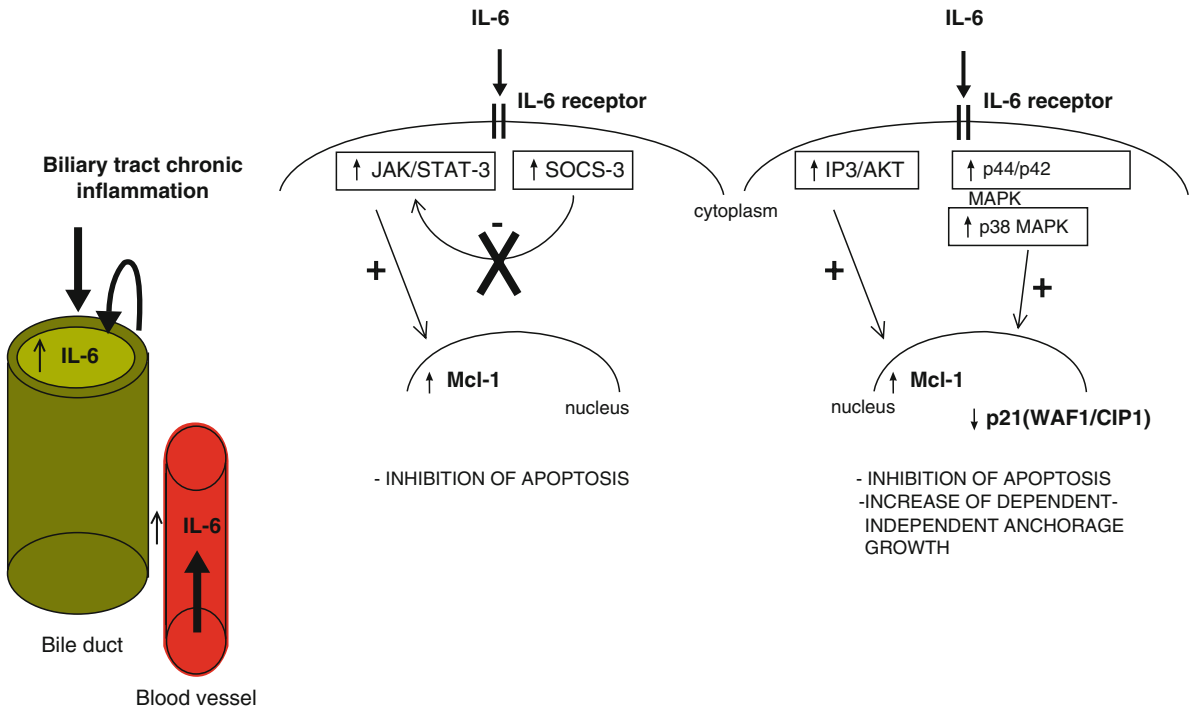

Fig. 59.2 Mechanisms of action of IL-6 on malignant cholangiocyte. Biliary tract chronic inflammation, a condition predisposing to cholangiocarcinoma, is associated with increased concentration of IL-6 in bile and serum. IL-6, through an activation of STAT-3, upregulates Mcl-1 transcription, a potent key antiapoptotic Bcl-2 family member protein, which increases cancer cell resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). IL-6 also activates p38 and p44/p42 mitogen activated protein kinases (MAPK) in cholangiocarcinoma (CC) cells, thus stimulating their dependent and independent anchorage growth

inhibiting CC cell growth [37]. The condition of inflammation, and in particular IL-6 itself, has been associated with epigenetic silencing of genes through CpG island hypermethylation [54]. Therefore, it has been described that IL-6 is capable of maintaining promoter methylation, thus representing one of the possible links between inflammation and growth and survival of tumors [54].

Transforming Growth Factor-β

Transforming growth factor beta, or TGF-β, is a cytokine involved in the network of functions such as cell growth, differentiation, migration, apoptosis, adhesion, survival, and immunity. Several cell types including Kupffer, endothelial and stellate cells synthesize TGF-β [4], whereas cholangiocytes express such a cytokine during cholestasis, but not in normal conditions [55]. In general, TGF-β possesses inhibitory effects. For example, it reduces human CC cells proliferation through regulation of the p21 cyclin-dependent kinase inhibitor [56]. On the contrary, this antiproliferative effect in CC cells is eluded because of the mutations of its receptor and the alterations of intracellular signaling mediators (e.g., Smad4), together with the intracellular overexpression of cyclin D1 [57, 58]. In this scenario, malignant cholangiocytes elude the inhibitory effect of TGF-β signaling [59] cells. The lack of TGF-β signaling also stimulates the deposition of fibrotic tissue, abundantly expressed by the CC [1]. The dysregulation of TGF-β signaling leads to an increase of cell proliferation and formation of fibrosis, both hallmarks of highly aggressive cancers such as CC [59].

DCP4/Smad4

DCP4/Smad4 is a TSG and also a downstream of TGF-β cascade [60]. Alterations of TGF-β/Smad pathway are thought to play an important role in the regulation of CC growth [61]. Also, loss of Smad4 correlates with the pTNM stage of ICC [61]. Moreover, Smad4 interacts with the other tumor suppressor gene PTEN to maintain a physiologic cellular balance and block the process of cholangiocarcinogenesis [62]. This explains why the blockage of these TSGs favors the development of biliary malignancies [62].

c-Met/Hepatocyte Growth Factor

c-Met is a proto-oncogene located on chromosome 7q that codes for a tyrosine kinase growth factor receptor, highly expressed on the surface of CC cells [63, 64]. The ligand for Met is the hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF-Met pathways are implicated in embryonic development. However, abnormal Met signaling has been strongly implicated in the process of tumorigenesis, in particular in the growth of aggressive and metastatic cancers [65].

CC cells express high levels of HGF, both in vitro and in vivo, together with the upregulation and hyper-phosphorylation of *c-Met,* its specific surface receptor [66]. This data suggests the existence of an autocrine-loop that governs the growth of malignant cholangiocytes [66]. Increased expression of the *c-Met* gene in CC cells is associated with increased cell migration and invasion. On the contrary, inhibition of *c-Met* expression is followed by reduced cellular growth and invasiveness [66]. HGF binds to *c-Met* and induces the autophosphorylation of an intracellular tyrosine kinase on the β-subunit of the receptor [65]. This process is followed by the activation of a network of signaling molecules such as Src, P13K, Gab1, SOS, Grb2, and MEK1/2 [67], all involved in the regulation of cell invasion, angiogenesis and tumor differentiation/proliferation [68, 69].

ErbB-2

ErbB-2 is a molecule highly expressed in several CC cell lines [70, 71], which plays a substantial role in the development and progression of biliary malignancies [72]. In addition to the direct effect on CC cells, ErbB-2 stimulates the production of COX-2, which forms a complex with a subunit of the IL-6 receptor [73]. This effect suggests a close link between IL-6 and ErbB-2 signaling [1, 73].

ErbB-2 overexpression in malignant cholangiocytes maintains cell growth and survival. Recently, Lai and colleagues [74] showed that when normal cholangiocytes are transfected with the neu (the rat homologue of ErbB-2) oncogene, they undergo a malignant transformation that closely reproduces the molecular features of human CC.

Glutathione

Reduced GSH is the principal intracellular defense against oxidative stress during inflammation [75]. The capacity of GSH is to favor the reduced state of intracellular molecules and to participate in the detoxification of many molecules [75]. Several studies demonstrated that GSH is synthesized by cholangiocytes, which can also obtain it from bile after secretion by hepatocytes [7]. The reduction of intracellular GSH is associated with: (1) increased cholangiocyte apoptosis; (2) decreased Bcl-2 protein expression due to increased

Bcl-2 degradation, (3) without changes in Bax and Bcl-2 protein expression [75]. These data explain how GSH depletion predisposes cholangiocytes to apoptotic cell death.

COX-2

Cyclooxygenase (COX) is the enzyme involved in the biosynthesis of prostaglandins, which play a fundamental role in the course of inflammation. Two specific isoforms of COX are well-known: COX-1, that is constitutively expressed in many cell types and participates in the homeostatic functions of prostaglandins, and the inducible COX-2, which expression can be stimulated by a variety of stimuli, such as cytokines and lipopolysaccharides [76].

Several studies showed the fundamental role of COX-2, which is increased during the course of inflammation, in predisposing and maintaining CC growth [77]. In a murine model of biliary adenocarcinoma, developed upon overexpression of ErbB-2, increase of COX-2 was initially observed [78], and in rat CC cells overexpression of COX-2 enhanced cell growth [79]. In contrast, antisense depletion of COX-2 inhibited cell proliferation [79]. The pivotal role of COX-2 in enhancing CC proliferation suggests that the inhibition of COX-2 could represent a strategy to prevent CC development. In fact, recent studies showed that selective COX-2 inhibitors (e.g., celecoxib) inhibit CC-cell proliferation by inducing apoptotic process [70, 79–81]. These findings demonstrated that the inhibitory effect of celecoxib in CC cell growth was accompanied by an inhibition of PDK1 and PTEN, followed by a consequent decrease of Akt phosphorylation [82]. Furthermore, celecoxib treatment also inhibits CC cell proliferation through activation of cyclin-dependent kinase inhibitors p21waf1/cip1 and p27kip1, with consequent cell cycle arrest at G1/S phase [83]. However, not all COX-2 inhibitors were shown to have a benefit in inhibiting CC cell growth [79].

In the last years, important progresses have been made in defining the mechanisms that stimulate production of COX-2 during the course of inflammation. Oxisterols, derivatives from cholesterol, are present in bile of patients with inflammatory processes of the biliary duct system and activate COX-2 in CC cells in vitro [84]. Their possible role in biliary carcinogenesis was raised by the observation that they are present in bile during cholestasis, the other condition predisposing to CC development [85] and that they enhance COX-2 expression in human CC cells in vitro [84].

COX-2 expression was moderately low in cholangiocytes from livers affected by primary biliary cirrhosis (PBC), whereas cholangiocytes from livers affected by PSC showed a very high immunoreactivity [54]. In a recent review, Sirica et al. noted that such a discrepancy between PBC and PSC might account for different incidences of CC in these two conditions [9, 70].

Nitric Oxide

Another protein implicated in inflammatory processes and expressed in high concentration during the course of inflammations and malignancies of the biliary tract is inducible nitric oxide (NO) synthase (iNOS) [86]. Several studies indicated that this protein, similarly to COX-2, is involved in a variety of cellular events such as proliferation, survival, and angiogenesis [87]. Although iNOS and COX-2 may be induced independently [88], Ishimura et al. [86] recently showed that iNOS expression stimulates COX-2 expression through NO production. This suggests a strong link between these two fundamental proteins in the genesis and in the development of CC. In particular, in mouse cholangiocytes, iNOS enhances COX-2 expression likely through activation of p38 MAPK and JNK1/2 [86]. Inflammatory cytokines activate iNOS with consequent increase of intracellular NO, which exerts a double effect in the process of cholangiocarcinogenesis: one part allows accumulation of DNA mutations by inhibiting DNA repair [89] and the other part induces COX-2 expression [86, 90]. The malignant transformation of cholangiocytes is accompanied by an increase of iNOS [11] that stimulates NO expression, which counteracts the mechanisms of DNA repair, thus favoring its damage and mutagenesis [7]. Similar to what happens in pancreatic cancer, the effect of NO in promoting CC development is due to the upregulation of Notch-1 [57]. To strengthen this hypothesis, evidence shows that Notch-1 is hyperexpressed in cholangiocytes of patients affected by PSC as well as in CC cells and it co-localizes with iNOS [91]. Notch-1 is stimulated by NO and its expression can be inhibited after cell transfection with iNOS antisense constructs [91].

Bile Acids

Several studies demonstrate that bile acids accumulating during cholestasis regulate CC cell growth. In particular, deoxycholic acid activates the epidermal growth factor receptor (EGFR), which triggers survival and proliferation by activation of phosphoinositide 3-kinase (PI3-K) [92, 93]. The mechanism by which this occurs is complex and has been demonstrated through a series of studies. The involvement of TNF-α and metalloproteinase in such a mechanism is explained by the fact that bile acid-dependent activation of EGFR is blocked by TNF-α antiserum and by inhibitors of metalloproteinases [92]. In addition to the PI3-K signaling,

the bile acid-dependent activation of EGFR also enhances the expression of anti-apoptotic molecules of malignant cholangiocytes [92]. However, the actions of bile acids on CC development are even more complicated. For example, bile acid-induced EGFR activation also leads to the stimulation of the MAPK cascade with activation of ERK1/2, p38, and JNK proteins [84]. In addition, the activation of ERK1/2 and p38 is then responsible for COX-2 expression [84]. This aspect could suggest that bile acids may play a substantial role in CC development in a non-inflammatory background. Therefore, bile acids affect a multitude of intracellular events implicated in CC and members of the wide family of bile acids may exert different effects, even opposite, on malignant cholangiocytes. In fact, data from our laboratory has demonstrated that tauroursodeoxycholate inhibits human cholangiocarcinoma growth via Ca^{2+}, PKC-α, and the MAPK-dependent pathways [94].

Apoptosis and Cholangiocarcinoma

Apoptosis, or programmed cell death, represents an essential physiological process that plays a critical role in maintaining the homeostasis of the biliary epithelium [48]. This event is used by the organism to remove the cells that are not able to repair their damaged DNA [48]. A reduction or dysregulation of apoptotic processes leads to the survival of mutated cholangiocytes, which have the potential to go through a multistep process of mutations leading to malignant transformation. Increase in the expression of Bcl-2, mutation of K-ras, and/or dysregulation of p53 have been associated with inhibition of apoptosis [47].

Bcl-2 represents a superfamily of antiapoptotic proteins. Bcl-2, the prototype of this family [49], is overexpressed in CC cells, which possess an apoptotic threshold significantly increased compared to normal cholangiocytes [50], and exerts its antiapoptotic activity by reducing caspase 3 activation by preventing cytochrome c release from the mitochondria [50].

Several other factors contribute to the dysregulation of cholangiocyte apoptosis. In addition to being implicated in the induction of cholangiocarcinogenesis processes [7], NO also inhibits apoptosis of biliary epithelial cells. Indeed, Torok et al. demonstrated that the transfection of CC cells with nitric-oxide-synthase (NOS)-cDNA, leads to a resistance for etoposide-induced apoptosis, an event that occurs through the nitrosylation of caspase 9 [122].

Notch-1 and COX-2 attenuate TRAIL-mediated apoptosis [12, 53]. Recently, it has been described that high levels of COX-2 inhibit Fas-induced apoptosis in CC cells [53]. The relevant role of COX-2 in regulating CC growth was also confirmed by the fact that the selective COX-2 inhibitor celecoxib triggered cell death by apoptosis, inhibiting the PI3-kinase signaling [17]. However, COX-2 expression correlates with tumor differentiation and is increased in highly differentiated biliary cancers [29].

It has been demonstrated that COX-2 represents downstream regulations for iNOS-NO in the promotion of biliary carcinogenesis [55]. In fact, in immortalized murine cholangiocytes, iNOS inhibition markedly diminished COX-2 mRNA and protein expression [55]. This event was reversed by the introduction of NO donors [55]. These data acquire a major relevance since high immunoreactivity of both iNOS and COX-2 has been demonstrated in bile ducts of patients affected by PSC [54, 55].

There is growing interest on the cytokine TRAIL [56]. The activation of such a ligand selectively stimulates apoptosis only in malignant cells, having no toxicity in normal tissues [56]. Therefore, TRAIL could be a target to establish a novel therapeutic strategy for the management of biliary tumors [56]. High levels of Mcl-1 in CC cells lead to a resistance to TRAIL-induced apoptosis [56]. When Mcl-1, but not Bcl-2, expression is blocked by specific small-interfering mRNA or stable transfection with Mcl-1-small-hairpin-RNA, CC cells become sensitive to TRAIL-induced apoptosis [56]. The expression of Mcl-1 is also regulated by bile acids accumulating in the course of biliary obstruction and cholestasis. Deoxycholic acid, for example, markedly increases the cellular Mcl-1 by blocking protein degradation via activation of an EGFR/Raf-1 cascade. Furthermore, Raf-1 inhibitors block the increase of Mcl-1, rendering the cells much more sensitive to Fas-induced apoptosis [25, 56].

Invasion and Progression of Cholangiocarcinoma

CC cells enhance the development of a rich vascular structure, which sustains the metabolic needs and ensures an adequate support of oxygen and nutrients to the malignant cells [1]. Tumor vascularization is enhanced by high levels of vascular endothelial growth factor (VEGF) [1, 5], which is stimulated by β-catenin [66] and TGF-β, and which is expressed by the surrounding mesenchymal cells and by the malignant cholangiocytes themselves. This suggests an autocrine/paracrine mechanism which stimulates the production of VEGF necessary for tumor development and growth [67].

The rapid invasiveness and tendency to metastasize are two important features of CC cells. Terada et al. showed that CC expressed a large amount of matrix metalloproteinases (MMP) and a correlation between high levels of MMP and increase in clinical invasiveness was reported [63]. Human aspartyl (asparaginyl) β-hydroxylase (HAAH) and proteins

related to the connective tissue growth factor family are also highly expressed in CC cells [69]. Some reports indicate that their concentration is proportional to the increased motility and invasiveness of malignant cholangiocyte [69].

