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Cellular and Molecular Mechanisms of Liver Injury

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Abstract

Derangements in apoptosis of liver cells are mechanistically important in the pathogenesis of end-stage liver disease. Vulnerable hepatocytes can undergo apoptosis via an extrinsic, death receptor-mediated pathway, or alternatively intracellular stress can activate the intrinsic pathway of apoptosis. Both pathways converge on mitochondria, and mitochondrial dysfunction is a prerequisite for hepatocyte apoptosis. Persistent apoptosis is a feature of chronic liver diseases, and massive apoptosis is a feature of acute liver diseases. Fibrogenesis is stimulated by ongoing hepatocyte apoptosis, eventually resulting in cirrhosis of the liver in chronic liver diseases. Endothelial cell apoptosis occurs in ischemia-reperfusion injury. Natural killer and natural killer T cells remove virus-infected hepatocytes by death receptor-mediated fibrosis. Lastly, activated stellate cell apoptosis leads to slowing and resolution of apoptosis. This review summarizes recent cellular and molecular advances in the understanding of the injury mechanisms leading to end-stage liver disease.

Liver injury encountered in clinical practice is arbitrarily divided into acute and chronic, based on the duration or persistence of liver injury. Acute insults are mostly surmountable with rapid resolution upon elimination of the injurious agent and complete restitution of normal liver architecture and function without enduring evidence of the preceding insult. Progressive fibrosis is the hallmark of chronic liver injury; it can eventually result in cirrhosis, liver failure, or hepatocellular carcinoma. This distinction between acute and chronic liver injury is a mechanistic oversimplification. Chronic liver injury reflects, in part, continuous acute liver injury extended over time. The consequences of continuous acute liver injury are what drive hepatic fibrogenesis. This process became especially apparent when effective therapy for chronic hepatitis B became available. Many patients with end-stage liver disease thought to warrant liver transplantation for survival had significant recovery with antiviral therapy and no longer required urgent transplantation. Furthermore, with the recognition that hepatic fibrogenesis has a reversible component; inhibition of liver injury has become a potential therapeutic strategy for advanced liver disease. Thus, an understanding of the mechanisms mediating liver injury is of biomedical and clinical relevance. Recent advances in understanding the cellular processes and molecular signaling that mediate liver injury are summarized in this review. The first half focuses on mechanistic insights, and in this section references to nonliver systems serve as paradigms; the latter half focuses on select liver-specific disease processes.

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Mechanisms of Liver Cell Death

Apoptosis and Necrosis

Nomenclature in the literature refers to apoptotic cell death and necrotic cell death in diseased livers. Apoptosis is defined morphologically on the basis of cellular rounding up, cytoplasmic shrinkage (pyknosis), chromatin condensation, and nuclear fragmentation (karyorrhexis). Effector caspase (proteases that cleave at aspartate residues) activation is required for the acquisition of this morphology. Necrotic cell death has the morphology of oncosis (cell swelling due to the inability to maintain cellular ion gradients), karyolysis, and rupture of the plasma membrane. While definitions are useful as broad categories, understanding the minute mechanisms that lead to cell death and ensuing injury are more important than allotting modes of cell death to a particular liver disease. Suffice it to say that in the liver, morphologically observed cell death can be apoptotic or necrotic or a combination of the two. Furthermore, the same stimulus can result in either morphology.^{1,2} It is conceivable that on a cellular basis, necrosis in the liver is the result of overwhelming or dysregulated apoptosis. For example, exaggerated mitochondrial dysfunction from “apoptotic” signaling cascades can result in cellular adenosine triphosphate depletion and necrotic morphology. Hepatocytes are the most numerous cell type in the liver, and their apoptosis is prominent in liver injury.^{3–5} Councilman bodies, described by the pathologist William T. Councilman (1854 – 1933), in the liver of patients with yellow fever result from apoptotic death of individual hepatocytes.⁶ On careful examination, hepatocyte apoptosis can be identified in virtually all forms of liver injury.^{4,7–10} Apoptosis of other cellular compartments is also important. For example, sinusoidal endothelial cell apoptosis is observed in ischemia-reperfusion injury, and failure of activated stellate cell apoptosis promotes fibrosis. The M30 neoantigen is one example of an emerging clinical applicability of the apoptosis cascade.¹¹ This epitope is formed by proteolytic cleavage of cytokeratin 18 by caspase 3 at Asp396 position. It is readily detectable in plasma by enzyme-linked immunosorbent assay. Circulating levels are increased in patients with chronic liver disease, and highest levels are found in patients with cholestasis or cholangitis.¹² Levels in hepatic graft-versus-host disease are elevated and correlate with response to therapy.¹³ In patients with steatohepatitis, serum levels of M30 correlate with liver levels and inflammation.¹⁴ Thus, a biomarker reflecting hepatocyte apoptosis may eventually be important in establishing and monitoring therapy in human liver diseases. The appearance of serum cytokeratin 18 degradation products in virtually all liver diseases also highlights the role of caspases in liver tissue injury. Apoptosis can be initiated from any membrane-defined organelle in the cell. In this review, we emphasize this mechanistic concept.

