Cell Death in Biology and Diseases

Wen-Xing Ding
Xiao-Ming Yin *Editors*

Molecules, Systems and Signaling in Liver Injury

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Molecules, Systems and Signaling in Liver Injury

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Series Preface

Cell death, or conversely cell survival, is a major biological phenomenon. Just as with cell proliferation and cell differentiation, cell death is a choice that a cell has to make, sometimes voluntarily, other times accidentally. As such, cell death serves a purpose in the biology of a multicellular organism. The machinery of cell death and that of cell protection are evolutionarily conserved, and their elements can even be found in single-celled organism. The disruption of cell death mechanisms can often cause developmental abnormalities. Factors that can trigger cell death are diverse, and the cell death process is intricately connected with other biological processes. Cell death directly contributes to the pathogenesis of many diseases, including cancer, neurodegenerative diseases, and tissue injury in organ failure.

The study of cell death and cell survival has become a multidisciplinary subject, which requires expertise from all fields of the modern biology. Exploring the role of cell death in disease development and the modulation of cell death for the prevention and treatment of devastating disease demands constant updating of our knowledge through the broadest interactions among all investigators, basic and clinical. The rapid expansion of our knowledge in this field has gone beyond what could be summarized in a single book. Thus, this timely series Cell Death in Biology and Diseases summarizes new developments in different areas of cell death research in an elaborate and systemic way. Each volume of this series addresses a particular topic of cell death that either has a broad impact on the field or has an in-depth development in a unique direction. As a whole, this series provides a current and encyclopedic view of cell death.

We would like to sincerely thank the editors of each volume in the series and the authors of each chapter in these volumes for their strong commitment and great effort towards making this mission possible. We are also grateful to our team of professional Springer editors. They have worked with us diligently and creatively from the initiation and are continuing this on the development and production of each volume of the series. Finally, we hope the readers will enjoy the reading, find the content helpful to their work, and consider this series an invaluable resource.

Indianapolis, IN, USA — Xiao-Ming Yin
Augusta, GA, USA — Zheng Dong

Preface

Cell death is a fundamental biological phenomenon. It is evolutionarily conserved but can assume different forms under different conditions. While apoptosis, necrosis, and necroptosis are perhaps best studied, less known forms of cell death such as pyroptosis, ferroptosis, parthanatos, and entosis are increasingly found in various conditions or organisms. In multicellular organisms, cell death is important for development, shaping organ size, reforming tissue architecture, promoting functional differentiation, and determining mitochondrial inheritance. In the post--development stage, cell death determines the severity in tissue injury and the degree of subsequent response in inflammation, fibrosis, repair, regeneration, and tumorigenesis. The pathological changes in a complex organ, such as the liver, can be greatly affected by the death program in its cellular components. As the major organ for metabolism and detoxification, the liver is constantly under challenges from both internal and external sources. Viral infection and xenobiotics are the two major environmental stimuli that can cause significant hepatocyte death. Metabolic disturbance (such as in autophagy function) and special food components (such as lipids, ethanol, cholesterol, sugars) are the major internal stress that can also take a significant token on the hepatocytes that leads to cell death. Furthermore, the liver may experience traumatic injury as in cholestasis or ischemia, which often leads to tissue injury to various degrees. The liver is composed of hepatocytes (the majority), cholangiocytes, stellate cells, fibroblasts, macrophages/Kupffer cells, sinusoidal cells, and many more. They respond to these insults with different sensitivities and contribute to the overall liver pathology in various ways. Thus different signaling pathways may be involved in a cell type-specific and/or in a stimulus-specific way, which triggers subcellular organelle (mitochondria and endoplasmic reticulum as well as lysosomes) stress to induce cell death. Significant progresses have been made in the past decades to characterize these cell death pathways and the cells that are involved in liver injury. We are thus preparing two books devoted to the mechanisms of cell death in the liver and liver diseases. While the first volume mainly discusses liver injury and cell death caused by the various external and internal stimuli, the second volume discusses in great detail the type of cells and intracellular organelles involved in cell death as well as several major signaling pathways

involved. We have paid particular attention to the interaction of different cells and aimed to provide a global view of the liver pathology that connects injury/cell death to other pathological changes, such as inflammation, fibrosis, and tumor development. Notably we will also discuss the interaction between the liver and the intestine where microbiota can both influence and be influenced by the liver pathology. Authors who are invited to contribute to the two volumes are respected experts in the subject, which should greatly enhance the authority of the chapter and the book. The volume editors are thus indebted to the authors for their outstanding contributions that make these two books so informative and thought-provoking.

Kansas City, KS, USA — Wen-Xing Ding
Indianapolis, IN, USA — Xiao-Ming Yin

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Chapter 1
Lipotoxicity in Non-parenchymal Liver Cells

Edward N. Harris and Justin L. Mott

Abbreviations

α-SMA	Alpha-smooth muscle actin
DR5	TRAIL death receptor 5
eNOS	Endothelial nitric oxide synthase
FasL	Fas ligand
GGT	Gamma-glutamyl transpeptidase
Hh	Hedgehog
HSCs	Hepatic stellate cells
KCs	Kupffer cells
LSECs	Liver sinusoidal endothelial cells
LXRs	Liver X receptors
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
PD-1	Programmed death 1 receptor
PD-L1/2	Programmed death 1 receptor ligands
PNPLA3	Patatin-like phosholipase domain-containing 3

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TGF-β	Transforming growth factor-β
TNFR	Tumor necrosis factor receptors
VEGF	Vascular endothelial growth factor