"Cell adhesion molecules" (CAMs) indicate a network of molecules that play a critical role in sustaining cancer invasion and metastasis [70]. Indeed, membranous expression of E-cadherin, α-catenin, and β-catenin is reduced in a majority of biliary tract cancers and this downregulation correlates with a high grade in tumors. On the contrary, it doesn't correlate with vascular invasion, metastasis, and p53 expression [70].

WISP1v is a member of the connective tissue growth factor family, a group of proteins involved in progression and invasion of human tumors [73]. It has been described that the expression of WISP1v was significantly associated with lymphatic and perineural invasion of tumor cells, as well as a poor clinical prognosis [74]. Furthermore, WISP1v stimulated the invasive phenotype of CC cells with activation of both p38 and p42/p44 MAPKs [74].

Epithelial-to-Mesenchymal Transition of Cholangiocarcinoma Cells

CC cells undergo sarcomatoid change, termed epithelial-to-mesenchymal transition (EMT) [95], which favors a more aggressive tumor invasion and diffusion. The incidence of sarcomatous change in CC is approximately 5% and this phenomenon is related with a poorer prognosis of patients affected by this neoplasia than ordinary CC [96]. These transformed cells present a predominantly spindle, bizarre giant shape and express cytokeratins (KRTs) as an epithelial marker and vimentin as a mesenchymal marker [96]. This cellular phenomenon is favored by the interplay of extracellular signaling including components of the extracellular matrix, as well as growth factors. Overall, HGF and its receptor, Met tyrosine kinase, are predominant stimulators of EMT [97]. Other molecules, such as TGF-α, TGF-β, EGF, and fibroblast growth factor (FGF) activate specific intracellular mechanisms thus contributing to ETM [98–101]. Moreover, several transcriptional activators are involved in controlling the EMT process. For example, Fos induces EMT by activating a Wnt-like pathway [102, 103] and the Jun/Fos transcriptional complex lies downstream of the Ras pathway and Jun might exert its effect in EMT by mediating the translocation of Rac to the plasma membrane, which is a prerequisite for its activation and subsequent cytoskeletal rearrangements required for cell motility [104]. Other transcription factors, including the snail family, another snail family slug, and the Ets family, are also candidates for playing a role in EMT during embryonic life and they may also participate in tumor progression [105–107].

Recently, it has been described that EGF and EGFR are involved in the signaling pathways that regulate EMT [108, 109]. Lee et al. [95] showed that EGF transcriptionally downregulates the cytoplasmic protein ANXA8 during CC tumor dedifferentiation, leading to the morphological changes of EMT. This means that downregulation of ANXA8 is associated with tumor progression and metastasis [95]. In addition, EGF-mediated FOXO4 modulation is involved in an upstream signaling pathway of ANXA8 expression, thus suggesting that both FOXO4 and ANXA8 may be involved in EGF-mediated tumor progression and EMT in CC [95].

The molecular mechanisms whereby cancer cells generate a cell-autonomous EMT-like process, via oncogenic activation are still not completely clear [96]. Recently, Yoo et al. [96] identified by cDNA microarray technique, some up or downregulated genes in the sarcomatous cells compared to non sarcomatous CC cells. Among them, the deletion mutation of p53 may be involved in the sarcomatous change or the tumor progression of CC cells [96]. Moreover, down-regulation of Fas and FasL is described in ICC and is correlated with histologic dedifferentiation and invasion [96].

MicroRNA and Cholangiocarcinoma

In recent years, some authors have studied and partially described the miRNAs gene expression profiles in CCs [110]. The currently available data is incomplete since most groups have studied methylation of one or at most, only a limited number of genes in CC [110]. The oncomiRs miR-141, miR-21, miR-23a, miR-27a, let-7a, and miR-200b are upregulated, while the tumor suppressor miRNAs miR-29b and miR-370 are downregulated in malignant cholangiocytes [111–115].

Moreover, in CC cells the upregulated miR-141 may target the CLOCK gene, which modulates circadian rhythms and acts as a tumor suppressor [113]. Its involvement in CC growth is underlined by the fact that inhibition of miR-141 decreases cell growth of CC cells [113].

The role of miR-21 and miR-200b in stimulating CC proliferation is given by the fact that miR-21 regulates PTEN and is an anti-apoptotic and pro-survival factor [115], and both miR-21 and miR-200b are inhibited by gemcitabine, the chemotherapic drug specific for CC.

It has been suggested that a target gene for miR-200b could be PTPN12, which may contribute to tumor cell survival and carcinogenesis in case of its dysregulation [113]. Moreover, the same miR-200b could inhibit ZFHX1B, which is involved in the TGF-β signaling pathway and in processes of EMT via regulation of E-cadherin [116]. Similar to a variety of cancers where the expression of miR-29b is reduced [117–119], CC express low levels of this molecule [111].

Mott et al. [111] showed an inverse relationship between miR-29b and Mcl-1 expression in CC cells. In fact, the reduction of miR-29b is accompanied with an increase of the anti-apoptotic Mcl-1.

miR-370 expression is reduced in malignant compared to normal cholangiocytes [112]. Some evidences show that miR-370 targets MAP3K8, which is consequently upregulated in CC cell lines as well as in tumor cell xenografts in vivo [112, 115]. Epigenetic regulation of miR-370 occurs by hypermethylation and through IL-6 [112, 115].

Alterations in miRNA expression contribute to tumor growth and response to chemotherapy. Enhanced miR-370 expression suppresses growth of malignant human cholangiocytes and may therefore be suited as a novel target for molecular therapeutic strategies using small RNAs. Aberrantly expressed miRNA or their targets will provide mechanistic insight and therapeutic targets for CC, and additional detailed investigations about the miRNA expression using human CC samples are needed in the future to elucidate the mechanisms involved in the oncogenesis of these enigmatic tumors.

Molecular Differences Between Intra and Extrahepatic CC

A variation of molecular and genetic alterations has been reported in CC differing for morphology, pathology, and clinical presentations. For example, *K-ras* proto-oncogene mutations are common in biliary malignancies showing a periductal extension and hilar distribution, and seem to be occurring at an increasingly higher incidence in bile duct cancers arising distally in the common bile duct [51, 60]. Furthermore, *K-ras* mutations have been shown higher in the case of CC with lymph node metastasis than in those without lymph node metastasis [61].

Mutations determining inactivation of DPC4/*Smad4,* the TSG involved in the transduction of signals from the TGF-β family of cytokines, are also more frequent in distal bile duct cancers than in more proximal bile duct and intrahepatic tumors [63].

Karamitopoulou et al. elegantly showed that p16 protein expression varies in accordance to the location of the tumor [120]. In fact, this protein is more expressed in intrahepatic than in ECC cells [120]. Moreover, p16 has a prognostic value in patients affected by CC since its expression relates with a better survival; on the contrary, loss of p16 protein is associated with a poor prognosis [120]. Bcl-2 was found to be overexpressed in ICC, whereas p53 was exclusively expressed by extrahepatic and gallbladder carcinomas. Although p53 mutations have been described in CC, they have been detected in only 5–35% of ICC [16, 121]. This data suggests that the inactivation and/or accumulation of inactive p53 in ICCs may lead to an insufficient level for the immunohistochemical detection of the protein [120]. In general, all these described proteins possess an important role in the pathogenesis of CC thus being very useful to the pathologist for the diagnosis of CC and also between extrahepatic and ICCs in difficult cases. These data demonstrate a clearly distinctive immunoreactive pattern between intrahepatic and ECCs. The different immunophenotype of intrahepatic (absence of p53 and increased Bcl-2 and p16 expression) and extrahepatic (increased p53 and very low Bcl-2 and p16 expression) CCs suggests different pathogenetic processes and genetics for these tumors. However, further studies are required.

Conclusion

The multitude of factors released in the environment during the course of cholestasis and chronic inflammation induce an accumulation of genomic and epigenetic damages leading to malignant transformation and uncontrolled proliferation of cholangiocytes. CC is a highly lethal disease with an extremely poor response to conventional anticancer therapies and a poor survival rate. Only the complete identification of molecular pathways involved in the pathogenesis of CC will permit the discovery of novel tools for an early diagnosis, and the detection of specific molecular targets for specific therapies.

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Chapter 60
Neoplasms of Extrahepatic Bile Ducts

Nora Katabi, Juan Carlos Roa, and N. Volkan Adsay

In order to appreciate the molecular pathology of carcinomas occurring in the extrahepatic bile ducts (EBD), it is important to understand the epidemiology and risk factors for these cancers. A chapter on cholangiocarcinoma with focus on intrahepatic biliary neoplasms is included independently in this textbook (Chap. 59).

Epidemiology

EBD cancer is a rare malignant neoplasm. It is far less common than gallbladder cancer (see Chap. 61) presumably because of the lack of association with cholelithiasis [1]. In the United States, the ratio of bile duct cancer to gallbladder cancer is 1/2, but the incidence increases with the age of the patient [2]. The reported incidence of EBD cancer in large autopsy series varies from 0.01 to 0.2%, constituting around 2% of all cancer cases [3]. The average patient age is 68 years, with a peak incidence in the 70s [2, 3]. Unlike gallbladder cancer, EBD cancer occurs more frequently in males [4]. In Japan, the male to female ratio is 1.9:1 [1, 2, 5]. Caucasians and African Americans appear to be affected equally by EBDs cancer [6].

Risk Factors

Most cases of EBD cancer are sporadic with unknown etiology. The following factors however have been considered to increase the risk of developing bile duct cancer.

Primary Sclerosing Cholangitis and Ulcerative Colitis

Primary sclerosing cholangitis (PSC) is a rare chronic biliary disorder (see Chap. 50). It is characterized by persistent periductal chronic inflammation and fibrosis involving the extrahepatic and most often also the intrahepatic bile ducts, which ultimately causes multifocal strictures. The true incidence of bile duct carcinoma in association with PSC is unknown [3]. Historically, the prevalence of bile duct carcinoma in patients with PSC was given as 5–15% [7–9]; however, recent studies suggest a much higher association of 40% autopsy cases and 36% of liver explants in PSC patients [3, 10–12]. About 70–80% of PSC patients have ulcerative colitis (UC), but only few a patients with UC develop PSC [1, 3]. A similar inflammatory process occurs in bile ducts and colon, and presumably as a result of the inflammation and the immune response, the biliary epithelium becomes more susceptible to malignancy. Moreover, the incidence of UC is higher in patients with EBD carcinoma compared to those without carcinoma [5]. Patients with EBD carcinoma and UC usually have a long-standing history of colitis, and are relatively young with a mean age of 42 years [5]. Carcinoma of the bile ducts can develop years after the colonic resection, and neither medical nor surgical treatment of the coexisting UC appears to change the risk of developing cancer in the bile ducts [5, 12].

Congenital Biliary Cyst Disease and Abnormal Choledochal-pancreatic Duct Junction

Congenital biliary cysts or choledochal cysts are pathologic dilatations of the biliary tract. They are predominantly located in the common bile duct but can also involve any part of the biliary tract, including the intrahepatic bile ducts (Caroli's disease) [2]. They are classified on the basis of their anatomic location into five types (Todani classification) [1, 5, 13]. Choledochal cysts are more common in Asian

N.V. Adsay (✉)
Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA
e-mail: volkan.adsay@emory.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_60, © Springer Science+Business Media, LLC 2011

population with a female to male ratio of 3.5:1. They show predominance in pediatric population, with only 20–30% of the patients being older than 20 years [2]. Choledochal cysts have been reported to be associated with increased risk of malignancy. The same mechanism might explain the reported increased risk of bile duct cancer in the patients who undergo transduodenal sphincteroplasty and endoscopic sphincterotomy [14, 15]. The reported incidence of carcinoma in choledochal cysts increases progressively with patient age; it is less than 1% in patients younger than 10 years and 6.8% in patients between 10 and 20, and up to 15% in patients older than 20 years [2, 5]. Adenocarcinoma is the most common malignant tumor associated with choledochal cysts, although adenosquamous carcinoma, squamous cell carcinoma, and rhabomyosarcoma have also been reported [2, 5, 16–18].

Abnormal choledochal-pancreatic duct junction, in which the pancreatic duct enters the common bile duct outside the duodenum, has been reported in 64–97% of patients with choledochal cysts [5]. This anomalous arrangement predisposes to reflux of the pancreatic enzymes into the bile duct, which damages its wall and causes secondary chronic inflammation and fibrosis. The resulting changes over time can promote malignant transformation of the biliary epithelium [2, 3, 5, 14, 15, 19].

Hepatolithiasis

Hepatolithiasis and recurrent pyogenic cholangiohepatitis or oriental cholangiohepatitis, are widespread in Japan and parts of south Asia. Hepatolithiasis has been associated with bile duct cancer in 10% of the cases [3, 8, 20–23]. It is thought to cause portal phlebitis and chronic bacterial overgrowth. This might give rise to pigmented stone formations, causing obstruction of bile ducts, and eventually leading to recurrent inflammation and stricture formation [3].

Infections

Hepatobiliary flukes (*Clonorchis sinensis* or *Opisthorchis viverrine*) are prevalent in parts of East Asia and are associated with increased risk of bile duct cancer [3, 5, 21, 24, 25]. The correlation between fluke infection and bile duct carcinomas has been confirmed in several animal models and case-control studies [8, 26, 27]. In Thailand, where around seven million people are estimated to be infested with *C. sinensis*, the annual incidence of bile ducts carcinoma is 87 per 100,000 [3, 28]. With persistent infections, neoplastic changes and carcinoma may develop. In addition, long term carriers of *Salmonella typhi* have been found to show higher frequency of developing bile duct cancer. These chronic carriers might develop PSC, which can progress to carcinoma [5].

Miscellaneous

Several radionuclides and chemical carcinogens, such as thorotrast, radon, nitrosamines, dioxin, and asbestos have been correlated with an increased risk of bile duct carcinoma. In addition, bile duct cancers have been reported in association with familial adenomatous polyposis coli and hereditary non-polyposis colorectal cancer (HNPCC) [5, 29–34]. This might be related to underlying genetic abnormalities that occur in these diseases. The association of bile duct carcinoma with Crohn's disease has been suspected, but not fully verified [5, 35–37].

Histogenesis

Similar to gallbladder cancer, EBD cancers appear to develop through multiple steps of tumor progression. As illustrated by the established risk factors discussed above, chronic injury and cholestasis are the key factors in the pathogenesis of bile duct carcinoma. They create an environment that might promote molecular alterations, leading to premalignant and malignant transformation. There are two main precursor lesions or preinvasive neoplastic lesions that have been associated with the development of bile duct carcinoma: flat lesions (biliary intraepithelial neoplasia (BilIN)) and biliary intraductal biliary papillary neoplasms. Both types of precursor lesions may occur in patients with PSC, choledochal cysts or hepatolithiasis, which are conditions associated with increased frequency of bile duct carcinoma [38–41].

Flat Biliary Dysplasia (Biliary Intraepithelial Neoplasia)

EBD carcinoma appears to follow the same histogenesis as gallbladder carcinoma, which might explain the similar histologic features of both carcinomas. Similar to gallbladder, the pathogenesis of EBD carcinoma is thought to result from an evolutionary sequence from metaplasia to dysplasia to carcinoma. Two major types of metaplasia occur in the biliary tract: gastric and intestinal [2, 5, 42]. Gastric metaplasia is the most common type of metaplasia, in which the metaplastic epithelium recapitulates the gastric pyloric or antral

mucosa. Intestinal metaplasia consists of variable amounts of goblet or columnar cells with a brush border. Squamous cell metaplasia can also be seen in the biliary tract. In a retrospective study by Albores-Saavedera, metaplasia has been noted in 48% of the 42 studied EBDs lesions (32 neoplastic and 10 inflammatory), with 80% pyloric gland metaplasia, 5% intestinal metaplasia, and 5% squamous cell metaplasia [43]. It has been noted that dysplasia and carcinoma often arise in a background of metaplasia [5].

Flat biliary dysplasia or intraepithelial neoplasia, also called BilIN [40], is commonly identified adjacent to the invasive carcinoma, and occasionally as an incidental finding in specimens obtained for other reasons [3, 5, 31, 36, 44, 45]. BilIN and carcinoma in situ were seen in 10–45% of EBD carcinomas [13, 36, 46]. Although the common coexistence of dysplasia in patients with carcinoma supports the evolutionary dysplasia-carcinoma progression, the natural history of dysplasia in biliary tract remains unknown. When dysplasia is incidental, it is often a microscopic lesion that does not form a mass and is unrecognizable grossly and radiographically. Histologically, this lesion is often "flat", but can have abortive papillae and is characterized by cytoarchitectural atypia including atypical disordered proliferation of the biliary epithelium with loss of polarity, pseudostratification, nuclear enlargement and mitotic activity. Dysplasia is categorized as low, moderate or high grade based on the degree of the atypia. Carcinoma in situ is considered as part of the spectrum of high grade dysplasia, representing the fully established end of this spectrum.

The term "biliary intraepithelial neoplasia" (BilIN), a three-grade classification system, was proposed instead of the term "biliary dysplasia," to describe the flat or the low-papillary dysplastic biliary epithelium [4, 40]

In the biliary tract, dysplasia is most common and best studied in the gallbladder and its incidence appears to be much lower in the rest of the biliary tract [3, 5]. Furthermore, histologic distinction of true dysplasia from reactive atypia or colonization of the biliary epithelium by invasive carcinoma might be difficult, which makes the true incidence of biliary dysplasia difficult to determine.