Mitochondria

Mitochondrial dysfunction is the commitment step in hepatocyte cell death, and hepatocyte cell death is dependent on mitochondria.¹⁵ Apart from the well-recognized metabolic functions of mitochondria such as the respiratory chain, the inner and outer mitochondrial membranes also isolate a number of proapoptotic proteins within the intermembrane space. Mitochondrial outer membrane permeabilization leads to the release of these apoptosis mediators, cytochrome *c*, second mitochondrial activator of caspase/direct IAP binding protein with low pI (SMAC/DIABLO), HtrA2/Omi, apoptosis-inducing factor, and endonuclease G.¹⁶ Activation of downstream effector caspases ensues, resulting in the typical morphologic changes of apoptosis. Mitochondrial outer membrane permeabilization occurs selectively, mediated via activated Bax or Bak (vide infra) or secondary to mitochondrial permeability transition.¹⁷ For example, in ischemia-reperfusion injury, the mitochondrial permeability transition pore (which is composed of the voltage-dependent anion channel, adenosine nucleotide transporter, and cyclophilin D) upon activation leads to influx of solutes and ions, swelling of the mitochondrial matrix, and rupture of the outer mitochondrial membrane, releasing proapoptotic proteins into

the cytosol. Of the 3 proteins that comprise the mitochondrial permeability transition pore, cyclophilin D is essential for the permeability transition pore.¹⁸

Mitochondrial outer membrane permeabilization can occur downstream of death receptor-triggered signaling cascades (extrinsic pathway), lysosomal permeabilization, endoplasmic reticulum (ER) stress pathways, or activation of intracellular stress kinases, such as c-jun N-terminal kinase (JNK) (intrinsic pathway) (Figure 1). The Bcl-2 family proteins (Table 1) are best described as mediators of mitochondrial dysfunction.¹⁹ They are divided into proapoptotic and antiapoptotic proteins. The proapoptotic proteins are structurally divided into multidomain (Bak and Bax) and BH-3 domain only (Bid, Noxa, Puma, Bim, Bmf, Bik, Hrk, and Bad), and Bcl-2, Bcl-xL, A1, and Mcl-1 are the important antiapoptotic proteins. Bax is cytosolic and Bak is located on the mitochondrial membrane; when Bax is activated, it too translocates to mitochondria. These 2 multidomain proapoptotic proteins can, on activation, form pores on the outer mitochondrial membrane, inducing mitochondrial permeabilization. Bax and Bak are either directly or indirectly activated by the BH-3-only proteins. For example, Bid is cytosolic and is activated by caspase 8-mediated cleavage, downstream of death receptor activation, and in turn activates Bax/Bak. The antiapoptotic proteins sequester Bax and Bak, preventing apoptosis. When disabled by excessive binding of BH-3-only proteins, they become overwhelmed releasing Bax and Bak and cell death ensues.

Lysosomes

When it occurs, lysosomal involvement in cell death is an early event, observed before mitochondrial permeabilization or caspase activation. In general, lysosomes can be activated by the extrinsic or death receptor-mediated pathway or myriad intracellular stimuli, such as free fatty acids, sphingosine, ceramide, reactive oxygen species, photodamage, or lysosomotropic agents (weakly basic amines that can accumulate in lysosomes and raise intravesicular pH).²⁰ Release of lysosomal proteases (cathepsins) mediates downstream effects. Cathepsin B (one of 11 known human cathepsins) is active at neutral pH and has been studied in several models of liver injury. Lysosomal permeabilization can result in necrotic cell death or apoptotic cell death. Massive release of cathepsins from total lysosomal permeabilization leads to necrotic cell death. Selective lysosomal permeabilization leads to apoptosis, in some instances independent of caspase activation; however, in liver injury models, caspase activation occurs downstream of lysosomal permeabilization. Lysosomal ultrastructural abnormalities are seen in many chronic liver disorders. Studies utilizing mice deficient in cathepsin B, which develop normally, show that the lysosomal pathway of apoptosis is important in steatohepatitis, cholestatic liver injury, and tumor necrosis factor (TNF)- α -mediated liver injury.^{21–23}