1.1 Introduction

Liver injury in the presence of metabolic alterations is becoming a major cause of morbidity and mortality. Nonalcoholic fatty liver disease (NAFLD) includes simple steatosis and the more severe NASH (Goh and McCullough 2016; Demir et al. 2015). NASH-associated cirrhosis and end-stage liver disease are currently the second leading indication for liver transplantation and are the fastest-growing cause for transplantation in the USA (Wong et al. 2015; Charlton et al. 2011). Altered metabolic states associated with NAFLD and NASH are obesity, insulin resistance, metabolic syndrome, and type 2 diabetes. Obviously, there is considerable overlap among these conditions. Development of metabolic syndrome is commonly associated with obesity, and increasing rates of obesity have paralleled increased NASH incidence. It is estimated that 34–40% of the adult population in the USA is obese (body mass index over 30 kg/m^2). Over 9 million adults in the USA have NASH, and over 100 million have NAFLD (Angulo 2002). The burden of this disease is hard to overstate.

Histologic findings of NASH include lipid droplet accumulation in hepatocytes (steatosis), hepatocellular injury and ballooning, and lobular inflammation. While not required for the diagnosis of NASH, fibrosis is the best predictor of disease outcomes (Angulo et al. 2015; Ekstedt et al. 2015). In stage 2 and higher fibrosis, there is portal fibrosis in addition to perisinusoidal lobular fibrosis. Another unscored histologic finding is ductular reaction with proliferation of hepatic progenitor cells in the lobule expressing markers of biliary epithelial cells and ductular reaction with a related expansion of biliary epithelial cells in the portal tract (Richardson et al. 2007). The degree of ductular reaction correlated with fibrosis stage in NASH with the greatest area of ductular reaction in stage 4 fibrotic NASH livers (Richardson et al. 2007; Gadd et al. 2014). Portal inflammation with CD68-positive macrophages and CD3- or CD8-positive lymphocytes also correlated with stage of fibrosis (Gadd et al. 2014). Ultrastructurally, liver sinusoidal endothelial cells (LSECs) lose their fenestrae and become capillarized.

Metabolic changes associated with NASH suggest a role for lipid signaling in disease. Indeed, genome-wide association studies have demonstrated that the lipase patatin-like phosholipase domain-containing 3 (PNPLA3) is associated with NAFLD (Romeo et al. 2008). Circulating free fatty acids are increased in NASH patients, and patients with stage 3 or 4 fibrosis had significantly higher free fatty acid levels than patients with stage 0–2 fibrosis. Interestingly, serum free fatty acids did not correlate with the degree of steatosis (Nehra et al. 2001). The predominant circulating fatty acid is palmitic acid, a 16-carbon saturated fatty acid. Saturated free fatty acids such as palmitic and stearic acid are cytotoxic to several cell types, termed lipoapoptosis. Monounsaturated free fatty acids such as oleate or palmitoleate are generally not toxic and even offer protection from lipoapoptosis (Welters

et al. 2004; Akazawa et al. 2010; Mei et al. 2011; Ahn et al. 2013). Monounsaturated free fatty acids still promote lipid droplet accumulation, consistent with the concept that steatosis per se is not cytotoxic.

Here, we will discuss pathologic changes in NASH associated with lipid mediators. For some liver cell types, this includes lipoapoptosis; for others the lipotoxicity is manifested by altered function. The various cell types in the liver each contribute to NASH, and these roles will be discussed.

1.2 Hepatocytes

Apoptosis of hepatocytes in NASH is covered in depth elsewhere in this volume and will not be covered here at length. We note that hepatocyte apoptosis is a hallmark of NASH (Feldstein et al. 2003; Wieckowska et al. 2006) and free fatty acids can induce lipoapoptosis in cultured hepatocyte-derived cells (Ji et al. 2005; Malhi et al. 2006a; Cazanave et al. 2014). The term lipoapoptosis is used here to indicate apoptotic cell death caused by a lipid mediator and is not necessarily dependent on cellular accumulation of lipid droplets, a condition called steatosis. Steatosis is histologically striking in NAFLD and NASH, though may be absent in later stages of NASH, resulting in the historical term "cryptogenic cirrhosis" (Brunt 2005). On the other hand, apoptotic cell death is not readily apparent on general stains (such as H&E staining). Apoptotic hepatocytes can be identified on occasion in histologic sections, and their prevalence is better estimated using special staining such as TUNEL to detect cleaved DNA ends or cleaved caspase-3 to identify cells with activated caspases. In NASH, hepatocyte apoptosis is generally in the range of 2–5%, representing about a fivefold increase over normal liver (Feldstein et al. 2003).

1.3 Cholangiocytes

Evidence for biliary involvement in NASH is currently circumstantial. Pathological assessment of NASH is scored by steatosis, lobular inflammation, and hepatocyte ballooning, as well as unscored histologic findings such as fibrosis (Brunt et al. 2011). Biliary changes are not part of the histologic criteria for NAFLD or NASH. Bile duct epithelial cells do not accumulate histologically significant amounts of triglycerides in the form of lipid droplets. Because cholangiocytes do not become steatotic, it is tempting to overlook their potential role in nonalcoholic fatty liver disease. However, storing neutral lipids in the form of triglycerides and cholesterol esters in lipid droplets is not required to sense and respond to circulating lipids like free fatty acids.

Though not part of the scoring criteria, there are biliary histologic changes in NASH. For example, a common finding in NASH is the occurrence of the ductular reaction, a marked expansion of biliary epithelial cells (Roskams et al. 2003; Richardson et al. 2007; Gadd et al. 2014). Ductular reaction in NASH was most commonly associated with worse fibrosis (Richardson et al. 2007; Gadd et al. 2014).