Intraductal Papillary Neoplasms of Bile Ducts

Polypoid lesions are uncommon in the biliary tract compared to the gallbladder [3, 5]. In the EBDs, these lesions form intraductal masses that are grossly and radiographically visible, and can be solitary or multiple. Histologically, they may have papillary, tubular, or tubulopapillary growth pattern, showing a variable degree of cytoarchitectural atypia [1, 3]. The amount of papillary architecture correlates with worse prognosis [1]. When the entire intraductal tumor shows high grade dysplasia, it is regarded as intraductal "carcinoma." Invasive carcinoma is usually found in association with intraductal neoplasm with high grade dysplasia. However, it is also possible to find invasive carcinoma in association with an intraductal lesion that is devoid of any significant cytologic atypia, i.e., adenoma [3].

The nomenclature for intraductal biliary lesions has not been standardized, and a variety of terms has been used to describe these lesions in the literature such as papilloma, villous adenoma, papillary adenoma, and mucin hypersecreting bile duct tumor. The term papillary carcinoma has been used to describe a variety of lesions ranging from those intraductal lesions showing focal cytologic atypia to a conventional invasive adenocarcinoma with a prominent intraductal papillary component. However, most authors currently employ the term "papillary carcinoma" to tumors with prominent papillary growth pattern associated with diffuse high grade dysplasia [3, 43]. Papillary carcinoma is associated with a favorable clinical outcome [1, 47], especially if it is not associated with invasive carcinoma. Papillomatosis is a term that is applied to multicentric non-invasive papillary neoplasms [3, 43]. These lesions are more common in the EBDs compared to the gallbladder [1]. A high rate of recurrence and a strong association with invasive carcinoma have been demonstrated [1].

Some authors have begun to employ the term "intraductal papillary neoplasm" (IPNB) to describe papillary biliary lesions because of their clinical and pathologic similarities to the pancreatic intraductal papillary mucinous neoplasm (IPMN) [3, 48]. Despite their similarity to IPMNs, differences exist in frequency, morphology, and types of associated invasive carcinoma between the two lesions. Further studies are needed to clarify the relation of intraductal biliary neoplasms to IPMNs.

General Anatomic-Clinical Consideration

EBD cancer can involve the major proximal bile ducts in the hepatic hilum or can arise in the non-hilar EBDs. For therapeutic and prognostic purposes, extrahepatic biliary carcinomas are divided into upper, middle, and distal thirds based on the location of the tumor. Over half of the EBD carcinomas are located in the upper third, within 5 mm of the cystic duct, 25% in the middle, and 15% in the lower third [1, 2]. This is considered to be of significance, pertinent to the possible mechanisms of carcinogenesis. Multifocal or diffuse involvement of the biliary tract by carcinoma can occur in less than 10% of the patients [3, 5, 6]. The term Klatskin's tumor refers to those arising in the confluence of right and left hepatic ducts. Moreover, hilar and perihilar tumors have been separated according to their involvement of the hepatic ducts

(the modified Bismuth–Corlette classification) as follows: type I, below the confluence of the right and left hepatic ducts; type II, at the confluence of the right and left hepatic ducts; type III, at the common hepatic duct and type IV, multicentric tumors at the confluence of both right and left hepatic ducts [49]. Hilar carcinoma commonly involves the liver or the perihepatic structures by direct extension [50].

Regardless of their location, all EBDs carcinomas display similar histologic features, which resemble the histological features of gallbladder carcinomas as well as to those of pancreatic-ductal adenocarcinomas, and thus termed "pancreatobiliary." Three growth patterns of EBD carcinoma have been discerned: periductal infiltrating, papillary or intraductal, and mass forming.

Clinically, 90% of patients present with jaundice and 10% present with cholangitis [8, 51, 52]. Endoscopic retrograde cholangiography (ERCP), magnetic resonance cholangipancreatography (MRCP) and percutaneous transhepatic cholangiography (PTC) are considered the most important diagnostic modalities [1, 8, 28]. Most of the EBD carcinomas are resected at specialized care centers since these operations are seldom performed without a strong suspicion of malignancy [1].

Histologically, most EBD carcinomas display the morphologic features of pancreatobiliary type adenocarcinoma, often showing desmoplastic stroma and widely separated irregular and well formed glands. Papillary carcinoma is the term employed to describe lesions with a predominant papillary (in situ) growth pattern. However, this term has also been applied to a variety of lesions as discussed previously. Other histologic variants include adenosquamous and squamous cell carcinoma, intestinal type adenocarcinoma, mucinous carcinoma, clear cell carcinoma, signet ring cell carcinoma, undifferentiated carcinoma, and small cell carcinoma [4, 5]. Similar to gallbladder carcinomas, EBD carcinomas are graded by the histologic grading system advocated by the World Health Organization into well, moderate, or poorly differentiated. Bile duct carcinomas arising in association with PSC often show peribiliary gland hyperplasia [1, 53].

EBDs carcinomas are highly aggressive. Most patients present with a high stage tumor at the time of diagnosis [1]. The 5-year survival rate for EBD carcinoma is approximately 10% although minor improvements have been recorded recently [1, 2, 5, 8, 54]. Distal bile duct examples have the best reported prognosis among the EBDs tumors, probably due to their earlier detection and their resectability by pancreatoduodenectomy [1]. Important prognostic factors include the extent of tumor, histologic type, perineural and vascular invasion [4, 46, 55]. Papillary carcinomas have a favorable outcome especially when they are noninvasive or minimally invasive [1, 43]. Surgical resection, if clinically and surgically applicable, is the treatment of choice for EBD carcinoma, with pancreaticoduodenectomy performed for distal bile duct tumors and Roux-en Y hepaticojejunostomy for the proximal lesions [1]. It is being debated whether "debulking" operations are beneficial [8, 56]. Patients with PSC and bile duct carcinoma might be better treated with liver transplantation due to advanced liver disease and increased risk of de novo carcinoma [8, 57, 58]. The value of chemotherapy, radiation therapy, and photodynamic therapy are under discussion; they are commonly utilized in most major centers [8, 59–61].

EBD carcinomas are classified by the American Joint Committee Cancer (AJCC), which uses histologic definitions of the anatomic layers to differentiate the T stage of carcinoma, representing the extent of invasion. However, the landmarks of the extrahepatic biliary tract are not well defined, and the wall of the bile ducts consists of variable amount of fibromuscular tissue along the length of EBDs. For instance, the distal part of the EBDs walls is composed of thick smooth muscle bundles, whereas the proximal part has only few or no smooth muscle fibers. Moreover, the presence of invasive carcinoma and its stromal response often destroys the histologic landmarks of the EBDs. In addition, chronic inflammatory and fibrotic background, especially in patients with PSC, adds more distortion of the bile ducts wall histology. Furthermore, the TNM staging system does not correlate with tumor resectability or provide information about hepatic atrophy. It requires obtaining the surgical tissue in order to evaluate staging parameters. As a result, TNM classification does not seem to have the degree of expected predictive value because of the anatomy and the histologic features of the extrahepatic biliary tract. For these reasons, improved staging schemes are being proposed, including the one from Memorial Sloan Kettering Cancer Center for hilar carcinoma, based on the extent of biliary and vascular involvement [8, 62–64], and by Hong et al. [65] for EBD carcinoma, based on the microscopic depth of invasion of carcinoma.

Molecular and Genetic Alteration

The molecular aberrations associated with EBD cancer are poorly characterized. Inflammation and cholestasis along with bile acids, growth factors, and cytokines create a milieu that alters the epithelial cells and deregulates the expression of the DNA mismatch repair (MMR) proteins, proto-oncogenes, and tumor suppressor genes [59, 66]. Cytokines stimulate the expression of inducible nitric oxide synthase (iNOS) in the biliary epithelium. iNOS has been found to be upregulated in the setting of malignancy [59, 67]. Increased iNOS activity results in the generation of nitric oxide and reactive nitrogen oxide species (RNOS), which induce mutation in the double stranded DNA leading to DNA breaks [59]. A variety of oncogenic mutations have been identified in EBD carcinoma.

However, in contrast to intrahepatic cholangiocarcinoma, the histologic nature of the extrahepatic biliary tract along with the desmoplastic stromal tumor response hamper successful genetic analysis of these tumors which require laser capture microdissection of the carcinoma cellular elements [50]. As a result, most of the published literature on molecular and genetic alterations of biliary tract does not differentiate between intrahepatic and EBDs, although there are likely to be numerous differences. Nevertheless, understanding the molecular and genetic alteration of biliary carcinogenesis is essential to improve screening techniques of high risk patients and the dismal prognosis of this rare tumor.

Using immunohistochemistry, biliary carcinoma shows similar immunoprofile to pancreatic and gastroesophageal, namely, foregut-derived carcinomas with positive CK7 immunostaining in almost all cases [1, 2]. CK20 might be also positive in EBD carcinoma [1]. Several glycoproteins such as MUC1, MUC5AC, CEA, B72.3, and CA19–9 are expressed in EBD carcinomas, though their expression might be focal [1, 3, 68, 69]. Expression of CEA and MUC1 was reported to increase progressively from precursor lesions to invasive carcinoma [1, 70], with invasive poorly-differentiated carcinomas showing intense cytoplasmic labeling. Furthermore, both precursor lesions of EBD carcinoma, i.e., BilIN and IPNBs seem to share similarities with their pancreatic counterparts, PanIN and IPMN [70]. Similar to the pancreatic or gallbladder precursor lesions, the molecular pathways in the carcinogenesis of BilIN differ somewhat from those of intraductal biliary neoplasm [70]. BilIN or intraepithelial neoplasia develops as a result of a gradual progression of mutation in cyclin D1, p21, and p53, with greater loss of DPC4 and molecular adhesion expression [70]. Additionally, BilIN shows much higher expression of matrix proteins (MMP-7 and MT1-MMP) and mucin core protein MUC1 than the intraductal biliary neoplasms, which corresponds with its worse prognosis and greater potential of invasion and metastases. In contrast, intraductal biliary neoplasm or IPNB displays a gradual increase in cyclin D1 and P21 expression with a parallel decline in adhesion molecules and DPC4 expression [70]. Lower levels of matrix proteins (MMP-7 and MT1-MMP) and expression of the intestinal MUC2 in the intraductal biliary neoplasms might account for the favorable outcome of these lesions [70]. Many studies are needed to further understand the molecular alterations in EBD carcinoma and its precursor lesions.

Cell Cycle Proteins

Cyclin D1 is an important proto-oncogene that regulates cell cycle progression. Cyclin D1 protein forms a complex with cyclin dependant kinase 4 and 6 (CDK4 and CDK6), which inhibits the Rb (retinoblastoma) pathway and allows cells to progress from the G1 to the S phase [71]. Nakanishi et al. [72] have detected altered expression of cyclin D1 levels in parallel to progression of dysplasia from mild to moderate, both in flat dysplasia and intraductal biliary neoplasms. A similar finding has been reported for PanIN and IPMN [73]. Of interest, in a study by Itatsu and colleagues [74], cyclin D1 was observed to be overexpressed in intraductal biliary neoplasms in higher levels as compared to flat dysplastic biliary lesions. This indicates the importance of cyclin D1 upregulation in these lesions. However, no difference of cyclin D1 expression was found in invasive carcinoma arising in either flat biliary dysplasia or intraductal biliary neoplasms [72, 73].

P21 is a cyclin dependent kinase inhibitor that prevents cell-cycle progression. Expression of p21 was observed to increase with histologic progression of dysplasia in both BilIN and intraductal biliary neoplasms [72].

Tumor Suppressor Genes

DPC4 (Smad4) is a tumor suppressor gene located on chromosome 18q, which has been implicated in carcinogenesis. Mutations in DPC4 gene were observed in 16% of resected biliary tract cancers [75]. Inactivation of DPC4 gene occurs late in biliary-tumor progression [76]. Additionally, loss of DPC4 expression in bile duct carcinoma increases from low in proximal to high in distal bile ducts and is more frequent in EBD carcinoma (55%) compared to intrahepatic cholangiocarcinoma (13%) or hilar bile ducts carcinoma (15%) [77]. Furthermore, DPC4 expression by immunohistochemistry was noted to decrease gradually with tumor progression from BilIN to invasive carcinoma. This is similar to the reduction in DPC4 expression in invasive pancreatic carcinoma compared to its expression in PanIN [72, 78].

P53 overexpression was detected in 50% of bile duct carcinomas, with the highest frequency reported in the distal common bile ducts [1]. Some data has suggested that p53 mutations are fairly specific to late stages of BilIN and its associated invasive carcinoma [1, 72]. Overexpression of p53 has been identified in 75% of invasive bile ducts carcinoma arising from BilIN and in 11% of high grade flat biliary dysplasia (BillN-3) [72]. In contrast, it is rarely identified in mild to moderate grade flat biliary dysplasia (BillN1 and BillN2) or normal biliary epithelium. P53 was found to be upregulated differently in intraductal biliary neoplasms in which overexpression of p53 was in early low grade and not in high grade lesions [72].

C-myc is a transcription factor that inhibits cellular apoptosis and increases cellular proliferation. C-myc overexpression was identified in higher levels in intraductal biliary

neoplasms compared to BilIN, and was not detected in normal biliary epithelium [74]. Overexpression of C-myc in intraductal biliary neoplasms correlates with decreased membranous expression of β-catenin and E-cadherin in these lesions [70]. This observation suggests that C-myc might play an important role in development of intraductal biliary neoplasms and less critical role in BilIN.

Cell Adhesion Proteins

E-cadherin is a tumor suppressor gene that encodes a transmembrane protein involved in epithelial cell adhesion and tumor progression [70, 79]. Alterations in E-cadherin play a role in the loss of cellular differentiation and increase the potential of invasion [70]. Decreased expression or mutations in E-cadherin was identified in 46% of EBD carcinomas and was associated with poorly differentiated carcinomas [70, 79]. Furthermore, decreased E-cadherin expression was found to correlate with lymphatic and perineural invasion and was associated with short overall survival [79]. Therefore, loss of E-cadherin may be a late event in biliary tumor progression that correlates with tumor recurrence and prognosis [70].

Syndecan-1 is part of the Syndecan family of transmembrane proteins, which have an important role in cell proliferation, cell adhesion, and cell–matrix interaction [80–82]. Syndecan-1 is the major Syndecan in epithelial cells [79]. Reduced Syndecan-1 expression was noted in 69% of the EBD carcinomas and was found to correlate with lymphatic, venous, and perineural invasion, indicating tumor recurrence and prognosis [79].

β-catenin is a cytoplasmic-membraneous protein that mediates cell adhesion and has been implicated in tumor progression [70]. Decreased membranous expression of β-catenin, which disrupts cell adhesions and promotes tumor invasion, has been reported in bile duct carcinoma in 58–82% of the cases [83–85]. Itatsu and colleagues have found a gradual decrease in membranous expression of β-catenin with tumor progression from mild to high grade dysplasia and to invasive carcinoma [72]. This latter finding was identified in both BillN and intraductal mass forming lesions (IPNB) [72]. However, nuclear staining of β-catenin by immunohistochemistry was noted only in the intraductal precursor lesions (22%), which probably reflects activation of Wnt signaling pathway in these lesions [72].

Mucin-Related Glycoproteins

Mucin core proteins (MUCs) are high molecular weight glycoproteins produced by many epithelial tissues. They play an important role in a variety of cell functions, including protection of the epithelium by anti-adhesive activity that disrupt the normal adhesive interaction between cellular integrins and extracellular matrix [70, 86]. A number of human MUC proteins has been identified and can be subdivided in two groups: *membrane bound*, examples of which include MUC1, MUC3, and MUC4; and *secretory type* that forms extracellular protective gels, of which MUC2, MUC5AC, MUC5B, and MUC6 are the most well known [86]. Alterations of MUC protein expression were found to correlate with prognosis in many carcinomas including EBD carcinoma [70, 86, 87].

MUC1 expression has been associated with signs of aggressiveness including poor clinical outcome, poor differentiation, infiltrative pattern and deep tumor invasion, lymphatic and perineural invasion, and liver metastasis [70, 86, 87]. MUC1 expression was detected in up to 30% of BillN and in most of the invasive carcinoma associated with BillN. These findings suggest that MUC1 expression is a late step in the transformation of BillN into invasive carcinoma [41, 72]

Furthermore, MUC1 expression appears to regulate the differentiation of invasive carcinoma associated with intraductal biliary neoplasms (IPNB) into tubular vs. mucinous type [70].

The expression of intestinal differentiation markers, MUC2 and CDX2 is relatively low in EHBD carcinomas. MUC2, intestinal type secretory mucin, is not expressed in normal biliary epithelium. [36, 42, 88] CDX2 gene is a member of the caudal-related homeobox gene family which plays an important role in intestinal programming [88–92]. Both CDX2 and MUC2 have been detected in high levels, in addition to normal intestinal mucosa, also in intestinal metaplasia seen elsewhere in the GI tract. In the EBD, Hong et al. [88], reported CDX2 and MUC2 expression in 38 and 42% of EBD carcinomas by immunohistochemistry, respectively, although the degree of expression appeared to be defined differently than in other studies, and may reflect a low level of expression. Moreover, this expression was seen mostly in carcinomas with intestinal differentiation and their synchronous expression correlated with better overall survival [88]. Other studies have also found that MUC2 and CDX2 expression is associated with better survival [70, 93, 94]. It has been suggested that MUC2 expression decrease tumor invasion and metastases [95]. In contrast, MUC2 expression does not seem to play an important role in this transformation [70]. On the other hand, MUC1 expression was less commonly identified in intraductal biliary neoplasms (IPNB) and its associated invasive carcinoma (12.5 and 60% respectively) [72].