Endoplasmic Reticulum

ER stress is an active area of research in the pathogenesis of chronic liver injury (Figure 2), although most of the current understanding of the known mediators of the ER stress pathway comes from other experimental systems. The stressed ER exhibits an imbalance between unfolded proteins and mature proteins, activating a series of compensatory responses, collectively termed the unfolded protein response (UPR).²⁴ ER stress can also be induced by myriad stimuli, such as calcium depletion, glycosylation inhibition (tunicamycin), UV radiation, and insulin resistance. The ER stress response is a 3-pronged attempt at correcting the accumulation of unfolded proteins, and in situations of inadequate correction or sustained ER stress, it signals cell death. There is a global reduction in protein synthesis, decreasing the amount of newly synthesized proteins that have to enter the ER, and selective induction of a set of genes referred to as UPR target genes. Three membrane sensors have been identified in the ER that function as signal transducers of ER stress: inositol-requiring protein 1 (IRE1), activating transcription factor (ATF) 6, and protein kinase RNA-like endoplasmic reticulum

kinase (PERK) (Figure 2). IRE1 activation leads to endoribonucleolytic excision of an intron within X-box binding protein 1 (XBP1) messenger RNA. Uncleaved XBP1 inhibits gene transcription of a set of genes, whereas on its IRE1-mediated processing XBP1 acts as an activator of transcription of the same genes. IRE1 can also signal via JNK; this pathway is implicated in insulin resistance. ATF6 activates UPR target genes. PERK activation leads to phosphorylation of eukaryotic translation initiation factor 2 α , reducing its activity and thus leading to a global decrease in protein synthesis, and selective translation of ATF4, transcription of C/EBP-homologous protein (CHOP), and activation of nuclear factor κ B. ER stress can activate Bim, the potent proapoptotic BH-3-only protein, via CHOP-induced transcription, leading to its increased expression and decreasing its proteasomal degradation.²⁵ Additionally, CHOP can also increase expression of death receptors.²⁶ Thus, based on data in nonliver tissues, the ER stress pathway of cell death can regulate both the intrinsic and extrinsic cell death machinery and is an exciting area of research in liver injury.

Plasma Membrane Death Receptors

TNF- α , Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) are death ligands that signal via binding their cognate receptors, the death receptors.²⁷ Death receptors belong to the TNF/nerve growth factor superfamily and are essential for death ligand-mediated cell death. The death receptors Fas, tumor necrosis factor receptor 1 (TNFR1), and TRAIL receptors have recognized roles in liver injury. Receptor-ligand binding leads to receptor trimerization and activation of intracellular signaling cascades. The intracellular portion of these proteins contains death domains that recruit adaptor proteins leading to the activation of caspase 8 (an initiator caspase), cleavage of Bid to tBid, which in turn translocates to mitochondria and leads to mitochondrial permeabilization. Also, cell death can occur via TNF- α -induced Bid-dependent and TRAIL-induced Bax-dependent lysosomal permeabilization.²⁸ While death receptors do not induce ER stress, ER stress-mediated regulation of TRAIL receptor expression has been described.²⁶

Fas

Hepatocytes, cholangiocytes, sinusoidal endothelial cells, stellate cells, and Kupffer cells express Fas (CD95/Apo-1).^{29–31} Fas is activated on binding with membrane-bound FasL or soluble FasL. FasL is expressed on cytotoxic T lymphocytes and natural killer (NK) cells.³² Fas is important in hepatic health and disease. Mice genetically deficient in Fas exhibit hepatic hyperplasia.³³ Fas-induced cell death leads to removal of unwanted hepatocytes, such as virus-infected hepatocytes and cancer cells, by NK cells, NK T cells, and T lymphocytes.^{34–36} The exquisite sensitivity of the liver to Fas was shown by the induction of fulminant hepatic failure on injection of an Fas agonistic antibody in mice in 1993.¹ Furthermore, in a series of elegant experiments, it was shown that this toxicity is regulated by the Bcl-2 family proteins, prevented by Bcl-2,³⁷ and dependent on the proapoptotic protein Bid.³⁸ Significant elevations of soluble FasL occur in patients with acute liver failure, such as drug-induced liver injury or acetaminophen-induced liver injury.^{39,40} FasL and Fas receptor expression is up-regulated in many chronic liver diseases.^{4,35,41,42} Fas signaling cascades are shown in Figure 3.