Increasing fibrosis stages correlated with increasing area of ductular reaction (Richardson et al. 2007). Interpreting the significance of the ductular reaction is complicated by the observation that ductular reaction may be present from different mechanisms. For instance, ductular reaction may indicate hepatocyte injury in combination with impaired hepatocyte proliferation (Roskams et al. 2003; Falkowski et al. 2003; Clouston et al. 2005). Alternatively, ductular reaction may indicate a proliferative response to direct biliary damage (Xu et al. 2004; Munshi et al. 2011). Some contribution from each is also feasible.

Is there evidence of direct biliary damage? The biliary marker gamma-glutamyl transpeptidase (GGT) is released into the serum in biliary injury. Elevated serum GGT is correlated with metabolic syndrome (Rantala et al. 2000), risk of developing type 2 diabetes (Perry et al. 1998), histologic fibrosis (Tahan et al. 2008), and likelihood of NASH (Neuschwander-Tetri et al. 2010). However, GGT is not specific for injury to bile duct epithelial cells and can be found at canaliculi of hepatocytes as well (Hanigan and Frierson 1996; Irie et al. 2007). Patients with a cholestatic phenotype of NAFLD (elevated GGT, elevated alkaline phosphatase) were matched to patients with non-cholestatic NAFLD for body mass index and markers of hepatocyte injury (alanine and aspartate aminotransferases) and were assessed for microscopic evidence of biliary injury. Histologic assessment demonstrated bile duct swelling, duct loss, ductular proliferation (ductular reaction), and portal fibrosis. Notably, those patients with increased markers of biliary injury were more likely to have worse fibrosis or even cirrhosis than the matched cohort (Sorrentino et al. 2005). To date, these markers of injury may still reflect either primary biliary injury or secondary biliary damage due to signaling in other cells in the liver. Notably, primary biliary cholangitis was worsened by concomitant overweight, with more steatosis, higher inflammatory grade, and worse bile duct damage found in overweight patients suggesting that lipotoxicity may contribute in the biliary tree, although other metabolic traits were not correlated with severity (Hindi et al. 2013). Additionally, the same single nucleotide polymorphism in PNPLA3 (I148M) that increases NAFLD risk was found to be associated with worse transplant-free survival in patients with primary sclerosing cholangitis. This effect was found in men with severe fibrotic disease manifested by a dominant stenosis (Friedrich et al. 2013). The molecular mechanism behind worsening of disease is unknown. The communication between cells that is necessary for normal liver function means that perturbation in one cell type can, and often does, alter another cell type.

The location of fibrosis in early NASH is generally pericentral, suggesting that cells in the portal tract may contribute less to disease (Brunt et al. 2011). Certainly, hepatocyte injury, altered endothelial function, and activation of Kupffer and hepatic stellate cells result in fibrosis. Still, in pediatric NASH, fibrosis is commonly found predominantly in the portal tract, sometimes referred to as type 2 NASH (Schwimmer et al. 2005; Takahashi et al. 2011). Sequential liver biopsies have demonstrated that patients with periportal fibrosis (stage 2 or 3) can transition to a pattern of pericentral fibrosis (Adams et al. 2005), demonstrating that the region of fibrosis may not be constant or may be different depending on sampling of the liver by biopsy. Whether bile duct epithelial injury promotes portal fibrosis in NASH remains an

open question, but biliary fibrosis in other conditions implies that the cholangiocyte could contribute.

Developmentally, hepatocytes and cholangiocytes arise from a bipotential common precursor cell. The cells generally are few in number in the healthy adult liver but likely contribute to the ductular reaction under conditions of injury. Because of this shared developmental origin, it is likely hepatocytes and cholangiocytes retain common signaling pathways. Also because of this shared development, and the fact that the albumin promoter becomes active in precursor cells, it is important to carefully interpret studies in which the albumin promoter has been used to drive liver-specific genetic changes. For example, it is common to employ a cell-type-specific promoter to express Cre recombinase allowing for genetic rearrangement at loxP sites in a subset of somatic cells, but not in all cells. These conditional genetic systems can be used to selectively delete genes that have floxed exons only in cells expressing Cre. Alternatively, the Cre system can be used to selectively induce expression of a constitutively silent transgene that contains a lox-stop-lox cassette upstream of the transcriptional start site. Recombination in Cre-positive cells then removes the stop cassette and allows expression in a cell-type-specific manner. In the liver, either the albumin promoter (Alb-Cre) or the albumin promoter and alpha-fetoprotein enhancers (Alfp-Cre) have been used. These systems are liver specific (Postic et al. 1999; Kellendonk et al. 2000) but should not be considered hepatocyte specific. Both Alb-Cre and Alfp-Cre result in recombination in cholangiocytes as well as the expected hepatocytes (Tchorz et al. 2009; Kellendonk et al. 2000), i.e., in both epithelial cell types in the liver.

Given these caveats, deletion of the apoptosis-related TRAIL death receptor (DR5) in liver epithelial cells reduced NASH injury in a mouse model (Idrissova et al. 2015). The TRIAL death receptor is upstream of the initiator caspase-8. Cre-mediated deletion of caspase-8 in liver epithelial cells also decreased injury and fibrosis in methionine−/choline-deficient mice (Hatting et al. 2013). These findings implicate death receptor signaling in lipoapoptosis and severity of liver injury. In hepatocyte-derived cell lines, interfering with mRNA for DR5, caspase-8, or FADD reduced lipoapoptosis induced by treating hepatocytes with saturated free fatty acids (Cazanave et al. 2011). The same study also found that human cholangiocyte-derived cell lines underwent apoptosis upon exposure to saturated free fatty acids and that inhibition of DR5 or caspase-8 prevented lipoapoptosis (Cazanave et al. 2011).