MUC5AC is a gel forming mucin that is expressed in gastric foveolar and transbronchial epithelium [86]. Abnormal expression of MUC5AC has been identified in intrahepatic and EBDs [95]. A recent study by Park and colleagues [86] has shown that MUC5AC is more frequently expressed in advanced tumors. The same group has also found a higher

expression of MUC5AC in EBD carcinoma (70%) than in intrahepatic cholangiocarcinoma (47.1%) [86]. Furthermore, it has been suggested that serum MUC5AC expression might be a sensitive and specific indicator of carcinoma and a predictor of poor outcome [95–97].

MUC6 is normally expressed by a variety of epithelium including gastric pyloric glands and duodenal Brunner's glands [98]. MUC6 has been detected in 14% of EBD carcinomas in one study [64]. In addition, MUC6 expression was noted more frequently in well differentiated carcinomas as well as in pyloric gland type tumors with less aggressive behavior [64], the latter is exceedingly uncommon in EBD.

Matrix Proteins

Matrix metalloproteinases (MMPs) are a family of zinc-dependant proteinases that digest extracellular matrix, which is an important barrier to tumor invasion and metastases [70, 99]. Increased expression of MMPs was detected in EBD carcinomas in several studies [99, 100]. Membrane type metalloproteinase-1 MT1-MMP degrades type I collagen and extracellular adhesion molecules [70]. MMP-7 degrades extracellular collagen, laminin, fibronectin, and proteoglycans. Normal biliary epithelium does not express MMPs [99]. Overexpression of both MMP-7 and MT1-MMP was detected in EBD carcinoma in 75 and 55% of the cases, respectively, and was found to be expressed more frequently in conventional non-papillary carcinoma than in papillary carcinoma [99]. Moreover, expression of both MMP-7 and MT1-MMP was found to correlate with higher grade tumors, perineural invasion and advanced TNM stage [99]. Itatsu et al. [74] have found low expression of MMP-7 in intraductal biliary neoplasms (IPNB). In contrast, expression of MMP-7 and MT1-MMP1 was found to persist consistently from early to late BillN and in biliary dysplasia-associated invasive carcinoma [74]. These findings might explain the less aggressive behavior of intraductal biliary neoplasm/papillary carcinoma.

Miscellaneous

The incidence of *K-ras* mutations in EBD carcinomas is around 56%, which is significantly higher than gallbladder cancer [101]. Similar to the expression of p53 and loss of DPC4, the frequency of K-ras mutations increases from proximal to distal EBD, but even in the most distal part, it is still lower than pancreatic tumors . It has been suggested that K-ras mutations arise early in biliary carcinogenesis since identical mutations were found in both intraductal biliary neoplasm and its associated invasive component [48]. Interestingly, a high incidence of K-ras mutations was reported in tumors associated with abnormal choledochal-pancreatic duct junction, which might be related to reflux of the pancreatic enzymes into the bile duct [102].

IMP-3 (insulin like growth factor II mRNA binding protein 3) is an oncofetal protein, expressed during embryogenesis and carcinogenesis [103]. In a study by Riener et al. [103], IMP3 expression by immunohistochemistry was identified in 50% of EBD carcinomas and was found to be an independent adverse prognostic factor. Furthermore, the same study disclosed strong positive IMP3 immunostaining in BillN-3 compared to low-grade dysplasia and reactive biliary epithelium, which might be diagnostically useful to differentiate these lesions in small sample material [103].

Recent studies have suggested that reduction of the length of telomere, which helps maintain chromosomal stability, may contribute to the development and progression of carcinomas in various organs. A study by Hansel et al. [104] using the fluorescence in situ hybridization (FISH) method showed that telomere shortening is a consistent and an early event in biliary carcinogenesis. An intratumoral heterogeneity of telomere lengths was found in invasive carcinoma of bile ducts by the same group.

EGFR (epidermal growth factor), Her2 (human epidermal growth factor 2), and VEGF (vascular endothelial growth factor) are members of a crucial family of growth factors that have been implicated to have a role in biliary carcinogenesis as well. EGFR and Her2 are members of the ErbB receptor tryosine kinase family, which plays an important role in progression, migration, proliferation, and survival of tumor cells [104–106]. VEGF binds to VEGF-receptor and leads to migration, proliferation, and survival of endothelial cells, playing a key role in tumor associated neoangiogenesis [107]. Recently, agents targeting molecules, including EGFR, Her2 and VEGF, have been used clinically to treat a wide variety of cancers [108]. In a study by Yoshikawa and colleagues, EBDs carcinomas showed immunohistochemical overexpression for EGFR, VEGF, and Her2 in 19, 59, and 9% of the cases, respectively [106]. The same study found overexpression of EGFR in EBC carcinoma to be a significant prognostic factor that correlates with macroscopic lymph node metastasis, lymphatic and perineural invasion, tumor recurrence, and tumor stage [106].

Fascin is an actin binding protein that plays a role in mesenchymal and neuronal cell motility. Fascin was identified to be upregulated in many neoplasms and its overexpression correlates with poor prognosis [109]. In EBD carcinomas, the overexpression of Fascin was noted as an independent poor prognostic factor [109].

MicroRNAs (miRs) are recently discovered, non-coding proteins that regulate the expression of specific target genes by binding to target messenger (m) RNA [110, 111].

Overexpression of miR21 was detected in human bile ducts carcinoma [111]. Additionally, miRs overexpression was found to be 95% sensitive and 100% specific in distinguishing between carcinoma and normal biliary epithelium [110]. Moreover, it has been suggested that miR21 might play an oncogenic role by inhibiting PDCD4, a potential tumor suppressor gene, as well as TIMP3, a member of MMP [110].

MMR/MSI: DNA MMR (mismatch repair) genes are responsible for recognition and correction of base-pairing errors, which occur during DNA replications [110]. Microsatellite instability (MSI) is the term used to describe microsatellite alterations characterized by deletions and insertion mutations in short tandem repeats in DNA, which serves as a surrogate of MMR gene mutations. MSI is considered to occur as a result of defective DNA MMR genes. Many studies using both immunohistochemical and PCR based methods have suggested that genetic defects in the DNA MMR system and MSI do not play a prominent role in biliary carcinogenesis [112]. On the other hand, some investigators have found that the allelic loss at chromosome 1p36 plays an important role in MSI which might be an indicator of tumor prognosis [113]. They have suggested that MSI at chromosome 1p36 might contribute to biliary carcinogenesis associated with liver flukes [113].

Summary

Biliary tract carcinogenesis is a complex event in which many pathways and genes/molecules appear to participate. There are numerous genes and molecules other than the ones discussed above that are under investigation. It is likely that our understanding of biliary carcinogenesis will improve significantly in the coming years.

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Chapter 61
Neoplasms of the Gallbladder

Juan Carlos Roa, Nora Katabi, and N. Volkan Adsay

Epidemiology

Gallbladder cancer (GBC) is an uncommon disease in most developed countries, with the exception of some geographical areas. The highest gallbladder cancer incidence rates have been reported in women from North India (21.5/100,000), Chile (18.1/100,000), Pakistan (13.8/100,000), and Ecuador (12.9/100,000). High incidences have also been found in Korea and Japan and some central and eastern European countries such as Poland, the Czech Republic, and Slovakia [1, 2]. GBC is up to three times higher among women than men in most countries and up to six times in select populations [1, 3].

Risk Factors

Gallstones

Gallstones are the most important and most common risk factors for the development of gallbladder cancer, present in between 60 and 90% of GB cancer patients [3–5]. A large population-based study not only supports the role of gallstones in biliary carcinogenesis, but also suggests that the underlying genetic or lifestyle determinants of stones within families contribute to the risk of biliary tract cancer outside the gall bladder [6]. It has recently been reported that an increase in the size of the stones could be related to a greater risk of GBC [7, 8], and this information could be used as a decision-making factor in performing a cholecystectomy in high-risk GBC areas [7]. However, others suggest that the increase in the number and size of the stones among patients with gallbladder carcinoma could simply be an effect of aging or a reflection of the long-term presence of stones in the gallbladder [9]. The composition and chemical mutagenicity of gallstones have been previously studied with inconclusive results [10].

Diet and Obesity

Obesity is associated with an increased risk of GBC (as it is with many other cancers) [11]. It is possible that the association with obesity is through its predisposition to lithiasis. In the Japan Collaborative Cohort Study (JACC), people with a high intake of fried food and those with bowel movements less than once every 6 days were found to have a significantly elevated risk of GBC. Conversely, the same study indicated that a high intake of boiled beans and fish had a significant preventive role in females [12].

Pollutants and Environmental Factors

A recent meta-analysis reviewed these environmental factors [13]. A number of heavy metals such as nickel, cadmium, copper, and zinc have been implicated, but the evidence is not sufficiently robust to confirm an association. Studies have shown that 3 methylcholanthrene and nitrosamines cause GBC in experimental animal models [14]. The role of radiation and pesticides in water has also been evaluated without any conclusive evidence of an association with GBC.

Gender

There is no evidence to suggest that the higher incidence in women is related to any factors other than gallstones and obesity, which are well known to be significantly more common in females. Almost total absence of estrogen and progesterone receptor expression in GBC cells, practically discard their role in GBC histogenesis as well [15].

N.V. Adsay (✉)
Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA
e-mail: volkan.adsay@emory.edu

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_61, © Springer Science+Business Media, LLC 2011

Bile Infections

Studies have shown a strong association of gallbladder cancer with some bacteria, in particular, chronic carriers of Salmonella are considered to be high risk for GBC, especially in areas of high typhoid endemicity, especially India [16, 17]. Chronic bacterial infection of bile leading to the production of carcinogenic precursors has been postulated as a factor in the pathogenesis of gallbladder carcinoma [16–18]. More recently, Helicobacter species has received particular attention in this regard, and an association to its presence in areas at high risk of GBC [19–22]; however, some have failed to confirm a relationship [23, 24].

Porcelain Gallbladder

Historically, a strong association has been reported between porcelain gallbladder and GBC [25]. It appears that calcified gallbladders may have some degree of association with an increased risk of GBC, but at a much lower rate than previously estimated [26]. In fact, more recent epidemiologic studies have challenged whether there is such an association at all [27].

Anomalous Pancreaticobiliary Ductal Junction

Anomalous pancreaticobiliary ductal junction (APDJ) is a rare congenital anomaly considered to be an etiological factor in the development of carcinoma of the biliary tract [28], especially in relatively young female patients with no gallbladder stones [29]. This has been attributed to the refluxing of pancreatic juice into the biliary tract, which induces chronic inflammation and increased cell proliferation, thus leading to epithelial hyperplasia, metaplasia, and eventually to carcinoma. This view is further substantiated by the very high incidence of K-ras mutations in biliary tract carcinomas associated with APD [30].

Histogenesis – Precancers

In the gallbladder, as in most other epithelial/glandular organs, two biologically distinct carcinogenetic models are recognized, through which the malignant transformation takes place: the metaplasia-dysplasia-carcinoma sequence and the adenoma-carcinoma sequence (Fig. 61.1) [31].

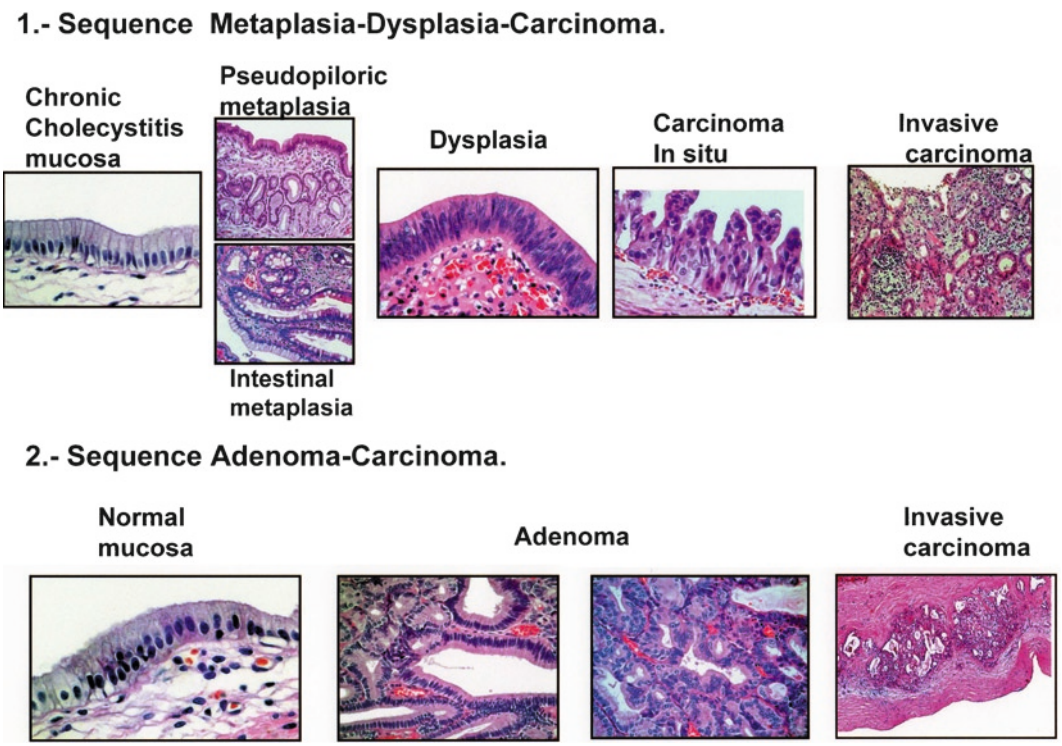

Fig. 61.1 Morphological progression of gallbladder carcinogenesis. From Roa et al. [31]. Used with permission

The first is based on microscopic alterations in the epithelium, starting with metaplasia as an adaptive process secondary to chronic irritation, on top of which develops dysplasia, which then progresses to carcinoma in situ, and subsequently into an invasive carcinoma [32–34]. In the second pathway, which represents the adenoma-carcinoma sequence, the neoplastic cells form exophytic (papillary and polypoid) intramucosal neoplasms growing towards the lumen of the gallbladder. These cells keep proliferating and form clinically detectable intraluminal masses measuring up to several centimeters before they transform into in situ, and finally, invasive carcinoma [32–36]. The evidence at the genetic-molecular level demonstrates that these two histogenetic pathways correspond to two different biological events, with different molecular make up [37, 38]. Below, we will discuss these in further detail.

Metaplasia-Dysplasia-Carcinoma Sequence (Flat Dysplasia; Intraepithelial Neoplasia)

In this model, there is no exophytic lesions formed and the precursor lesions are typically detected adjacent to carcinoma [33], or incidentally during microscopic examination of cholecystectomies performed for other causes, such as cholecystitis. Chronic inflammation is presumed to be the main instigator [5]. Metaplasia in the chronically inflamed gallbladder mucosa is a very common finding, detected in over 50% of the cases in some studies. Two types of metaplasia are observed in the gallbladder mucosa: the pyloric (or gastric) type and the intestinal type [39–42]. Gallbladder cancers are associated with both types of metaplasia; however, the association with intestinal type appears to be far stronger [40, 43]. It is believed that metaplasia serves as the precursor of carcinogenetic transformation, progressing into dysplasia, and culminating in invasive carcinoma [32, 39, 44, 45]. Dysplasia is commonly encountered in areas adjacent to carcinoma in situ, and furthermore, over 80% of invasive gallbladder carcinomas have associated foci of dysplasia or CIS in the neighboring mucosa [33, 40, 45, 46]. Recent molecular evidence is also keeping with this progression model. Another surrogate evidence for this progression phenomenon is the age at which these lesions are encountered. Naturally, it is not feasible to observe this transformation clinically, since most dysplasia/CIS are detected incidentally in cholecystectomies performed for inflammation or gallstones, at which time the natural history of the process has already been intervened. Thus, the age at which the histological diagnosis is established represents one of the approximations possible to estimate the period in which the lesions occur. In one major study, from a total of 1,326 patients (201 dysplasias and 1,125 cancers), the average age of the patients with dysplasia not associated with cancer was 52 years, early cancer was 57, and advanced carcinoma, 63 years. Accordingly, the time span from dysplasia to invasive carcinoma is estimated to be about 10 years [47].

Adenoma-Carcinoma Sequence (Mass-Forming Preinvasive Neoplasia)

Conventional adenomas usually present as solitary, sessile, and echodense polyps [48, 49]. Although it is well documented that adenomas can progress into invasive carcinoma, the precise incidence and rate of this progression has not been fully characterized, in particular, because there is no uniform definition of adenoma properly established in this organ. The adenoma-carcinoma sequence was originally observed by Kozuka et al. [36], who reviewed the histology of 1,605 gallbladder cancers and found 11 benign adenomas, 7 with signs of malignancy, and 79 with invasive carcinomas. His theory was also supported by the following observations: (a) The presence of histological transition from adenoma to carcinoma; (b) The close relation between the size of the lesion and the malignant change; malignant transformation observed only in those >12 mm, and invasive carcinoma in those >30 mm. (c) The correlation with age: 50 years for benign adenomas, 58 for the adenomas with malignant changes, and 64 for the invasive carcinoma. This is in parallel with what has been observed in adenoma-carcinoma sequence occurring in the colorectal region, where adenomatous residue is observed in up to 32% of cases, and in ampullary cancer, where an adenomatous component can be seen in up to 90% of cases.