TNF- α

TNFR1 and TNFR2 are both expressed on hepatocytes, although only TNFR1 expresses a death domain and executes the apoptotic program.⁴³ TNFR1 can activate both prosurvival and proapoptotic signals.^{44,45} TNF- α /TNFR1 signaling occurs in 2 steps (Figure 4); immediate recruitment of TNFR-associated protein (TRAF2) and receptor interacting protein leads to activation of nuclear factor κ B and transcriptional activation of prosurvival genes (eg, Bcl-xL, A1, XIAP, and cFLIP).⁴⁶ Apoptotic signaling occurs subsequently via the adaptor protein TNFR-associated death domain (TRADD) mediated caspase 8/Fas-associated death domain

(FADD) activation in a receptor-initiated, albeit receptor-independent, cytosolic complex. In this dual signaling process, prosurvival signals predominate; indeed, to unmask the proapoptotic signaling, in experimental models inhibitors of protein synthesis are used. The biological role is even more complex because in addition to its role as an inflammatory cytokine, TNF- α also mediates caspase-independent death via formation of reactive oxygen species. Receptor interacting protein 1 is recruited to TNFR1 and activates Nox1 reduced nicotinamide adenine dinucleotide phosphate oxidase, leading to reactive oxygen species formation and sustained activation of JNK leading to necrotic cell death.⁴⁷ Further studies are needed to elucidate the role of this pathway in liver diseases. While cultured primary hepatocytes are resistant to TNF- α toxicity, diseased hepatocytes can be sensitized to its toxicity. Very recently, the loss of cellular inhibitors of apoptosis proteins 1 and 2 has been shown to sensitize cancer cells to TNF- α -mediated cytotoxicity.⁴⁸ The effect of liver diseases on expression of cellular inhibitors of apoptosis proteins 1 and 2 is an interesting area of study.

TRAIL

TRAIL receptor 1 (death receptor 4) and TRAIL receptor 2 (death receptor 5/killer/TRICK2) are of intense interest and emerging significance in the pathogenesis of chronic liver diseases.⁴⁹ TRAIL receptors 1 and 2 can induce apoptosis via caspase activation, similar to Fas. Death receptor 3 (Apo-3/TRAMP/WSL-1/LARD) and death receptor 6 are incomplete receptors and cannot stimulate apoptotic signaling. While some controversy exists, the majority agrees that TRAIL is harmless to normal human hepatocytes.⁵⁰ Emerging data point to the sensitization of diseased hepatocytes to TRAIL-mediated apoptosis in several models of hepatitis, both experimental and human chronic viral hepatitis as well as nonalcoholic fatty liver disease.^{51, 52}

JNK

Membrane/organelle-initiated cytotoxic signaling pathways often converge on JNK; therefore, a brief discussion of JNK is relevant to all of the above mechanisms of cell death. Of 3 known mammalian JNK genes, 2 are expressed in the liver: JNK1 and JNK2.⁵³ Both can be activated by death receptor and ER stress pathways of apoptosis and may also be the pathway of caspase-independent reactive oxygen species-mediated cell death. While transient JNK activation is prosurvival, sustained JNK activation leads to cell death. This can occur via modulation of Bcl-2 family proteins, with subsequent mitochondrial permeabilization. For example, JNK can phosphorylate Bim, resulting in its activation and triggering mitochondrial dysfunction,⁵⁴ or lead to caspase 8 activation, Bid cleavage, and mitochondrial cytochrome *c* release.⁵⁵ Sustained JNK1 activation can promote degradation of cFLIP (an endogenous inhibitor of death receptor signaling), thereby promoting cell death by TNFR1, Fas, or TRAIL receptor 1/TRAIL receptor 2.⁵⁶ JNK can also promote transcription-dependent cell death by activating the transcription factor AP-1. Both JNK1 and JNK2 have been implicated in liver injury. In dietary obesity, JNK1 plays a predominant role⁵⁷; however, in free fatty acid-based cellular models of hepatocyte lipoapoptosis and toxin-mediated TNF-dependent liver injury, JNK2 plays a predominant role.^{55, 58} In acetaminophen-induced acute liver injury JNK2 is predominant, although both JNK1 and JNK2 partly mediate its toxicity.⁵⁹ Thus, JNK plays a pivotal role in many models of cell death.