Based on this study, and the shared developmental origin of cholangiocytes and hepatocytes, we have begun to study cholangiocyte lipoapoptosis. Cultured cholangiocyte-derived cell lines undergo caspase-dependent lipoapoptosis upon treatment with stearate or palmitate. Apoptosis increased with increasing concentration of these fatty acids and was partially dependent upon activation of the transcription factor FoxO3 (Natarajan et al. 2014). In contrast to hepatocyte lipoapoptosis (Malhi et al. 2006a), cholangiocytes did not require JNK activation for cell death. This cell culture model employed cultured cells and individual fatty acids at relatively high concentrations. The serum free fatty acid content is increased in obesity and metabolic syndrome as well as in NASH patients, so the free fatty acid concentrations were chosen to mimic pathophysiologic, not physiologic, levels. These limitations

should be recognized in considering the potential role of cholangiocyte lipoapoptosis in NASH. We hypothesize that cholangiocyte lipoapoptosis, in combination with activation of the surviving cholangiocytes, can promote or worsen fibrogenesis, inflammation, and NASH progression.

1.4 Kupffer Cells

Kupffer cell apoptosis does not appear to cause worsening of NASH. However, it is apparent Kupffer cells are an active cell type determining the severity of inflammation and injury. Specifically, in a series of needle biopsy samples, Kupffer cells were found to be aggregated in NASH but not in healthy liver samples or livers with steatosis (Lefkowitch et al. 2002). There was even sporadic evidence of intracellular lipids in Kupffer cells, which may have represented Kupffer cell lipid droplets or phagocytosed lipid-laden hepatocytes (Lefkowitch et al. 2002).

Experimental depletion of Kupffer cells, preferentially large periportal Kupffer cells, can be accomplished by treating animals with gadolinium chloride which forms aggregates above pH 6 and is taken up by KCs causing their death (Hardonk et al. 1992). Alternatively, the hydrophilic bisphosphonate clodronate can be packaged into liposomes facilitating uptake into macrophages by phagocytosis where it induced apoptosis (Van Rooijen and Sanders 1994). Using these tools revealed that Kupffer cells are necessary for short-term steatosis and hepatic insulin resistance. Depletion of Kupffer cells for 1 week prevented hepatic steatosis due to high-fat feeding (Stienstra et al. 2010). An acute, 3-day high-fat diet induced hepatic insulin resistance that was prevented by Kupffer cell depletion (Lanthier et al. 2010). Gadolinium chloride-mediated depletion of Kupffer cells prevented steatosis induced by 2 weeks of either high-fat or high-sucrose feeding (Huang et al. 2010). Additionally, Kupffer cell depletion prevented hepatic insulin resistance tested by insulin clamp. During this short dietary intervention, there was no change in fasting insulin or fasting glucose levels (Huang et al. 2010). Weekly Kupffer cell depletion with liposomal clodronate prevented glucose intolerance during a 4-week high-fat diet (Lanthier et al. 2011). Similarly, twice-weekly gadolinium improved systemic insulin resistance and hepatic steatosis in mice fed with a high-fat diet for 3 weeks (Neyrinck et al. 2009). Weekly clodronate-mediated depletion in mice fed with a methionine−/choline-deficient diet for 3 weeks that causes NASH-like liver changes without insulin resistance or obesity reduced steatosis and markers of fibrosis and inflammation (Rivera et al. 2007). However, a 16-week dietary challenge with high fat did not benefit from Kupffer cell depletion during the last 10 days (Lanthier et al. 2011). This suggests that once metabolic changes are present, Kupffer cells are not required to maintain these alterations.

In summary, lipid-induced apoptosis of liver macrophages is unlikely to contribute to NASH, while prevention of Kupffer cell activation (by killing off these cells) has a beneficial, preventative effect. Therapeutic benefit from Kupffer cell depletion after metabolic changes lacks experimental support.

1.5 Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSECs) are unique endothelial-type cells lining the capillary bed of the liver and serve as the blood barrier between general circulation and the space of Disse which allows access to stellate cells and hepatocytes. The three defining characteristics that make LSECs unique from other endothelia are (1) the lack of an underlying basal lamina (Wisse et al. 1985), (2) numerous cytoplasmic holes or fenestrae organized as sieve plates, and (3) a very high endocytic (fluid phase) capacity. Each fenestra is 100–175 nm in diameter and allows plasma components including small chylomicron and chylomicron remnants to pass through this single-cell layer and come in direct contact with hepatocytes (Braet and Wisse 2002; Wisse 1970). Formation of fenestrae occurs by an actin-dependent fusion between opposite sheets of the plasma membrane when there is a lack of intramembrane particles (Taira 1994). In addition, formation and overall numbers of fenestrae are maintained by adequate levels of vascular endothelial growth factor (VEGF), and loss of VEGF reduces fenestrae (Xie et al. 2012). Due to the dynamic nature of fenestrae, various external stimuli cause the number of fenestra to increase, including hypoxia (Frenzel et al. 1976) and pressure (Nopanitaya et al. 1976; Fraser et al. 1980), or decrease including ethanol (Mak et al. 1984; Jacobs et al. 2009; Sarphie et al. 1997) and nicotine (Fraser et al. 1988). It has been observed that normal aging decreases the size and number of fenestrae, termed pseudo-capillarization, and affects lipid metabolism due to reduced chylomicron contact with the hepatocytes (Le Couteur et al. 2001; McLean et al. 2003; Warren et al. 2005). LSECs are very efficient in the internalization of extracellular matrix and waste materials from blood. Rapid ligand internalization and receptor recycling are owed in part to a net-like distribution of clathrin heavy chains associated with clathrin-coated vesicles in the endocytic processes of internalization, vesicle maturation, and ligand trafficking (Falkowska-Hansen et al. 2007). It is believed that few cells of the mammalian body can match or exceed the endocytic capacity of LSECs.