There appears to be populational differences in the incidence of adenomas although lack of proper definition of adenoma and application of variable criteria hampers accurate comparison of data. In a Chilean series, only 32 cases in 21,412 cholecystectomies (0.14%) were reported to have "adenomas," suggesting the incidence of adenoma to be significantly lower in this population. However, 96% of these adenomas were of tubular type, which indicates that papillary type adenomas were either very uncommon or were excluded by these authors. On the other hand, most of the adenomas reported were small with an average size of 7.2 mm, and almost half being <5 mm, and only 28% >10 mm. Some authors would prefer to regard such small lesions (<5 mm) as polypoid metaplasia (precursor of precursor) and not classify them as "adenoma." These definitional issues aside, this data provides an interesting analysis of these lesions: Most cases were asymptomatic and only discovered by an ultrasound finding or during the pathological study of the specimen. The average age of patients with adenomas was not statistically different from those patients with non-neoplastic polyps (43 vs. 50 years). Two-thirds of the adenomas were solitary, and

localized to the distal half of the gallbladder. Twenty-five percent was associated with adenocarcinoma. The average age of patients with malignant change in adenoma was significantly greater than those without any malignant transformation (65 vs. 44 years), supporting the existence of a progression phenomenon. Five cases with intramucosal carcinomas were >10 mm and two <5 mm.

In summary, all the evidence suggests that the metaplasia-dysplasia pathway is the main culprit for most gallbladder cancers whereas the "adenoma-carcinoma" sequence accounts for only a small proportion of cancers arising in the gallbladder [50–52]. As in large intestine, the size of adenoma appears to be an important factor [50–54]. It is clear that molecular mechanisms of these two pathways appear to be distinct [37, 55]. For example, while p53 and p16 mutations are common in the conventional dysplasia pathway, they are fairly uncommon in adenomas. In contrast, adenomas often show beta-catenin pathway alterations (see below for detailed discussion).

General Considerations for Invasive Carcinoma

An important and distinctive aspect of invasive GB carcinoma is that it is difficult, if not impossible, to distinguish from cholecystitis or gallstones (with which it is commonly associated with), either clinically or macroscopically. A significant percentage of cases are undiagnosable as carcinoma until they are examined microscopically. For this reason, establishing appropriate protocols for examination of gallbladder specimens in pathology laboratories is crucial. The protocols employed have varied significantly in different countries, depending on the incidence of cancer in their population, which can vary from 0.14 to 6.1% [56–58], and accordingly have a tremendous impact on the medical practice. The approach established by one of us (JCR) in Temuco, Chile where GB carcinoma incidence is extremely high can be very helpful in this regard. According to this approach, any gallbladder specimen is examined by taking a full-thick section encompassing 80% of the gallbladder length, which allows the detection of more than 98% of neoplastic and preneoplastic lesions of the gallbladder [58]. This approach can then be modified depending on the increased risk based on the imaging findings, or the patient's risk factors. For example, more than half of the patients with PSC develop GB carcinoma and thus such cases should be examined more extensively.

Established prognostic factors in gallbladder cancer are: grade, depth of wall infiltration, and lymph node metastasis [59]. Tumor dissemination is via the lymphatogenous spread or peritoneal surfaces, but direct invasion into the liver is also common [60, 61]. This explains why the liver wedge resection plus lymphadenectomy with adjuvant chemotherapy is the best treatment currently known for PT2 (subserosal tumors) [62, 63].The most frequent histological type is adenocarcinoma, accounting for more than 98% of GBCs, among which almost two thirds are moderately or poorly differentiated. Other less frequent histological types have been described (as in the WHO classification), such as squamous/adenosquamous [64, 65], mucinous, sarcomatoid, and others. A spectrum of neuroendocrine neoplasms including carcinoids [66] and small-cell carcinomas [67, 68] are also seen. It should be kept in mind that lymphomas and secondary tumors such as metastatic melanomas and mesenchymal neoplasms also occur. Molecular aspects of these are beyond the scope of this chapter. Here, only the carcinomas specific to gallbladder and their precursor lesions will be discussed.

Molecular Genetic Alterations in Neoplastic Lesions

Existing information regarding the changes observed at the genetic and molecular level of gallbladder cancer is still limited. As in other organs, gallbladder carcinogenesis appears to be the product of the accumulation of multiple alterations at the genetic level [69–71]. Current data indicates the participation of oncogenes, tumor suppressor genes, and DNA repair genes among others. Most of these correspond to oncogene activation and the inactivation of suppressor genes through the loss of heterozygosity (LOH), and mutation, or methylation in the promoter genes. The most studied genes are: K-ras [72, 73], beta-catenin [55, 74] and the tumor suppressor genes p53 [75–77], $p16^{Ink4/CDKN2}$ [78, 79], Rb [80, 81], and more recently APC and DPC4 [79, 82, 83]. There are also studies on LOH, microsatellite instability (MSI), and DNA repair gene inactivation [78, 83]. Recently, data regarding epigenetic changes mostly in the form of altered methylation patterns have been reported. The search of biomarkers to help identify subgroups of patients with higher susceptibility to develop GBC is a real need. Studies on different polymorphisms have shown specific relationship with gallstone formation and GBC in different populations in high risk areas. However, more studies with larger numbers of cases are needed.

Oncogenes

K-ras

The ras family of genes (H-ras, N-ras and K-ras) is one of the most frequently altered groups of genes in human neoplasias, especially in glandular organs. The encoded proteins for

these genes are interrelated, conforming to a protein structure with a weight of 21 kDa that gives them the name p21. They have GTPase activity and participate in the signaling pathways for cell growth and differentiation [84]. Mutated p21 proteins are activated constitutively, and stimulate growth and differentiation autonomously [79, 82, 85, 86]. K-ras mutations typically (>80%) occur at codon 12, and correspond predominantly to transitions of guanine to adenine, and substitution of aspartic acid by glycine [72, 87]. Mutations in codons 60 and 61 are less common [52, 82, 88, 89]. The reported incidence of mutation has been variable, from negligible in few studies [78, 90] to 40–50% of the cases in others [78, 79, 82, 85, 86, 91]. Patients with an anomalous pancreatic-biliary union, present a greater frequency of K-ras mutation than in subjects without this condition, suggesting that the reflux of pancreatic juice might have a role in the carcinogenesis of this group [91, 92], since K-ras mutation is seen in >90% of pancreatic cancers.

p21/CDKN1A

The expression of cyclin-dependent kinase inhibitor p21 has been observed in about a third of gallbladder cancers. By itself, p21 has no known impact on the survival of patients; however, the immunohistochemical expression of p21 in combination with other factors such as p27 and p53 has correlated with survival, suggesting that p21 exerts its effects through interaction with other pathways. For example, patients with no p21 expression but p53 mutations, survive longer than those patients with coexisting p21 expression and p53 mutation. On the other hand, patients without expression of p21 but with expression for p27 have a better survival rate than those that co-express both p21 and p27 [93].

C-erb-B2

The C-erbB2 oncogene, located in chromosome 17, encodes an 185 kDa protein tyrosine kinase localized to the cytoplasmic membrane and exhibits great homology with the epidermal growth factor receptor. ERBB2 amplification and/or overexpression have been implicated in neoplastic progression in gallbladder carcinoma, associated with a worse clinical outcome. In a transgenic mouse model, overexpression of ERBB2 in the basal layer of biliary-tract epithelium led to the development of GBC in 100% of mice by 3 months of age. However, instead of conventional invasive carcinoma, the tumors developed in this model were characterized by adenoma and papillary neoplasms that filled the gallbladder lumen [94].

ERBB2 overexpression has been detected in 33–64% of GBCs [95–98], and gene amplification has been demonstrated in 70% of these tumors [96]. These findings indicate a role for HER-2 neu in a significant of GBCs. More studies are required on this topic, especially considering there are drugs available commercially that specifically block this protein.

Cyclin D1 and Cyclin E

These participate in tumorogenesis by promoting cell cycle progression, and they might have an important role in GBC carcinogenesis. Overexpression of the cyclin D1 and Cyclin E was detected by IHC in 41 and 49% of GBCs, respectively [71, 99, 100].

Tumor Suppressor Genes

TP53

TP53 has a key role in maintaining the integrity of the genome, and functions as an oncosuppressor by regulating the cell cycle, DNA synthesis and repair, cell differentiation and apoptosis. It has been shown that point mutations of TP53 typically occur in specific places (hot spots), and in vast majority of the cases, in exons 5–8. In general, TP53 mutation is considered to be a late event in carcinogenesis, detected much less frequently in precursor lesions, and progressively increasing in incidence from well to poorly differentiated carcinomas [75, 97, 101, 102]. The loss of heterozygosity of p53 has been shown in 92% of GBCs [81]. We have identified mutations in half of the cases we analyzed [77]. It may be interesting to note that, in the cases from Chile, only *transitions* were observed, as opposed to Japanese patients in whom both *transitions and transversions* were noted [75]. Accumulation of abnormal p53 protein that is demonstrable immunohistochemically, a surrogate evidence of TP53 mutation, has been identified in almost half of gallbladder carcinomas, with the reported incidence ranging from 27 to 70% [103, 104]. In contrast, p53 accumulation is seen in 20% of adenomas, and about 15% of cholecystitis cases. It should be noted that there are different cut-offs employed by different researchers to determine what constitutes "abnormal" accumulation of p53, and this may be responsible for some of the variances [105].

p16/CDKN2/INK4

The CDKN2A gene is a tumor suppressor gene located at 9p21, which encodes a protein known as p16 with active

participation in the control of the cell cycle. The progression of the cells from G1 to the S phase is regulated by the phosphorylation of the protein of the Rb gene (pRb) on the part of the cycline-cycline dependent kinase (CDK) complex. These complexes in turn are regulated by CDK inhibitors, such as the p16/^{INK4}. This protein acts on the cyclin D-CDK4 complex, preventing phosphorylation of the pRb and therefore the entry of the cell into the S phase. The inactivation of CDKN2A (p16) gene is a common event in human cancers [79] and occurs by three basic mechanisms: homozygous deletion, promoter hypermethylation, and mutation. In gallbladder carcinomas, deletions of this gene were observed in half of the cases [37, 78]. Kim et al. found mutation in exon 1–2 in 31% cases analyzed through PCR-SSCP, and it appeared to be more common in advanced stages [80, 103]. We have also observed inactivation of the p16 in 41%, either by LOH (11%) or methylation (24%) [52]. Immunohistochemically, p16 labeling was found in normal epithelial tissue of gallbladder cancer cases in 50–90% of cells, decreasing in the dysplasia-adenoma (about 50%), and lowest in carcinoma (10–50%) [78]. An absence of immunohistochemical labeling has been shown in tumors with methylation of the promoter area of the CDKN2A gene with allelic imbalance. Other studies have detected the loss in 75% of gallbladder cancers associated with an overexpression of the pRB [80]. The inactivation of the p16 gene has also been found to correlate with the mutation of the K-ras gene.

Fragile Histidine Triad Gene

The fragile histidine triad (FHIT) gene is located in chromosome 3p14.2, the common fragile site of FRA3B [106]. While intragene mutations in FHIT are very rare, abnormalities of 3p14.2 with allelic losses such as homozygous deletions have been identified in a variety of human cancers, rendering FHIT a candidate tumor suppresser gene [107–109]. In support of this, various groups have demonstrated that the introduction of wild-type FHIT gene suppresses tumorigenicity of cells [110]. In gallbladder cancer, recent studies have identified frequent allelic losses in the 3p region raising the possibility of FHIT involvement [111].

Other Tumor Suppressor Genes

Other candidate genes, some with known tumor suppressor function and some not yet characterized, have been detected in gallbladder cancers through allele typing. These include: 3p (20–52%), 5q21 (APC-MCC, 6–66%), 8p22–24 (22–44%), 13q14 (Rb, 20–30%), 18q22 (DCC 18–31%) [69, 112], and DPC4 (19%) [79, 111].

Microsatellite Instability (MSI)

Microsatellites are short and repetitive sequences of nucleotide bases that are widely distributed over the entire genome, especially in the areas that are not transcribed and with susceptibility for spontaneous mutations [113]. The correct replication of DNA including microsatellites is regulated through the DNA repair system (mismatch repair system; MMR) which is made up of at least six genes, the most important ones being MLH1, MSH2, PMS2, and MSH6 [114, 115]. When this system is deactivated, mainly by methylation or mutation, replication errors represented as MSI, occur. Inherited mutations of MLH1 and the MSH2 genes have been proven to be responsible for more than 90% of hereditary non-polyposis colorectal cancers (HNPCCs) [116]. MMR genes (mainly MLH1) are also involved in the development of a subset of sporadic colorectal, gastric, and endometrial neoplasms [117–120]. The majority of sporadic carcinomas with MSI have been demonstrated to be caused by somatic hypermethylation of the MLH1 promoter region, leading to its down-regulation [120, 121]. MSI is defined as any change in the length of a microsatellite compared to normal tissue due to insertions or deletions. The phenotypes for MSI has been defined as high-grade instability (MSI-H) when 30% or more of the markers show MSI, as low-grade (MSI-L) when <30%, and as microsatellite stability (MSS) when none of the markers studied show MSI. MSI can be important when microsatellites are found forming part of the genes related to cell proliferation and apoptosis, such as the TGFB RII, BAX, E2F4 and β2-microglobulin genes. The presence of MSI in these genes occurs in MSI-H cases and can be used as a screening test [122, 123].

As in other organs such as colon and stomach, MSI appears to be involved in only a small subset of gallbladder cancers [124–128]. In some studies, MSI was found in 20% of carcinomas, 40% of dysplasias, and in none of the adenoma cases [125, 129]. In our analysis in Temuco, Chile, by using the NCI panel of markers, we have found 10% (6/59) of the cases to be MSI-H. However, interestingly, in the patients with MSI-H carcinomas, MSI was also detected in the adjacent intestinal metaplasia and dysplasias, but not in normal epithelium. More interestingly, in over 90% of the instances, the pattern found in these preneoplastic lesions matched those found in the carcinoma, supporting the progression phenomenon [128].

Adhesion Molecules

Cadherin-catenin Complex

Beta-catenin is a key regulator of the Cadherin-Catenin cell adhesion system. Beta-catenin mutation and its altered immunohistochemical expression have been identified in

certain subsets of neoplasms in multiple organs. While it is very uncommon in gallbladder carcinomas and ordinary (flat) dysplasia, mutations involving exon 3 of this gene has been found in more than half of adenomas, further corroborating that adenomas represent a separate pathway of carcinogenesis than conventional dysplasia-carcinoma sequence [55, 130]. On the other hand, while mutations are uncommon in carcinomas, reduced expression of the cadherin-catenin complex has been observed in approximately a third of advanced gallbladder carcinomas [74] and cytoplasmic expression of beta-catenin has been found to correlate with better prognosis. This illustrates that regulation of cadherin-catenin system is highly complex and further studies are warranted to clarify their role in gallbladder carcinogenesis.

Cadherins are calcium-dependent proteins that form homotypic unions among the mucosal cells and are responsible for maintaining the normal architecture of the epithelium. Among these molecules, one of the best characterized is E-cadherin, a protein of 120 kDa that has been widely studied in gastric neoplasms [131]. In its cytoplasmatic portion, the E-cadherin is found joined to the cytoskeleton via a group of cytosolic proteins called α, β, γ catenins. Inherited mutation of E-cadherin is now identified as the cause of hereditary gastric cancer syndrome and responsible for a type of carcinoma characterized by poorly cohesive cells [132, 133]. In gallbladder neoplasia, including both types of precursor lesions of adenomas and dysplasia, the loss of E-cadherin and its cytolosolic companions have been found to occur, but the true incidence and significance of these events have yet to be characterized.

ICAM-1/CD54

The intercellular adhesion molecule (ICAM) is a glycoprotein of 90 kDa, located at the cell surface and belongs to the superfamily of immunoglobulins. It is believed to have an important role in cell–cell adherence, cell–matrix interactions as well modulation of inflammatory response [134]. While under normal conditions its expression is mostly in the cytokine-activated endothelial cells, it has also been detected in carcinoma cells of various organs including pancreas [135], colon [136], and stomach [137]; and in some studies, has been found to correlate with higher incidence of metastasis [137, 138]. In the gallbladder, its exrpession was detected in 14% of adenomas and in 39% of carcinomas, and this expression appeared to correlate with advanced stage [139].