Innate Immune System

The innate immune system is mentioned briefly because inflammation is an inseparable component of both acute and chronic liver injury. The readers are referred elsewhere for recent excellent reviews.⁶⁰ Suffice it to say that the liver, with its large population of Kupffer cells (tissue resident macrophages), dendritic cells, NK cells, and NK T cells, acts as an “immune organ” and has the unique milieu of close interaction between these immune cells and the

nonimmune cells of the liver. The cells of the innate immune system are not only the sources of inflammatory cytokines, especially TNF- α , but may well be the source of FasL and TRAIL in diseased livers. Indeed, it is the cells of the innate immune system that express the death ligands for death receptor-mediated liver injury. One of the more interesting features of liver injury in this context is the expression of stress ligands for the NK and NK T-cell receptor NKG2D; stressed hepatocytes may very well “invite” their destruction.⁶¹

Disease-Specific Mechanisms

Acute Liver Injury

Acetaminophen-induced acute liver injury is the leading cause of acute liver failure in the United States. Acetaminophen is metabolized to glucuronide and sulfate conjugates and excreted, and to a smaller extent (<5%) it is metabolized via cytochrome P450 2E1 to *N*-acetyl-p-benzoquinoneimine (NAPQI). NAPQI undergoes glutathione conjugation and is safely excreted. In acetaminophen-induced acute liver injury, glutathione depletion occurs and NAPQI accumulates within hepatocytes, presumably leading to irreversible binding to cellular and mitochondrial thiol proteins and mitochondrial permeabilization and cell death. This is the rationale for utilization of *N*-acetyl cysteine as an antidote. If given in a timely manner (within 16 hours of drug ingestion) it repletes cellular thiol stores, promoting NAPQI conjugation and excretion. However, if not administered early enough, the metabolism of acetaminophen is followed by an acute inflammatory response and liver cell death. Recently, several advances have been made into the mechanisms of acetaminophen-induced cytotoxicity. JNK activation and Bax translocation to mitochondria occur in models of acetaminophen toxicity, before onset of cell death.⁵⁹ Inhibition of JNK, both pharmacologically and genetically, results in improved survival, lower TNF- α Fas, and FasL levels, and cell death. Even delayed JNK inhibition was effective in reducing cell death, unlike *N*-acetyl cysteine.⁶² Furthermore, involvement of the innate immune system in acetaminophen-induced liver injury was shown by NK and NK T-cell depletion that abrogates acetaminophen-induced hepatotoxicity.⁶³

Elevated circulating levels of Fas, TNF- α , and TNFR are found in patients with acetaminophen-induced acute liver injury and fulminant hepatic failure of other causes.^{39,40,64} In addition to Fas and TNF- α , intrahepatic caspase activation, intrahepatic M30 immunostaining, and circulating levels of M30 are increased in patients with fulminant hepatic failure. Indeed, high levels of apoptosis (as measured by circulating M30) correlate with need for organ transplantation or death, and lower levels of apoptosis were found in spontaneous survivors. Whether the cytokine/death ligand balance in acute liver injury can be manipulated for therapeutic gain remains to be seen. Theoretically, inhibition of the innate immune response, Fas signaling, or JNK-induced apoptosis may be of benefit in decreasing massive hepatocyte apoptosis.^{65,66}

Cholestatic Liver Injury

Cholestatic liver injury is a descriptive term applied to liver injury occurring in the presence of bile flow impairment. It is characterized by elevated hepatocellular levels of toxic bile acids. Mechanisms of bile salt-induced liver injury have been elucidated using in vitro models as well as the bile duct-ligated mouse model. Bile salt toxicity correlates with their lipophilicity, conjugation state, and concentration, occurring well below the critical micellar concentration required for direct detergent activity; glycine-conjugated chenodeoxycholic acid is more toxic than the taurine-conjugated chenodeoxycholic acid. Elegant in vitro and in vivo studies have shown that hepatocyte apoptosis is a prominent feature of cholestatic liver diseases.⁶⁷ In cell culture models, glycine-conjugated chenodeoxycholic acid leads to Fas-induced cell death.⁶⁸ It can do so even in the absence of FasL by increasing cell surface trafficking of Fas and spontaneous oligomerization.⁶⁹ This ligand-independent oligomerization occurs in an