Tumor necrosis factor receptors (TNFRs) are very low to absent in the normal liver, but in lipotoxic conditions, TNFR expression is increased in hepatocytes, LSECs, cholangiocytes, and KCs (Volpes et al. 1992). Fas receptor, of the TNFR family, is a 45-kDa receptor on the plasma membrane belonging to the tumor necrosis factor family. Fas ligand (FasL) is expressed on activated immune cells (T cells, monocytes, and neutrophils) inducing apoptosis in Fas-expressing cells (Depraetere and Golstein 1997). Normally, the expression of Fas on LSECs is quite low, but that increases under conditions of NASH or lipotoxicity (Malhi et al. 2006b; Muschen et al. 1998). Despite the increase in Fas expression, LSECs are more resistant to apoptosis in the presence of FasL than hepatocytes and stellate cells, though higher physiological doses of FasL did, eventually, induce apoptosis in LSECs (Fig. 1.1). Low doses of FasL or anti-Fas antibodies during in vitro studies have demonstrated an increased expression of ICAM-1 and VCAM-1 instead of apoptosis suggesting that alternative pathways stimulated by Fas are operative in LSECs (Cardier et al. 1999). It is speculated that an increase in adhesion molecules may be a signal for

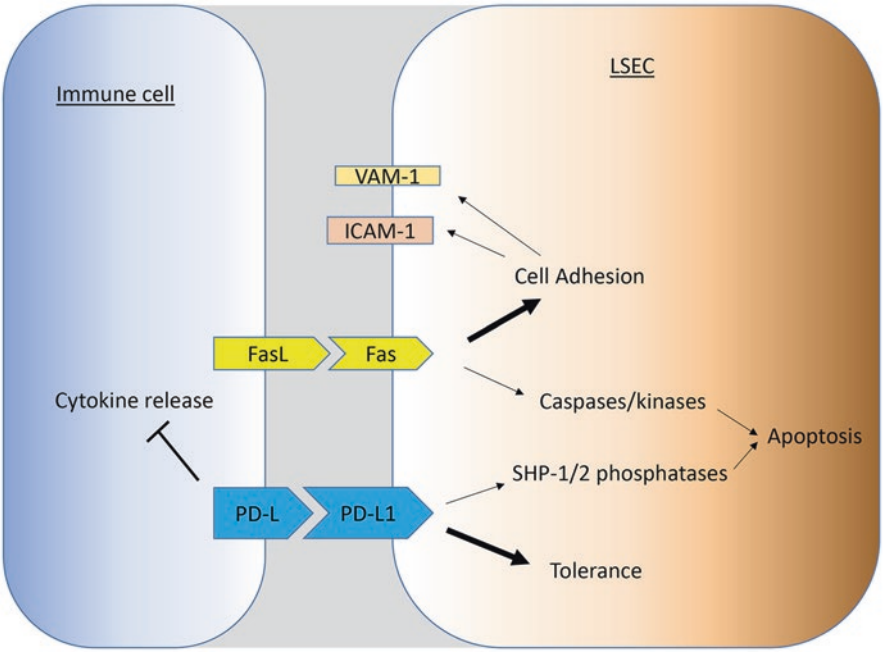

Fig. 1.1 Interaction of activated immune cells and LSECs. LSECs interact with both quiescent and activated immune cells. LSECs express Fas and PD-L1 receptors which induce parallel pathways that induce apoptosis. However, the dominant outcomes for each pathway are illustrated by the thicker arrow, and apoptosis is only induced after an ill-defined threshold of stimulation has been met. Overall, LSECs tend to dampen the inflammatory response in many environments including NAFLD/NASH

recruitment for liver-specific Kupffer cells (KCs). Clearance of cell debris and other particles is primarily performed by KCs as these cells are professional phagocytes, whereas LSECs internalize material through rapid pinocytosis. It is estimated that macromolecules suitable for LSEC internalization are less than 0.23 μm, which shifts the burden of phagocytosis to KCs (Shiratori et al. 1993; Park et al. 2008). KCs and LSECs coordinate controlled and tempered responses in chronic inflammatory responses that prove to be hepatoprotective in which both cell types are required to keep tissue damage in check. For instance, depletion of KCs prior to acetaminophen-induced liver injury results in exacerbated liver injury, particularly to the hepatic endothelium (Holt et al. 2010). Similarly in a sepsis model, LSEC apoptosis and Fas expression were increased after a depletion of KCs with the use of clodronate liposomes (Hutchins et al. 2013a). Both of these experimental examples show that KCs are necessary for the viability and well-being of LSECs in a background of escalating inflammation.