CD44

The CD44 gene, located in chromosome 11, encodes for a group of transmembrane glycoproteins involved in cell–cell and cell–matrix interactions. There are many isoforms of CD44, with the standard form of 89 kDa and found in normal epithelial and hemapoietic cells. Alternative variants that range in size from 120–150 kDa are predominantly expressed in epithelial organs, and called CD44v (variant). The expression of CD44v has been found to correlate with progression and higher incidence of metastasis in gastric [140] and breast cancer [141]. In the gallbladder, CD44 expression is relatively low; moderate increases are recorded in metaplastic lesions and adenomas, but not in carcinomas. In contrast, CD44v6 expression is not detected in normal tissues but is present in 15% of adenomas and up to 40% of carcinomas [142, 143]. More studies are warranted to investigate the role of CD44v6 in this organ.

CD99 (Mic2)

This is a transmembrane glycoprotein of 32 kDa that is involved in cell–cell adhesion during hematopoietic differentiation [144], apoptosis of immature thymocytes [145] as well as transmembrane protein transport [146]. It is expressed in only some human tissues, particularly in cortical thymocytes, pancreatic islets, and leydig and sertoli cells. MIC2 overexpression has been detected in sarcomas, in particular, those with neuroectodermal differentiation [147], as well as in some epithelial malignancies including ovarian and gastric [148]. In the gallbladder, it has been detected in 95% of normal gallbladders, 88% in metaplasia, 86% in adenoma, and 67% in carcinoma. These results suggest that the loss of CD99 expression is an event associated with gallbladder carcinogenesis at later stages.

CEA

Carcinoembryonic antigen (CEA) is a 180 kDa glycoprotein that can be detected in trace amounts in the cytoplasmic membrane of glandular epithelial cells. In the gallbladder, while it was detected uncommonly in normal epithelium, its expression increased progressively from 11% in metaplasia to 33% in low-grade dysplasia, and up to 71% in carcinoma [149, 150]. However, there have been few studies on the clinical significance of CEA in gallbladder cancer [139].

NCAM/CD56

Neural cell adhesion molecule, CD56, is a membrane glycoprotein whose primary function is to mediate interactions between neural cells; however, its expression is not limited to neuroendocrine system and has been detected in carcinomas of breast, lung, colon, and endometrium [151–153]. CD56 is

thought to have tumor suppressing activity [152] and reduction in its expression has been found to correlate with tumor metastasis [154]. In the gallbladder, Choi et al. [139] observed CD56 expression in a small subset of carcinoma (10.9%) and substantial percentage of adenoma (50%), but not in normal gallbladder. The significance of this is yet to be determined.

Epigenetic Alterations

Hypermethylation of promoter genes is a common epigenetic mechanism implicated in inactivation of tumor suppressor genes leading to neoplastic transformation. In some tumor types, it has been found to correlate with prognosis, and response to treatment. In a recent study of 109 advanced gallbladder cancers in our laboratory in Temuco, Chile (manuscript in preparation), we found significant association with survival in the methylation of genes p73 ($p<0.006$), *MGMT* ($p<0.006$), *DCL1* ($p<0.044$), as well as a trend for genes *CDH13* ($p<0.06$) and *FHIT* ($p<0.1$). In deeply invasive carcinomas, a methylation index of >0.4 was associated with worse clinical outcome ($p<0.001$). Separately, in multivariate analysis, hypermethylation of *MGMT* was found to be an independent prognostic factor ($p<0.01$) [7]. Other studies conducted on gallbladder cancer have shown a high frequency of methylation in *SEMA3B* (92%) and FHIT (66%), an intermediate incidence in *BLU* (26%) and *DUTT1* (22%), and a very low frequency in *RASSF1A* (8%) and *hMLH1* (4%) [155].

Genetic Susceptibility, Polymorphisms, and Biomarkers

Genetic susceptibility for and familial aspect of cancers are topics of great interest. This issue is of particular importance in high prevalence areas such as Temuco, Chile, where the incidence of gallbladder cancer is very high along with the high incidence of cholecystitis and cholelithiasis. It is a challenge to identify the group of patients that require more intense clinical attention, and perhaps prophylactic cholecystectomy, and genetic susceptibility analysis could potentially play a crucial role in the development of such management algorithms. Having serological biomarkers available would make it possible to prioritise surgical intervention in some selected groups.

Several groups have studied genetic polymorphisms and have found that CA242 and CA125, when used together, achieved best sensitivity and specificity for discriminating patients with gallbladder carcinoma from those with cholelithiasis [156]. Heparanase and HIF-1alpha are frequently expressed in gallbladder carcinoma and are associated with decreased survival [157]. It appears that "D" allele of lipoprotein receptor associated protein (LRPAP1), insertion/deletion polymorphism, NAT2 slow acetylator phenotype, and the X(+), D haplotype of APOB may have a role in susceptibility for GBC, with the risk being independent of the presence of gallstones [158–160]. The Val allele of the CYP1A1 Ile462Val polymorphism and the Pro allele of TP53 Arg72Pro polymorphism have also been implicated in increased risk of GBC among Japanese women and men, respectively [161], which shows that ile/val genotypes and val allele of GSTP1 are associated with increased risk of gallbladder cancer [162]. The hOGG1 Ser326Cys polymorphism has been found to be associated with an increased gallbladder cancer risk [163], but these isolated reports must be validated in larger studies.

Inflammation and Molecular Carcinogenesis

Studies have shown that more than 15% of cancer formation is related to infection and chronic inflammation, presumably due to infection factors that can activate oncogenes and change the biological features of cells [164].

In the gallbladder and biliary tract, this association appears to be even stronger than in any other organ. Inflammation and injurious agents, in particular, gallstones, primary sclerosing cholangitis, obstructive agents such as parasites, all have very strong ties to the cancer formation, both at epidemiologic and clinical levels. In fact, gallbladder and biliary tract cancers are probably the best examples of inflammation-carcinoma association, providing better support than any organ that carcinogenesis may in fact be reparation that has gone "wrong." For this reason, it is often quite difficult in this area to determine what constitutes inflammation-related alterations from those representing true neoplastic transformative changes. Thus, it is also not surprising that many of the molecular alterations discussed in this chapter often begin to show their faces in inflammatory lesions and regenerating gallbladders.

Mitochondrial DNA Mutations (mt DNA)

mt DNA mutations in humans are presumed to result from the action of reactive oxidized molecules produced during inflammation. Such mutations have been observed frequently in human neoplasia [165], in both coding and noncoding regions. A mononucleotide repeat (poly-C) between nucleotides 303–315 (D310), within the regulatory displacement loop, has been identified recently as a frequent hot spot of deletion/insertion mutations in neoplasms. D310 mutation at the mt DNA displacement loop appears to be a relatively

frequent and an early event in the sequential pathogenesis of GBC, detected in non-neoplastic epithelium from chronic cholecystitis [166].

Inducible Nitric Oxide Synthase (iNOS)

Macrophages are important for early immune responses to invading micro-organisms and the production of nitric oxide (NO) is central to this function. NO is generated by inducible nitric oxide synthase (iNOS) after exposure to certain cytokines, such as interferon-gamma (IFN-γ). iNOS is a soluble enzyme that does not require elevated intracellular levels of Ca2+ to be activated [167]. Recently, studies have demonstrated that NO has various functions in neoplastic development and has antitumor activity through the following mechanisms: (1) interferes with energy metabolism in the tumor cell [168], (2) inhibits protein synthesis [169], (3) damages DNA directly or indirectly, (4) induces apoptosis in tumor cells.

Jadeski et al. [170] found that NO derived from iNOS makes the cell cycle halt. In chronically injured tissue, the excessive NO produced by iNOS inhibits the proliferation of bacteria, viruses, as well as tumor cells. On the other hand, NO may also act as an important factor in tumor induction. Ohshima and Bartsch [171] found that NO produced by inflamed tissues can damage the cell, rupture the DNA and mutate the genes, followed by the development of cancer. Moreover, NO can promote neoplastic progression and metastasis by stimulating angiogenesis and adjusting the blood flow in the neoplastic tissue [172].

There are not many studies on iNOS expression in the gallbladder, but Zhang et al. [173] found that the degree of iNOS immunohistochemical expression was: 88% in chronic cholecystitis, 100% in cholecystitis with adenomyoma, and 71% in adenocarcinomas. It is speculated, based on this data, that the excessive NO induced by iNOS was an important instigating factor for neoplastic transformation. On the other hand, NO may have an antitumor effect as well; low expression of iNOS in gallbladder adenocarcinomas has been associated with early metastasis and poor prognosis. This illustrates the complex role of NO in cellular pathways, and its potential dual effect in both inhibition and progression gallbladder cancer. Clearly, more studies are needed.

Cyclooxigenase-2 (COX-2)

Prostaglandins (PGs) are arachidonic acid derivatives that act as autocrine or paracrine mediators contributing to tumorigenesis, by altering cell proliferation, differentiation, and adherence as well as by modifying the vascular response and immune surveillance. The crucial step in the conversion of free arachidonic acid into PGs is catalysis by the cyclooxigenase (COX) enzyme. Three COX isoforms have been identified (COX-1, COX-2, and COX-3). There is little information about the COX-3 isoform [174]. While COX-1 is constitutively expressed in the majority of cells, the expression of COX-2 is largely limited to the kidney, liver, and pancreatic islets. However, COX2 is typically activated as an early response to many injurious stimuli mediated by inflammatory cytokines, growth factors, and oncogenes. Evidence suggests that elevated level of PGs by overexpression of the inducible form of COX-2 is an important factor in the development of human cancer. Legan et al. [175] have shown that COX2 expression increases progressively from 14% in normal gallbladder epithelium to 45% in low-grade dysplasia and 88% in high-grade dysplasia. The incidence in invasive carcinomas in the same study was 59%. Other studies have detected even higher incidence in invasive carcinomas, at a range of 70% [147, 176]. COX-2 expression also appeared to correlate with higher grade tumors as well as p53, an important factor in gallbladder carcinogenesis [177]. There is evidence that the expression of COX-2 can be suppressed by p53, suggesting that the loss of p53 function can influence the overexpression of COX-2 [177]. Inversely, COX-2 can occasionally inhibit p53 depending on the type of transcriptional activity [178].

Others Genes

Angiogenesis and Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a growth factor that induces a mitogenic effect on endothelial cells [62] in response to ischemia or hypoxia and has also been implicated in tumor neo-angiogenesis, an important factor in tumor maintenance and progression. VEGF also causes increased vascular permeability, which leads to fibrinogen extravasation into the tumor stroma, which in turn facilitates formation of new vascularity [179]. This neovascularization is believed to have a role in lymphatic and hematogenous metastasis in carcinomas, including those of gallbladder [180, 181]. Tian et al. [104] have shown a progressive increase in VEGF expression from 17% in chronic cholecystitis, to 35% in adenomas, and up to 75% in carcinomas of gallbladder. Quan et al. have extended this observation to the stage of GBC, with 42% of Nevin stage S1–S2 cases showing VEGF expression, while the incidence of S4–S5 cases is at 77% [103]. The relation between VEGF and p53 has also been scrutinized and it was found that there is a correlation between the two [104, 168]. This relationship might be important, considering that p53 plays a vital role in suppressing the growth of the tumor by regulating its angiogenesis, and that VEGF is an active factor that works in coordination with p53.

hTERT/Telomere Aging and GBC

Telomeres are specialized structures located at the ends of eukaryotic chromosomes and have various functions, the two most important ones being protection of chromosome from exonuclease attack and prevention of errors during chromosomal DNA recombination [182]. In humans, they typically consist of 5,000–15,000 base pairs and are found in TTAGGG tandem repeat sequences, associated with proteins joined to the DNA. The telomerase is a ribonucleoprotein enzyme that catalyzes the addition of telomere repeats (TTAGGG) to telomeres. This activity takes place in germline cells, but also in a wide variety of malignant tumors. By contrast, it is usually repressed in many somatic cells, except in some tissues with a strong potential for regeneration, such as hematopoietic and epidermal cells [182, 183]. Three major subunits have been identified that comprise the telomerase complex in humans: the RNA component known as hTR (human telomerase RNA), an associated protein called TEP1 (telomerase-associated protein), and the catalytic subunit called hTERT (human Telomerase Reverse Transcriptase). hTR and TEP1 are expressed constitutively with no distinction as to the conditions of the tissue, unlike hTERT which is found predominantly in neoplastic tissues. Recent data suggests that hTERT is the determining factor in telomerase activity [182] and that the ectopic expression of hTERT in cells where no telomerase activity is detected (somatic cells), is sufficient to reconstitute holoenzyme activity [184], thus extending the life of normal human cells, even leading to immortality [185]. Presence of telomerase in various types of human cancers and its absence in many normal cells may offer an advantage in cancer treatment. Targeted therapies can be developed in which hindering telomerase could kill tumor cells (inducing a reduction in telomeres) without altering the operation of many normal cells [186].

In the gallbladder, Luzar et al. [187] have analyzed the presence of hTERT and detected progressive increase from 3% in the normal gallbladder to 4% in regenerative epithelium, 25% in low-grade dysplasia, 82% in high-grade dysplasia, and finally, 93% in adenocarcinomas. This was largely confirmed in other studies: the expression was very low in benign lesions while it was significantly high in invasive cancers, detected in more than two thirds of the cases [188, 189].

Conclusion

Gall bladder cancer presents various contrasting challenges. First, while it is relatively uncommon in developed countries, diagnosed mostly incidentally, in some geographic regions, such as Temuco, Chile, where one of the authors of this chapter practices (JCR), gallbladder cancer is a major public health problem. Questions remain as to whether the natural history and range of pathology is the same in these high and low prevalence areas. Comparative molecular and genetic susceptibility analysis on these cancers will likely shed new light to the mechanisms of carcinogenesis, not only in the gallbladder, but in other epithelial organs as well.

Another important aspect of gallbladder cancer is its close association with inflammation and injurious agents, in particular, gallstones. Perhaps, more so than in any other organ system, the gallbladder offers a unique model of inflammation-carcinoma association.

Gallbladder is also interesting by the type of precursor lesions occurring in this organ. Experimental and clinical evidence supports the existence of two distinct pathways of carcinogenesis in this organ. Most carcinomas develop through the "metaplasia-dysplasia-carcinoma sequence" ("flat" intraepithelial neoplasia). The second pathway is represented by exophytic, mass-forming preinvasive lesions (adenoma-carcinoma sequence), which is less well characterized and is responsible for a smaller subset of the cases. The molecular mechanisms of these two pathways appear to be highly distinct. Thus, they offer an interesting model of carcinogenesis for cancer researchers to scrutinize.

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Chapter 62
Current and Future Methods for Diagnosis of Neoplastic Liver Disease

Arief A. Suriawinata, Michael Tsapakos, and Gregory J. Tsongalis

Introduction

One of the largest of the visceral organs, the liver provides numerous critical functions for homeostatic balance in the human body. In this regard, variations to liver function through biochemical, infectious, autoimmune, and neoplastic mechanisms result in abnormal and/or disease phenotypes. Disease processes which affect the liver can result from a wide variety of insulting agents, and abnormal liver function can be detected through numerous diagnostic technologies and methods.

In this chapter, we will focus on the diagnosis of neoplastic liver disease as a challenging and multidisciplinary approach to patient management through traditional pathologic assessment and radiologic methods. The focus of these diagnostic methods will be exemplified by the discussion of two clinical cases followed by a discussion of future molecular diagnostic capabilities.

Case Presentations

Case 1

Clinical Background

This individual is a 50-year-old white female with previous history of a right renal cell carcinoma, which was resected with clear margins. She now presents with abdominal pain and diarrhea for the past several months which led to several emergency room visits. A subsequent computed tomography (CT) scan showed multiple lesions in the liver and a panendoscopy showed an infiltrative, ulcerative, submucosal mass. Chromogranin A levels were elevated to >10× the upper limit of normal.

Radiologic Assessment

Intravenously enhanced abdominal CT demonstrated the presence of multiple, varied sized enhancing hepatic masses, best seen on the arterial phase of imaging. Some of these masses demonstrated central decreased attenuation. These hypervascular liver lesions were on a background of a hypodense liver attenuation, compatible with diffuse fatty change (Fig. 62.1). The differential for these lesions would include, but not be limited to, metastatic carcinoid. Other possibilities would include metastatic neoplasms, such as melanoma, renal cell carcinoma, and breast cancer.

Pathologic Assessment

A fine needle aspiration (FNA) biopsy yielded cellular material with cytologic features of well-differentiated neuroendocrine or carcinoid tumor. This was confirmed by immunohistochemical stains performed on a cytology cell block (Fig. 62.2). The cellular aspirate contained cords of small uniform cells with visible cytoplasm and nuclei with stippled chromatin (Fig. 62.2a). Positive cytokeratin staining confirmed the epithelial origin of the tumor (Fig. 62.2b). Two common neuroendocrine differentiation markers, synaptophysin (Fig. 62.2c) and chromogranin (Fig. 62.2d), were also positive. These findings were diagnostic of well-differentiated neuroendocrine or carcinoid tumor.

Case 2

Clinical Background

This individual is a 65-year-old white male with a history of colon cancer for which he underwent a total abdominal colectomy. One of ten lymph nodes was positive for metastatic

G.J. Tsongalis (✉)
Department of Pathology, Dartmouth Medical School, Dartmouth Hitchcock Medical Center and Norris Cotton Cancer Center, Lebanon, NH, USA
e-mail: Gregory.j.tsongalis@hitchcock.org

S.P.S. Monga (ed.), *Molecular Pathology of Liver Diseases*, Molecular Pathology Library 5,
DOI 10.1007/978-1-4419-7107-4_62, © Springer Science+Business Media, LLC 2011

tumor. Seven months later, a follow-up CT scan showed a 4-cm mass in the right lobe of the liver. A positron emission tomography (PET) scan was performed that showed no evidence of additional lesions and a diagnostic biopsy was also performed.