epidermal growth factor receptor–dependent fashion involving Yes kinase and culminating in the phosphorylation of Fas.⁷⁰ Fas-deficient (*lpr*) mice show attenuated, but not absent, hepatocyte apoptosis following bile duct ligation.⁶⁷ This led to the demonstration that glycine-conjugated chenodeoxycholic acid also up-regulates TRAIL receptor 2 expression and sensitizes hepatocytes to TRAIL-induced apoptosis.^{71,72} Glycine-conjugated chenodeoxycholic acid–induced Fas-dependent and Fas-independent apoptosis can be blocked by inhibiting Bid expression.⁷³ This also prevents glycine-conjugated chenodeoxycholic acid–induced mitochondrial permeabilization and cytochrome *c* release.

Fas-deficient mice also display reduced fibrosis following bile duct ligation, pointing to a link between hepatocyte apoptosis and fibrogenesis.⁷⁴ Caspase inhibition with IDN-6556 (a commercially available, pan-caspase inhibitor), also led to attenuation of hepatocyte apoptosis and liver inflammation and was antifibrogenic.⁷⁵ Further studies promoting stellate cell apoptosis in these models have also shown decreased fibrosis.⁷⁶ Thus, in cholestatic disorders, apoptosis can be modulated in 2 ways for therapeutic gain: inhibition of hepatocyte apoptosis with the goal of decreasing inflammation and fibrosis and promotion of stellate cell apoptosis with the goal of halting fibrogenesis. The toxic bile salt sodium deoxycholate can activate the ER stress pathway in an in vitro model.⁷⁷ This is also observed in vivo; in transgenic mice overexpressing α_1 -antitrypsin Z protein in the ER, cholestatic injury is associated with activation of the ER stress pathway, although ER stress pathway activation is not observed in wild-type bile duct–ligated mice.⁷⁸

Chronic Viral Hepatitis

Chronic hepatitis C (HCV) is the most common cause of end-stage liver disease and the leading indication for liver transplantation in the United States. HCV causes persistent infection and fibrosis. The mechanisms that lead to the establishment of immune evasion and persistent infection are complex and beyond the scope of this review. Based on the paradigm of hepatocyte apoptosis begetting fibrosis as an injury response, up-regulation of hepatocyte apoptosis in chronic hepatitis C would be expected. Indeed, there is ongoing, low-grade, Fas-mediated apoptosis that correlates with the severity of inflammation.⁷⁹ Fas expression is enhanced on infected hepatocytes, leading to HCV-specific T cell–mediated apoptosis via expression of FasL.^{80,81} Circulating levels of soluble FasL are elevated in HCV infection and correlate with serum alanine aminotransferase level and histologic grade.³⁵ The cytokeratin 18 neoantigen (M30) is also elevated in patients with chronic hepatitis C.⁸² Even in patients who have normal serum alanine aminotransferase levels, M30 levels are elevated, reflecting ongoing hepatocyte apoptosis; the highest levels occur in patients with cirrhosis. This surrogate marker of apoptosis needs further validation and may eliminate the need for repeated liver biopsies.

Hepatitis C viral proteins regulate apoptosis. Both proapoptotic and antiapoptotic actions have been described. In different cellular models, the HCV core protein can sensitize TRAIL-resistant Huh7 cells to TRAIL-induced apoptosis⁸³; it can bind to the death domain of TNFR1 and sensitize to TNF- α –induced apoptosis by increasing expression of FADD⁸⁴ and sensitize to Fas-induced apoptosis in HepG2 cells.⁸⁵ NS3 protein can promote activation of caspase 8, sensitizing to Fas-induced apoptosis.⁸⁶ Synergistic Fas-mediated apoptosis occurs with alcohol consumption in hepatitis C and is associated with increased Fas levels.⁷⁹ Murine hepatocytes that express envelope protein 1 (E1) and envelope protein 2 (E2) can increase apoptosis of activated T cells, providing a mechanism for immune escape in chronic infection.⁸⁷

Hepatitis B virus (HBV) infection also regulates the apoptotic machinery to its benefit, establishing persistent infection and evading the immune system. Similar to chronic HCV infection, chronic HBV infection is also characterized by low levels of hepatocyte apoptosis.