LSECs are unique antigen-presenting cells and interact with immune cells including the resident KCs and transient immune cells via the programmed death 1 receptor (PD-1) and its ligands (PD-L1/2; Fig. 1.1) (Keir et al. 2008). LSECs express

PD-L1 which is the ligand for PD-1 expressed by cells within the sinusoids, KCs and CD8+ T cells. This interaction promotes immune tolerance and dampens liver inflammation which is important as the liver sinusoids are directly downstream from gut circulation, containing bacterial and food antigens, via the portal vein (Schurich et al. 2010). Furthermore, PD-1/PD-L1 interactions between LSECs and T_H1 and T_H17 suppress cytokine release from these activated T cells when comparing these activated T cells with dendritic cells, strengthening the hypothesis that LSECs are critical for immune tolerance within the liver (Carambia et al. 2013). PD-1/PD-L1 increases in expression to keep inflammation in check under a variety of simulants including viral infection (Xie et al. 2009; Larrubia et al. 2009), hepatitis, and hepatocellular carcinoma (Wang et al. 2011). Immune tolerance does have its limits in that an undefined threshold may be breached. In mice with sepsis-induced injury (punctured cecum), LSECs and KCs had increased PD-L1 and PD-1 expression, respectively. This resulted in LSEC cell death, with lower cell numbers overall when isolated from the liver, and the few harvested LSECs had lower VEGFR2 expression (a marker for angiogenesis and maintenance of healthy tissue) (Hutchins et al. 2013b). These animals succumbed to septic shock along the same pathways as those exhibiting acute liver failure. The characteristics of PD-1−/PD-L1-induced injury and cell death are very similar to Fas−/FasL-mediated injury model mentioned previously. These pathways are not connected but run in parallel and may synergize each other under the right conditions. The differences between the Fas/FasL and PD-1/PD-L1 are that the former utilizes death receptor-associated kinases and caspases (Wajant 2002) in contrast to the latter which is CD28 dependent and utilizes SHP-1 and SHP-2 phosphatases (Sheppard et al. 2004; Okazaki et al. 2001).

LSECs contain several scavenger receptors that "sense" the extrahepatic environment and respond accordingly (Weigel et al. 2012). These receptors include Stabilin-1, Stabilin-2, mannose receptor, and FC-gamma/CD32b receptors (Sorensen et al. 2015). One of the ligands for Stabilin-1 is SPARC or secreted protein acidic and rich in cysteine which may also serve as a ligand for VCAM-1 (Kzhyshkowska et al. 2006; Kelly et al. 2007). SPARC (also known as osteonectin and BM-40) is a secreted extracellular matrix-associated protein with a major role in the wound healing response, tissue remodeling (Bradshaw et al. 2001), and liver fibrosis (Atorrasagasti et al. 2013). SPARC expression is increased in acute-on-chronic alcohol-induced hepatitis. Using a SPARC knockout mouse, the investigators demonstrated decreased CD4+ T-cell infiltration in the liver and decreased apoptosis of the hepatic endothelium induced by TNF-alpha and IL-6 (Peixoto et al. 2015). This suggests that increased signaling through the Stabilin-1 receptor and, possibly VCAM-1, via SPARC activates LSECs, promotes leukocyte infiltration, and further stimulates death receptors such as Fas/TNFR.

Accumulation of free fatty acids and neutral fats arising from metabolic disorders promotes cellular damage and cell death in many tissues including the liver (Malhi and Gores 2008). It is well known that fat-storing hepatocytes suffer from lipotoxic conditions leading to ballooning and cell death; but how do these metabolic conditions affect LSECs? In most cell-based studies, free fatty acids activate several pathways that lead to cell death including BAX-dependent apoptosis and

lysosomal permeabilization, over-generation of reactive oxygen species, activation of p53, and hyperstimulation of the JNK pathway (Malhi and Gores 2008; Brenner et al. 2013). In line with this, rodents fed with a high-fat diet had LSECs with decreased numbers and porosity of fenestrae, also known as capillarization, compared with rodents fed with a balanced chow control diet (Cogger et al. 2016). In contrast, rats that were fasted for 48 h had an increase in LSEC fenestration by 10% (O'Reilly et al. 2010), and rats that were calorically restricted had an increase of 60% of LSEC fenestration and porosity (Jamieson et al. 2007) suggesting that deprivation of calories increases LSEC porosity for nutrient absorption by the hepatocytes, whereas overnutrition decreases LSEC porosity. However, LSECs do not store fatty acids in NAFLD and NASH conditions (author's unpublished observations), and high levels of certain free fatty acids may be neutral or beneficial for LSEC survival that is contrary for hepatocytes and cholangiocytes (Hang et al. 2012). Recent evidence supports this idea in that in vitro cultures of LSECs supplemented with oleic acid promote maintenance of fenestrae and modulate the Akt/PKB signaling followed by MAPK/ERK signaling to promote survival (Hang et al. 2012). Notably, oleic acid is largely nontoxic to hepatocytes and cholangiocytes except on very high concentrations. Overall, these results show that fenestrae of LSECs are a good marker for cell health and survival, and the amount and type of free fatty acids in vivo or in vitro will affect survival and health of LSECs.

Loss of fenestrae or capillarization is not the only marker for LSECs under stressful conditions. In both fatty liver rodent models and human patients, the size of the liver increases by up to ~20%. The increase in size is primarily attributed to the fat stored in the hepatocytes. Excessive fat storage compresses the sinusoidal space within the capillary bed of the liver and increases portal blood pressure (DeLeve et al. 2008b). Increased portal pressure and other associated factors of lipotoxicity will involve KCs and HSCs to recruit inflammatory cells into the liver and foster conditions for inflammation and deposition of extracellular matrix within the space of Disse (fibrosis) (Farrell et al. 2008; McCuskey et al. 2004). Physical parameters within the sinusoids are just as important in cellular health and maintenance as the biochemical signaling molecules. More research needs to be performed to determine the influence of geometric/physical conditions on LSEC autocrine/paracrine signaling.