Radiologic Assessment

The assessment was performed post-ablation with the image demonstrating a pig-tail catheter in the liquefied post-ablation site, which had become infected (Fig. 62.3).

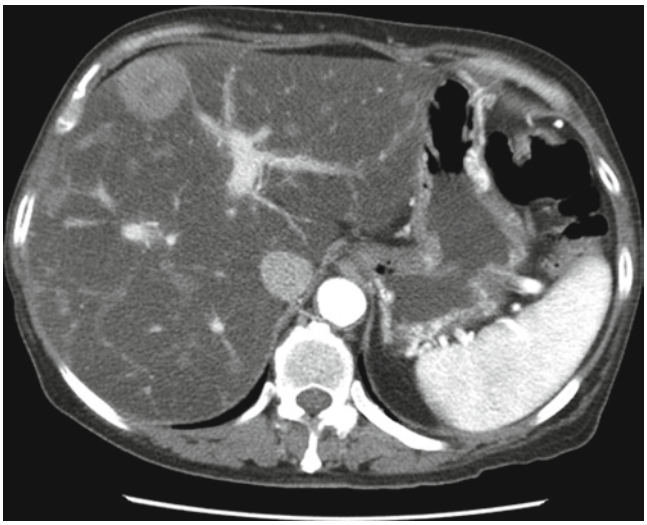

Fig. 62.1 Intravenously enhanced abdominal CT

Pathologic Assessment

A CT-guided needle biopsy revealed metastatic tumor with histologic features consistent with metastatic colonic adenocarcinoma and morphologically identical with the patient's primary colonic adenocarcinoma.

The needle biopsy showed complex neoplastic glandular structures consisting of cuboidal to tall columnar cells with mucin and necrotic/apoptotic debris in the luminal space (Fig. 62.4a). Benign liver parenchyma was seen in a separate fragment in the left field of the figure. Nuclear reactivity for CDX-2 by immunohistochemistry confirmed intestinal differentiation and origin of the tumor (Fig. 62.4b). Furthermore, coordinate negative staining of cytokeratin 7 (Fig. 62.4c) and positive staining for cytokeratin 20 (Fig. 62.4d) were consistent with metastatic colonic adenocarcinoma.

Radiologic Approaches to Diagnosing Neoplastic Liver Disease

Sonographically, the appearance of cancer in the liver, including hepatocellular carcinoma (HCC), is variable and nonspecific with small lesions exhibiting uniformly hypoechoic echotexture, and large lesions being more heterogeneous. Suggestive features of HCC include high arterial blood flow with a peripheral hypoechoic border.

With CT and magnetic resonance imaging (MRI), HCC can be best demonstrated during the arterial (30–35 s) and portal venous phase (65–70 s) after intravenous contrast

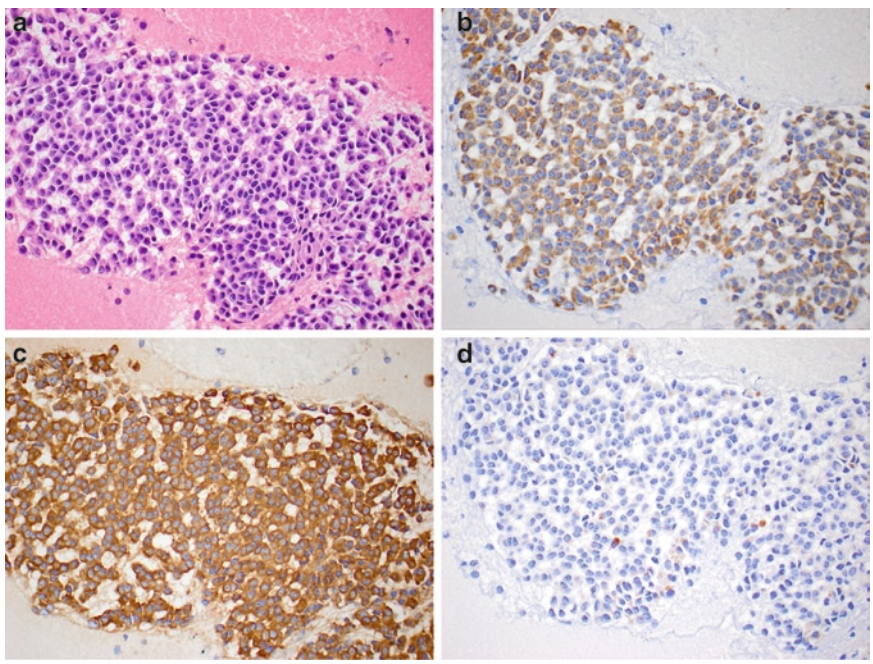

Fig. 62.2 (**a**) Cellular aspirate containing cords of small uniform cells with visible cytoplasm and nuclei with stippled chromatin. (**b**) Cytokeratin staining positivity confirmed epithelial tumor. (**c**, **d**) Positive immunhistochemical staining for two common neuroendocrine differentiation markers, synaptophysin (**c**) and chromogranin (**d**)

administration [1]. HCC is a tumor which receives its blood supply from the hepatic artery, resulting in rapid enhancement. As the grade of malignancy increases, increased enhancement during the arterial phase is seen, due to increasing tumor neovascularity and increased hepatic arterial blood supply [2, 3]. This has to do with the number of portal triads which are disrupted [4]. Well-differentiated HCCs tend to enhance poorly during the arterial phase, whereas poorly differentiated HCCs will enhance briskly [4]. Small (moderately or poorly differentiated) HCCs (< or equal to 2 cm) will enhance homogeneously, whereas larger tumors enhance more heterogeneously, often resulting in a mosaic pattern [5]. The mosaic pattern represents areas of necrosis in confluent small tumor nodules with areas of internal septation. In addition to heterogenous enhancement during the portal venous phases of CT or MRI post-intravenous (IV) contrast imaging, other suggestive features can include central fibrosis, capsular enhancement, and fatty change, the latter of which can be more clearly seen on MRI than on CT [6, 7]. Although central fibrosis and the presence of internal septations are not specific to HCC, their enhancement pattern can greatly aid in the discrimination of HCC from other hepatic benign lesions, such as focal nodular hyperplasia (FNH), hepatic adenoma, and hepatic hemangiomas [8]. HCC frequently invades the portal vein resulting in arterioportal shunting. This shunting is best identified on CT [9, 10] or MRI. Approximately 5–10% of HCCs can contain calcification [11, 12].

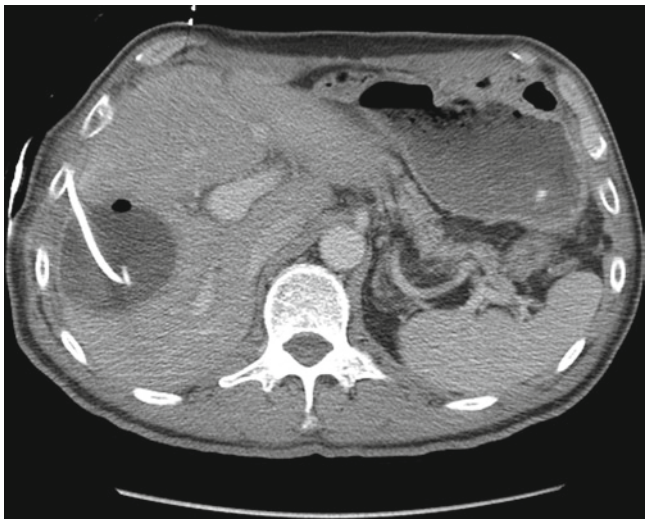

Fig. 62.3 Image demonstrating a pig-tail catheter in the liquefied post-ablation site

In the cirrhotic liver, CT and MRI have been reported to have approximately the same sensitivity and specificity for HCC detection, ranging from approximately 35 to 70% [13]. The introduction of 64-slice CT and similar improvements in MRI techniques have given rise to the problem of frequent detection of enhancing lesions that are too small to characterize in the cirrhotic patient. Early results suggest that the detection of HCC in cirrhotic livers is increased with MRI liver-specific contrast agents, specifically, gadobenate dimeglumine [14, 15]. In addition to having the vascular enhancement properties of other gadolinium-based MRI contrast agents, a small amount of gadobenate dimeglumine is secreted into the hepatobiliary tree [16], enabling delayed hepatobiliary

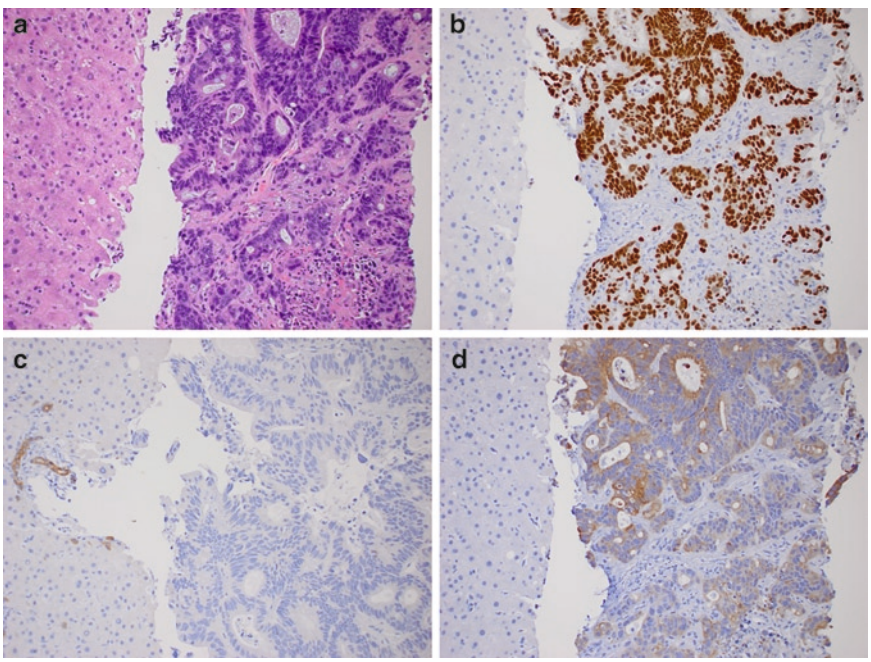

Fig. 62.4 (**a**) The needle biopsy showing complex neoplastic glandular structures and necrotic/apoptotic debris in the luminal space. (**b**) Immunohistochemical stain for CDX-2 showing a positive nuclear pattern. (**c**) Negative immunostaining for cytokeratin 7 and (**d**) positive staining for cytokeratin

phase imaging. Early studies have shown that the combined dynamic and hepatobiliary phase imaging with gadobenate dimeglumine in MRI results in improved lesion detection and characterization compared to either dynamic MRI or multidetector CT [14].

Pathologic Assessment of Neoplastic Liver Disease

Current Methods in Obtaining Liver Tissue for Diagnosis

The development of noninvasive tests in recent years, including serologic tests and clinical imaging, has increasingly been replacing liver biopsy in the diagnosis of liver disease [17]. Nevertheless, direct histopathologic examination of the liver morphology through liver biopsy remains as the "gold standard" in the diagnosis and management of liver diseases and space-occupying lesions. Furthermore, ancillary studies to enhance or confirm the diagnosis can be ordered at the same time of liver biopsy submission.

The utility of liver biopsy ranges from the evaluation of abnormal liver function tests to the diagnosis of space-occupying lesions (Table 62.1). Today, a significant proportion of liver biopsies are performed for the evaluation of chronic viral hepatitis and fatty liver disease to assess disease activity and the degree of liver damage (grading and staging) and/or response to therapy, prompted by increased awareness and the availability of new therapies for these diseases; whereas ultrasound/CT-guided liver biopsy is commonly performed for the diagnosis of space-occupying lesions [18]. Over the decades, the techniques of liver biopsy have evolved to include percutaneous, transjugular/transvenous, open, laparoscopic, and ultrasound/CT-guided biopsies (Table 62.2).

Although relatively safe, liver biopsy is an invasive procedure and therefore the indication, goal, techniques, and their limitations should be carefully considered to avoid performing unnecessary procedures and/or to avoid preparation of a sample that cannot provide the necessary answer. Major complications of liver biopsy include bleeding and bile leakage. In addition, as many as one-third of patients experience right upper-quadrant pain and/or shoulder pain, which can be severe in 1–3% of cases [19]. The mortality rate among different techniques in general is approximately 0.01%. Rare complications of liver biopsy include hemobilia, pneumoperitoneum, pneumoscrotum, pneumothorax, septic shock, subphrenic abscess, and intrahepatic arteriovenous fistula. There is a minimal risk of hematogenous dissemination of malignant cells after liver biopsy. Although rare, seeding in a needle tract has been reported in HCC and metastatic colorectal carcinoma [20, 21]. A major contraindication to percutaneous liver biopsy is significant coagulopathy. Relative contraindications to percutaneous liver biopsy are morbid obesity and severe ascites. In these cases, transjugular biopsy is an alternative.

Table 62.1 Utility of liver biopsy

Evaluation of abnormal liver function tests
Evaluation of fever of unknown origin and systemic disease
Evaluation of jaundice of unknown origin and cholestatic disorders
Evaluation of portal hypertension and ascites
Recognition of hereditary and metabolic disease
Evaluation of abnormal serum iron study
Grading and staging of chronic liver diseases
Monitoring the response and side effects of therapy
Diagnosis of space-occupying lesions
Evaluation of uninvolved liver in tumor cases to determine feasibility for resection
Pre- and post-transplant evaluation
Obtaining tissue for ancillary or molecular studies

The routine fixative for liver biopsies is 10% neutral buffered formalin (NBF). The advantages of routine formalin fixation are its stability, ability to penetrate and fix tissues well, low cost, and that it allows for subsequent application of most histochemical, immunohistochemical, and molecular pathologic analyses when necessary. In addition, the characteristics of tissues fixed in formalin are well known among pathologists. Immersion of biopsies in saline will lead to discohesion of cells; therefore, when fresh, unfixed tissue is needed for ancillary studies, tissue should be submitted separately in both saline and formalin. See Table 62.3 for a list of fixatives for ancillary studies.

Ultrasound/CT-guided FNA biopsy has become the preferred method for the diagnosis of a space-occupying lesion and confirmation of a suspected malignancy [22]. In addition to its diagnostic utility, FNA biopsy can also be used to drain a cyst or abscess for culture and fluid analysis, or it can be followed by therapeutic ablation for malignant tumors. FNA is considered safe, accurate, and cost effective as it can be performed on an outpatient basis. It provides immediate interpretation and assessment of adequacy of liver biopsy by using smears or touch preparation. Routine supplementation with a cell block of tissue fragments increases its diagnostic accuracy, particularly in benign conditions or benign hepatocellular tumors, and provides material for immunohistochemical stains and ancillary studies for primary or metastatic malignant tumors [23, 24].

Current Methods in the Diagnosis of Liver Diseases and Tumors

Hematoxylin and eosin (H&E) stain is the standard stain of almost all liver biopsy specimens. It provides overall impression of the liver parenchyma and in most cases allows accurate

Table 62.2 Liver biopsy methods

Methods	Technique	Comments
Percutaneous biopsy	Core of liver tissue obtained by suction needles (Menghini, Klatskin, and Jamshidi) or cutting needles (Vim-Silverman and Tru-cut)	The most common, least expensive, and least invasive liver biopsy providing an adequate specimen for review and ancillary studies. It is commonly used for diffuse liver disease or easily and safely accessible lesions
Transjugular/ transvenous biopsy	Core of liver tissue obtained by a catheter through the internal jugular vein and superior vena cava	Second-line procedure in patients with coagulation disorders, severe thrombocytopenia, gross ascites, infectious peritonitis, clinical severe obesity, or fulminant hepatic failure. The main disadvantage of the technique is smaller size and often fragmented specimens
Laparoscopic or open biopsy	Direct visualization of the liver and peritoneal cavity. Core needle or wedge biopsy	The largest specimen; therefore, it is more sensitive in the diagnosis of cirrhosis and chronic liver or biliary diseases, and is useful for the diagnosis or staging of a malignant neoplasm. Recent development of laparoscopic bariatric surgery for obesity has increased the number of core or wedge intraoperative biopsies for fatty liver disease evaluation. Wedge biopsy is useful for focal lesions at the liver capsule or immediately subcapsular
Computed tomography (CT) or ultrasound guided biopsy	<1 mm needle is usually used (fine needle aspiration), or larger needle. Ultrasound or CT is used for visualization of hepatic lesion	For histologic or cytological diagnosis of space-occupying lesion. Immediate interpretation and assessment of adequacy by smears or touch preparation can be performed by a cytopathologist

Table 62.3 Histochemical stains in liver pathology

Histochemical stains	Utility
Connective tissue	
Masson's trichrome	Identification of type I collagen (blue). Evaluation of fibrosis
Sirius red	Identification of type I collagen (red). Evaluation of fibrosis
Gordon and sweets reticulin	Identification of type III collagen (reticulin fibers – black). Evaluation of early fibrosis, architectural alteration, and nodule formation
PTAH (phosphotungstic acid hematoxylin)	Identification of fibrin (blue)
Microorganisms	
Ziehl–Neelsen	Mycobacterium (red)
Victoria blue	Hepatitis B surface antigen (blue)
Shikata's orcein	Hepatitis B surface antigen (dark brown)
Ammoniacal silver	Fungi, bacteria, mucin (black)
Pigments and minerals	
Perls' iron	Iron (blue). Semiquantitative evaluation in hereditary hemochromatosis and secondary iron overload
Hall's	Bilirubin (green). Identification of bile in cholestatic diseases
Rhodanine	Copper (reddish-orange). Identification of copper in chronic cholestasis and Wilson's disease
Glycogen	
Periodic acid Schiff	Glycogen, fungi (magenta)
Periodic acid Schiff with diastase digestion	Glycoprotein, basement membrane, alpha-1-antitrypsin, atypical Mycobacterium, ceroid laden macrophages (magenta)
Amyloid	
Congo red	Amyloid (red) detection in amyloidosis
Lipids	
Oil Red O	Fat detection (red)

histologic diagnoses of liver diseases and tumors. In addition to the routinely performed H&E stain, other immunohistochemical stains are frequently requested to confirm structures or findings seen or suspected on the H&E slide (Table 62.4).

In the last three decades, the development of monoclonal antibodies and highly sensitive immunohistochemical staining procedures has made it possible to demonstrate many antigens in routinely processed tissue sections. These immunologic reactions are performed in liver sections for (a) detection of viral antigens, (b) detection of normal and abnormal structures, (c) identification and classification of primary and metastatic liver tumors, (d) prognostic factors in malignant tumors, (e) lymphoma/leukemia immunophenotyping, (f) identification of bile duct epithelium, and (g) identification of pathological cellular structures. Table 62.4 lists commonly performed immunohistochemical stains in liver pathology. On the precautionary note, when the avidin–biotin–peroxidase complex method is used, an augmented cytoplasmic reaction may yield a false-positive result due to the significant amount of inherent endogenous biotin in hepatocytes.

In space-occupying lesions, biopsies are performed mainly to distinguish primary from metastatic liver tumors. Metastatic tumors to the liver are the most common malignancy in the liver, far exceeding primary tumors of the liver. The distinction between a primary and a metastatic tumor in the liver is both of therapeutic and prognostic significance, and knowledge of the primary tumor site and its morphology if available is important in evaluating and comparing it to its metastasis. When facing a tumor of unknown primary origin, immunohistochemical stains are

Table 62.4 Immunohistochemistry stains in liver pathology

Immunohistochemical stains	Interpretation
Detection of viral antigens	
Hepatitis B surface antigen (HBsAg) and Hepatitis B core antigen (HBcAg)	Confirmation of hepatitis B infection and evaluation of the status of HBV replication (HBcAg). Cytoplasmic and membranous HBcAg positivity indicate high levels of viral replication and corresponds to high disease activity. Visual aid to the identification of ground glass hepatocytes (HBsAg)
Hepatitis delta virus (HDV)	Confirmation of HDV superinfection in HBV-infected patients
Nonhepatotropic viruses (cytomegalovirus [CMV], herpes simplex, Epstein–Barr virus [EBV], adenovirus)	Identification of liver involvement by nonhepatotropic viruses, particularly in immunocompromised patients and infants
Hepatitis C virus	No reliable immunohistochemical stain currently available
Identification and classification of tumors	
Hepatocyte paraffin 1 (HepPar1)	Hepatocellular differentiation
Alpha fetoprotein (AFP)	Positive in approximately 40% hepatocellular carcinomas (HCC) and in all hepatoblastomas and fetal livers. Hepatocytes in cirrhotic nodules may occasionally show focal positive staining
Polyclonal CEA (pCEA)	Bile canaliculi staining or canalicular differentiation in HCC. Cytoplasmic and membranous staining in adenocarcinoma
CD10, villin	Bile canaliculi staining or canalicular differentiation in HCC
Monoclonal CEA (mCEA)	Positive in 60% adenocarcinoma. Negative in HCC
MOC-31	Positive in 80% adenocarcinoma. Negative in HCC
Cytokeratins	Coordinate CK7/CK20 staining is commonly used to suggest organ of origin, both are negative in HCC. Hepatocytes are positive for CK8 and CK18. CK7 and CK19 are positive in the presence of cholangiocellular differentiation
Thyroid transcription factor (TTF)-1	Cytoplasmic staining may indicate hepatocellular differentiation
Epithelial membrane antigen (EMA)	Negative in HCC. Positive in adenocarcinoma
Factor VIII-related antigen, CD31 and CD34	Vascular tumors. Sinusoidal endothelial cells are negative in normal liver, but positive in chronic liver diseases and HCC
Synaptophysin, chromogranin, and CD56	Neuroendocrine tumors, including carcinoid, small and large cell neuroendocrine carcinomas
Glypican 3	Positive in HCC and high-grade dysplastic nodules or early HCC. Negative in regenerative nodules or benign hepatocellular tumors
Glutamine synthetase	Positive in hepatocellular adenoma, focal nodular hyperplasia, and regenerative nodule
Beta catenin	Nuclear positivity in HCC and hepatocellular adenoma with activated beta catenin and risk of malignant transformation
Serum amyloid A	Positive in teleangiectatic/inflammatory hepatocellular adenoma
Organ or tissue-specific antigens	TTF-1 (nuclear staining) for metastatic lung; CDX-2 (nuclear staining) for intestinal differentiation; BRST-2, estrogen and progesterone receptors for breast; prostate-specific antigen for prostate; RCC for renal cell carcinoma; HMB-45 and S-100 for melanoma
Evaluation of bile ducts	
Cytokeratins 7 or 19	Identification of bile ducts and ductules. Useful to visualize bile duct presence to rule out ductopenia, vanishing bile duct syndrome or chronic ductopenic rejection
Prognostic factors	
AFP, Ki67, beta catenin, glypican 3	Differentiating HCC from high-grade dysplastic nodule or hepatocellular adenoma
Storage and hereditary diseases	
Alpha-1-antitrypsin	Predominantly periportal intracytoplasmic globules in alpha-1-antitrypsin deficiency
Fibrinogen	Intracytoplasmic fibrinogen deposition in fibrinogen storage disease
Miscellaneous	
Leukemia/leukemia phenotyping	Differentiating and/or confirming posttransplant lymphoproliferative disease (PTLD) from EBV hepatitis or acute rejection
Cytokeratins 8, 18, and 19	Embryonal hepatocytes, the expression of CK19 disappears by the tenth week of gestation
Synaptophysin, glial fibrillary acidic protein, and neural cell adhesion molecule	Neural/neuroectodermal differentiation markers can be used to identify resting hepatic stellate cells
Ubiquitin, cytokeratin 8 and 18, p62	Identification of Mallory-Denk bodies
Vimentin, desmin, and smooth muscle actin	Myofibroblastic differentiation in activated hepatic stellate cells (including vitamin A toxicity)

useful in narrowing down the possible organ system of origin. In addition, newer gene expression technologies are available to more specifically identify the primary tumor site. Among tumor types, adenocarcinoma, neuroendocrine tumor, and lymphoma are the most common affecting the liver. Common primary sites include colon (adenocarcinoma), pancreas (adenocarcinoma and pancreatic endocrine tumor), stomach and small intestine (adenocarcinoma, carcinoid, and gastrointestinal stromal tumor), lung (adenocarcinoma, neuroendocrine tumor, small cell carcinoma, and squamous cell carcinoma), breast, skin (melanoma), and kidney (renal cell carcinoma). On occasion, the liver biopsy may not include the targeted space-occupying lesion and only show nonspecific histologic changes due to local bile duct and blood flow obstruction [25]. In such a case, the oncologist should continue searching for a neoplasm, cyst, or abscess.

In recent years, clinicians have increasingly come to accept that in patients with cirrhosis and elevated alpha-fetoprotein, confirmation liver biopsy of a hypervascular tumor on imaging is not needed for the diagnosis of HCC [26]. Nevertheless, biopsy of HCC is often performed as a requirement of a treatment protocol.

Electron microscopy is not a routinely performed test in liver pathology. It has a limited but well-defined role in investigating hereditary and metabolic diseases of the liver, exotic viral infection not otherwise identified by light microscopy or serology, tumors of unknown histology, and certain drug-induced liver injuries. Therefore, consideration of differential diagnoses is important when submitting liver biopsy for processing, because tissue for electron microscopy studies should be submitted in 3% glutaraldehyde rather than the standard formalin. Table 62.5 lists tissue fixatives for various ancillary studies.

The combination of histopathologic examination, immunohistochemical stains, molecular studies, and imaging studies have provided insights to the pathogenesis and new subclassification of benign and malignant hepatocellular tumors. In benign hepatocellular tumors, new subclassification of hepatocellular adenoma was recently introduced which included (a) hepatocyte nuclear factor-1-alpha (HNF-1alpha)-inactivated, (b) beta-catenin-activated, and (c) inflammatory or telangiectatic hepatocellular adenoma. Please see Chap. 52 for additional details on molecular pathogenesis of hepatic adenoma. This new subclassification was based on molecular pathology characteristics accompanied by specific histopathologic and MRI features, which have a significant impact on the management of these patients [27–29]. In HCC, the pathogenesis of dysplastic nodules as well as early and small HCC continues to be studied for the purpose of treatment and prognosis [30, 31].

Table 62.5 Tissue fixatives for various ancillary studies

Ancillary studies	Fixative or procedure
Transmission electron microscopy	3% buffered glutaraldehyde
Scanning electron microscopy	Perfusion-fixation, gold or platinum coating
Viral, bacteria, and fungus	Fresh, unfixed tissue for culture or polymerase chain reaction identification
Fat stains, enzyme activity, protein analyses, viral DNA and RNA, in situ hybridization	Rapid freezing in liquid nitrogen or mixture of dry ice and isopentane
Glycogen storage diseases	Alcohol or 1% periodic acid in 10% neutral buffered formalin at 4°C for 48 h
Flow cytometry of lymphocytes	Fresh, unfixed tissue preferred
DNA analysis	10% neutral buffered formalin
RNA analysis	Fresh or alcohol based fixative (80% ethanol)
mRNA analysis	Fresh or 10% neutral buffered formalin
Protein analysis	Fresh or fresh-frozen, unfixed tissue
Laser capture microdissection	Conventional tissue section from paraffin-embedded tissue block

Molecular Pathology Assessment of Neoplastic Liver Disease

Molecular Evaluation of Neoplastic Liver Diseases

Much of the routine molecular diagnostic applications associated with liver disease are geared toward the assessment of viral targets such as hepatitis C (HCV). In this instance, molecular technologies have been developed for the qualitative and quantitative detection of the virus. In addition, technologies have been developed that allow for the accurate genotyping of the virus as a prognostic indicator and guidance of therapeutic options. With respect to neoplastic diseases of the liver, novel testing applications for establishing and/or confirming a diagnosis, identifying cancers of unknown primaries, and demonstrating the potential for response to therapeutic options are necessary.

MicroRNAS

Recently, the study of small molecule RNAs has become the center of much attention for clinical applications as these potential targets may offer levels of sensitivity and specificity

that are unprecedented for modern biomarkers. In addition, while these small molecules may be used in the diagnosis of disease, there is also a realism that they may eventually become the target of novel therapeutics.

MicroRNAs are a family of endogenous, small (~22 nucleotides in length), non-coding, functional RNAs [32]. It is estimated that there may be 1,000 miRNA genes in the human genome [33]. miRNAs are expressed in a tissue-specific manner, and changes in miRNA expression within a tissue type can be correlated with disease status.

The biogenesis of miRNAs begins with transcription by Pol II or Pol III into long primary miRNA (pri-miRNA) molecules, which are subsequently processed in the nucleus by the RNase III endonuclease Drosha and DGCR8 to form intermediate stem-loop structures ~70 nucleotides long known as precursor miRNAs (pre-miRNAs) [32]. These pre-miRNAs fold to form imperfect stem-loop structures that are transported from the nucleus to the cytoplasm by Exportin-5, where they undergo further processing by another RNase III endonuclease, Dicer. Dicer removes the loop of the pre-miRNA, producing an imperfect duplex made up of the mature miRNA sequence and a similar size fragment (miRNA*) derived from the opposing arm of the pre-miRNA. The miRNA strand of the duplex is loaded onto the RNA-induced silencing (RISC) complex; the miRNA* separates from the duplex and is degraded.

MicroRNAS and Neoplastic Liver Diseases

HCC is the fifth leading cause of cancer-related death in men in USA, with an estimated 21,000 new cases (men and women) diagnosed on an annual basis [34]. The major etiologies of HCC include viral infection, metabolic abnormalities, and immune-related disorders. Five-year survival rates approach 50–70%, with local recurrence rates in excess of 70% at 5 years. Neoplasms of the liver are clinically heterogeneous and have associated risks factors and genetic alterations. A more detailed chapter on the molecular biology of HCC is included in Chap. 56. Specific miRNAs have been shown to be aberrantly expressed in various liver tissues including HCC.

Murakami et al. were the first to profile miRNA expression in HCC using microarray technologies [35]. By analyzing miRNA expression profiles in 25 pairs of HCC and adjacent non-tumor tissue, they identified three miRNAs (miR-224, miR-18, and pre-miR-P18) that exhibited higher expression in the HCC samples than non-tumor tissue, and five miRNAs (miR-199a, miR-199a*, miR-200a, miR-125a, and miR-195) that showed lower expression in the HCC samples than non-tumor tissue. Using these eight miRNAs, an overall prediction accuracy of 97.8% could be achieved [35].

More recently, Ladeiro et al. demonstrated the utility of miRNA profiling for the differentiation of benign from malignant hepatocellular tumors [36]. Both benign and malignant hepatocellular tumors showed increased expression of miR-224 and decreased expression of miR-122a and 422b. HCC had increased expression levels of miR-21, miR-10b, and miR-222, while benign tumors showed decreased expression of miR-200c and miR-203.

Li et al. identified a profile of 69 miRNAs that differentiated noncancerous from cancer liver tissues [37]. miR-125b was shown to be down-regulated in HCC. However, overexpression of miR-125b was associated with good survival in HCC patients. Jiang et al. identified 19 miRNAs in HCC patients whose expression was associated with either poor survival (low expression) or good survival (high expression) [38]. These types of findings have significant implications for our understanding of liver pathophysiology and could shed light on novel therapeutic approaches.

Cancers of Unknown Primary

Cancers of unknown primary sites (CUP) are one of the leading causes of cancer deaths. CUPs are a common metastatic cancer with an annual incidence of approximately 50,000 patients in USA. CUPs are defined as histologically confirmed metastases in the absence of an identifiable primary tumor. CUPs are a diagnostic and a management challenge because they are characterized by the regression of the primary tumor, the development of early and uncommon systemic metastases, and are typically resistant to therapy [39]. The management of patients with malignant diseases is highly dependent upon the identification of the primary tumor, and not knowing this poses significant diagnostic and therapeutic problems. With respect to liver neoplasms, CUPs may be identified as the initial presentation of the disease with an underlying primary that is too small to detect in an alternative organ.

The use of molecular profiling to classify metastatic cancers with known primary sites has been accurate (76–89%), and this same approach utilizing an initial biopsy of the tumor may have potential as a test to diagnose the site of tumor origin in patients with unknown primary cancer. The ability to diagnose and classify unknown primary cancer more precisely would allow for more site-specific or targeted therapy, and likely improve patient outcomes.

Molecular Applications for Targeted Therapy in Neoplastic Liver Disease

Recently, the introduction of the epidermal growth factor receptor (EGFR) inhibitors has increased the treatment options available for patients with metastatic colorectal cancer (mCRC), including those metastatic lesions to the liver [40].

Two of these agents currently approved for the treatment of mCRC are the fully human monoclonal antibody panitumumab and the mouse-human chimeric monoclonal antibody cetuximab. Early studies using these targeted therapies suggested that clinical benefit was confined to a subset of patients who were treated. Mutation of the KRAS oncogene emerged as a powerful negative predictive biomarker to identify patients with mCRC who do not benefit from treatment with these two EGFR inhibitors [41, 42]. Other studies have now shown that clinical benefit from treatment with cetuximab and panitumumab is limited to patients with tumors harboring the wild-type KRAS gene. Therefore, it becomes critical for the clinical molecular diagnostics laboratory to be able to screen tumors for these mutations.

HCC is one of the most common malignant tumors worldwide for which there are limited therapeutic options. While surgery and percutaneous or transarterial interventions are effective for patients with limited or compensated underlying liver disease, more than 80% of patients present with multifocal HCC and/or advanced liver disease, or have comorbidities at the time of diagnosis. Treatment options for these patients have been limited to supportive care. The effectiveness of targeted therapy with monoclonal antibodies or small-molecule kinase inhibitors has been shown in the treatment of many different tumor types. The multitargeted kinase inhibitor, sorafenib, has been shown to prolong survival significantly for patients with advanced HCC [43]. Sorafenib was approved by the United States Food and Drug Administration as first-line therapy in HCC as the first agent demonstrating survival benefit in this disease. Although the survival benefit demonstrated by sorafenib is moderate, molecular targeted therapy has brought new hope in the management of HCC [40].

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DOI 10.1007/978-1-4419-7107-4, © Springer Science+Business Media, LLC 2011

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