Capillarization of LSECs is a hallmark of LSEC injury and a precursor for LSEC death. The mechanism for capillarization is beginning to be understood in that hedgehog (Hh) signaling is a primary driver of this phenomenon. Generally, Hh signaling regulates vascular development and increases during all types of liver injury. In addition to hepatocytes, LSECs express Hh receptor, ligands, and the ligand antagonist, Hhip. Using primary cells in vitro, capillarization was stimulated with increased Hh receptor signaling and inhibited with Hhip. Capillarization was compared in parallel with Hh target genes, Gli1/2 and Ptch1, LSEC migration, and tube formation (Xie et al. 2013). Since capillarization is the start of LSEC injury, does it occur before or after stellate cell activation which promotes inflammation and the pre-fibrotic phenotype in NASH? The current paradigm suggested that capillarization of LSECs precedes HSC activation, and decapillarization (healthy

fenestrae phenotype) promotes quiescence of HSCs (Deleve et al. 2008a). During liver injury, Hh signaling is increased as a normal repair response for organ regeneration. Injury associated with lipotoxicity (or chronic injury) induces aberrant Hh signaling which induces capillarization.

Liver X receptors (LXRs), oxysterol-activated nuclear receptors for lipogenesis, are found in the liver and other tissues with high lipid metabolism and may play a role in LSEC physiology. There is recent evidence indicating that LXR and Hh pathways cross talk in the development of LSEC capillarization. In LXR-deficient mice, Hh signaling was increased (measured by monitoring expression of Gli2 and CD31) in LSECs in parallel with LSEC capillarization in a carbon tetrachloride injury mouse model (Xing et al. 2016). The conclusions of all these experimental data are that LSEC fenestration is promoted by LXR under normal conditions and shifts to capillarization upon induction of the Hh signaling pathways during severe or chronic injury.

What are the outcomes of LSEC cell death and injury? In chronic injury situations, LSECs become capillarized reducing the oxygen content in the liver. Hypoxia stimulates VEGF production in hepatocytes, HSCs, and LSECs in both autocrine and paracrine manners. Overstimulation by VEGF increases the number of sinusoidal vessels, but these vessels have varying diameter, shape, and flow pattern for blood. While increased blood vessels may serve to decrease portal pressure by increasing avenues of blood flow, the full outcome of angiogenesis in the liver is correlated to portal hypertension and promotion of fibrogenesis (Thabut and Shah 2010). Activated HSCs are in intimate contact with capillarized LSECs which increase the number of HSCs around the blood vessel and also restricts the expansion of the blood vessel when expansion is needed to relieve fluid pressure. As HSCs tighten around the LSECs, portal pressure is increased which exacerbates chronic inflammation (Lee et al. 2007). All of these conditions are forward feeding in that a tenuous chronic inflammation may become more acute with the onset of fibrosis and cirrhosis from either physical or molecular injuries (Gentile and Pagliassotti 2008). Rats fed with a cafeteria diet high in saturated fat and sucrose developed hypertension, obesity, insulin resistance, and elevated triglycerides (i.e., features of metabolic syndrome). Plasma free fatty acids increased from 1600 to 3200 μM. Portal blood flow was decreased and hepatic vascular resistance more than doubled. The dietary intervention was continued for 30 days and resulted in steatosis, but fibrosis did not develop in this short time. In this early stage of disease, LSEC function was impaired with reduced vasodilation response and decreased endothelial nitric oxide synthase (eNOS) phosphorylation and enzymatic function (Pasarin et al. 2012).

In conclusion, LSECs are dynamic gatekeepers of the liver allowing select solutes of the blood to come in contact with the hepatocytes. As a regulatory function, they continually filter out macromolecules from the plasma. LSECs, in coordination with other non-parenchymal cells, keep a check on the immune system to prevent runaway inflammation and destruction of the tissue and promote immune tolerance. The loss of these cells is a precursor for liver failure.

1.6 Hepatic Stellate Cells

Hepatic stellate cells (HSCs) are the major site of storage for fat-soluble retinol esters and retinol in the body (Hendriks et al. 1985). HSCs reside in the space of Disse between LSECs and hepatocytes. HSCs have a transcription factor profile that is similar to adipocytes, consistent with their fat-storing function (She et al. 2005). Their name, stellate, comes from their shape with a triangular cell body containing the nucleus and long, delicate cytoplasmic processes extending into the space of Disse. Transdifferentiation of HSCs into a myofibroblastic phenotype results in loss of lipid droplets, expression of alpha-smooth muscle actin (α-SMA), development of contractile properties, and production of type I and III collagen. This process is referred to as activation of HSCs and is an integral part of hepatic fibrogenesis.

Activation of HSCs is due to cell-cell communication and changes in the extracellular matrix in NASH. For example, a major fibrogenic stimulus that activates HSCs is transforming growth factor-β (TGF-β) produced by HSCs and Kupffer cells (De Bleser et al. 1997; Friedman 2000; Bataller and Brenner 2005). Additionally, HSCs can be activated by conditioned medium from steatotic cultured hepatoma cells. A sub-toxic concentration of palmitic acid (200 μM) was added to primary human hepatocytes or immortalized hepatoma cell lines for 24 h and the conditioned culture medium then transferred to primary HSCs or to the HSC cell line hTERT-HSC, respectively. Conditioned medium increased HSC production of profibrogenic TGF-β, remodeling proteins matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-1 and metalloproteinase-2 and type I collagen, and the contractile protein α-SMA. Importantly, conditioned medium also promoted resistance of HSCs to apoptosis, suggesting that steatotic hepatocytes may promote survival of fibrogenic HSCs in the liver. Lack of toxicity in the palmitic acid-treated hepatocytes was confirmed by the absence of nuclear changes (Hoechst) and lack of release of lactate dehydrogenase or aminotransferases (Wobser et al. 2009). This study demonstrated that hepatocytes release an activating factor upon steatogenesis. The authors used size-exclusion chromatography with a 5-kD cutoff spin column and observed that the removal of proteins over 5 kDa abolished the activating effect. While free fatty acids are smaller than this cutoff, they are also generally bound to proteins in solution, so it is possible that some residual free fatty acids were in the conditioned medium.

Hepatocyte apoptosis is a hallmark of NASH and may provoke HSC activation. The HSC cell line LX-1 was found to have phagocytic capacity to take up apoptotic bodies from dead hepatocytes. This uptake was microtubule dependent and was specific to HSCs as hepatocytes themselves did not engulf apoptotic bodies. Uptake then resulted in HSC activation, measured by α-SMA and type I collagen expression as well as production of TGF-β (Canbay et al. 2003). In this study, apoptosis was induced in hepatoma cells (HepG2 cells) by exposure to UV light, eliminating the possible carry-over of the toxic stimulus in the crude preparation of apoptotic bodies. Apoptotic bodies were prepared by low-speed centrifugation of the collected medium (2000 *g*), so this preparation is not expected to contain soluble cell-free signaling mediators and probably represents a distinct profibrogenic signal.

Activation of HSCs by conditioned medium from steatotic hepatocytes and by hepatocyte apoptotic bodies is relevant in the liver where these two cell types are in close proximity. The predictable result is increased production of extracellular matrix proteins, especially types I and III collagen, resulting in fibrosis and liver dysfunction. Biopsy samples of liver samples from patients with NASH support a role for activated HSCs in fibrosis, with 97% of NASH patients in one study showing an increase in activated HSCs (Washington et al. 2000). Activated HSCs in that study were correlated with the severity of fibrosis but not the degree of steatosis or iron deposition. Resolution of fibrosis requires either deactivation of HSCs or apoptosis of activated HSCs. Evidence supports apoptosis of these cells during fibrosis resolution. Increased HSC apoptosis was observed, for example, in the first 2 days after surgical reversal of bile duct obstruction in the initial stages of fibrosis resolution (Issa et al. 2001). Similarly, non-parenchymal apoptosis was increased during days 3–7 after the last carbon tetrachloride injection in a chemical model of fibrosis and resolution (Iredale et al. 1998). Thus, induction of HSC apoptosis is expected to have a beneficial effect on fibrosis. Indeed, inducing HSC apoptosis in bile duct-ligated mice reduced markers of activated HSCs (Anan et al. 2006a) and reduced the severity of injury (Anan et al. 2006b).

Toxic lipid mediators in NASH appear to indirectly activate HSCs and indirectly promote the survival and fibrogenic potential of these cells. HSC apoptosis would presumably have a beneficial effect on NASH fibrosis, but regrettably lipid mediators seem to act indirectly on HSCs to promote their activation. Apoptosis in this cell compartment may be a therapeutic goal.

1.7 Conclusions

Lipotoxicity in the liver involves induction of cell death (lipoapoptosis) in hepatocytes and potentially cholangiocytes in NASH (Fig. 1.2). Cell death in these epithelial cells may lead to slight elevations of serum markers of hepatobiliary injury but more importantly promote activation of fibrogenic HSCs and inflammatory Kupffer cells. Additionally, lipotoxicity to LSECs leads to capillarization, impaired LSEC function, increased vascular resistance, and ultimately cell death. Increased LSEC and epithelial cell death plus activation and increased survival of Kupffer and stellate cells mean that global attempts to prevent cytotoxicity in the liver may not yield therapeutic benefits. Instead, increasing death of Kupffer cells and HSCs while protecting hepatocytes and cholangiocytes may be a better, if more difficult, goal.

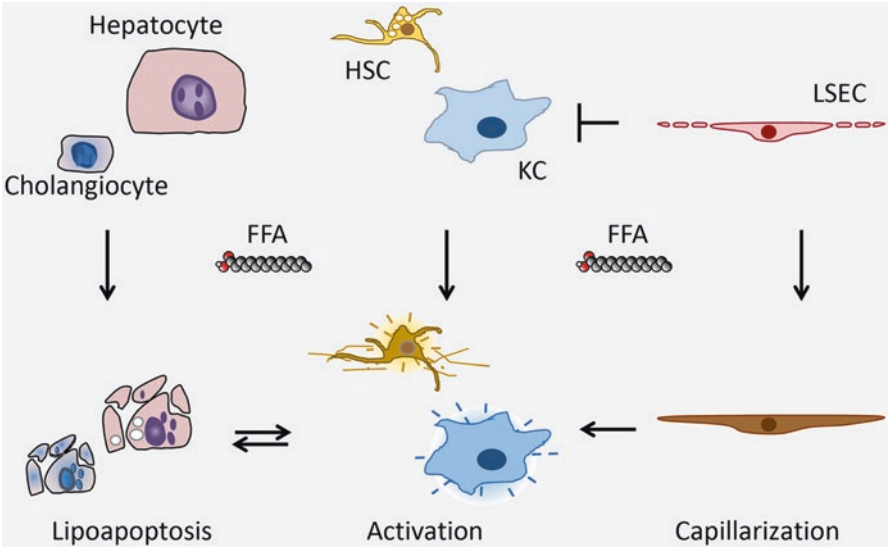

Fig. 1.2 Lipotoxicity in the liver. Healthy cells, hepatocyte, cholangiocyte, hepatic stellate cell (*HSC*), Kupffer cell (*KC*), and liver sinusoidal endothelial cell (*LSEC*) are shown at the top of the figure. Upon pathologic signaling, including by free fatty acids (*FFA*), the normal cell functions and interactions are disrupted. LSEC capillarization and injury as well as lipoapoptosis of hepatocytes and possibly cholangiocytes promote activation of KCs and HSCs. The combined effects include injury, inflammation, and fibrogenesis representing key features of nonalcoholic steatohepatitis

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