Liver injury is triggered by host immune response to viral protein expressing hepatocytes, because the virus is non-cytopathic. Serum Fas and hepatic TNFR1, TNF- α , and Fas expression are enhanced in chronic HBV infection and correlate with histologic activity^{36,45}; in patients with fulminant hepatic failure due to HBV, Fas-expressing infiltrating lymphocytes were shown in areas of apoptotic hepatocytes.⁴⁰ Chronic HBV infection is characterized by periodic flares. NK cell TRAIL expression increases in temporal correlation with serum alanine aminotransferase levels during flares, and TRAIL receptor 2 is found to be up-regulated in liver samples from patients with chronic HBV.⁵¹ In another study, Bax protein and messenger RNA were up-regulated in hepatocytes, and Bax expression positively correlated with the number of apoptotic nuclei⁸⁸; hepatocyte sensitization to TRAIL toxicity was also Bax dependent.⁸⁹ In experimental models, HBx protein can up-regulate TRAIL receptor 1 expression, leading to TRAIL-mediated apoptosis.⁹⁰

Alcoholic Steatohepatitis

Hepatocyte steatosis, ballooning, apoptosis, and a lobular inflammatory infiltrate are the characteristic histologic features of alcoholic steatohepatitis. In the absence of advanced fibrosis, these changes are reversible on cessation of alcohol consumption. In experimental models, ethanol produces characteristic changes of mitochondrial and microsomal dysfunction, oxidative stress,⁹³ Bax translocation to mitochondria, and mitochondrial permeability transition leading to cytochrome *c* release and caspase activation.⁹⁴ Conversely, antioxidants, inhibition of mitochondrial permeability transition, or inhibition of caspases prevents acute ethanol-induced apoptosis. Ethanol induces cytochrome P450 2E1 in hepatocytes, further promoting formation of reactive oxygen species. Ethanol ingestion also leads to activation of Kupffer cells.⁹⁵ Activated Kupffer cells secrete multiple inflammatory cytokines, such as TNF- α , interleukin-6, transforming growth factor β 1, that further promote liver inflammation, and inactivation of Kupffer cells prevents ethanol-induced liver injury. The importance of TNF- α in the pathogenesis of alcoholic steatohepatitis is well characterized.⁹⁶ It mediates both hepatocyte apoptosis and hepatic inflammation. Circulating levels of TNF- α and TNFR1 are elevated in alcoholic steatohepatitis, and the latter correlates with 3-month mortality^{97,98}; hepatic expression of TNFR is enhanced in chronic ethanol ingestion,³ and genetic studies in mice show that TNFR1 is essential for alcohol-induced liver injury.⁹⁶ In another study, TNFR1 only partially mediated ethanol-induced liver injury in mice, suggesting a TNF- α -independent role for the ER stress pathway (vide infra).⁹⁹ TNF- α neutralization decreases hepatic injury and inflammation in experimental models.¹⁰⁰ While data on infliximab are conflicting, initial studies using etanercept, a TNF-neutralizing antibody, have shown its safety in humans, and larger studies are under way.¹⁰¹

In the proinflammatory cytokine milieu of alcoholic steatohepatitis, the mode of cell death of hepatocytes is apoptotic.⁵ Apoptosis correlates with bilirubin level, aspartate aminotransferase level, inflammation, and Maddrey score, a prognostic indicator in acute alcoholic hepatitis. Hepatic Fas receptor expression is enhanced in alcoholic steatohepatitis, as are circulating levels of Fas, FasL, and TNF- α .³ Apoptotic hepatocytes colocalize with infiltrating neutrophils, suggesting that the characteristic inflammatory response partly occurs secondary to hepatocyte apoptosis and partly due to the direct activation of Kupffer cells by ethanol leading to cytokine production. Stellate cells are also regulated in a paracrine manner by acetaldehyde and reactive oxygen species, produced by the metabolism of ethanol in surrounding hepatocytes.¹⁰² One area of active research is ER stress. Acetaldehyde, an ethanol metabolite, induces ER stress in cell culture systems.¹⁰³ Mitochondrial dysfunction occurs as a consequence of ER stress. In animal models, ethanol induces a CHOP-dependent apoptosis.¹⁰⁴ Wild-type mice on ethanol feeding up-regulate cathepsin B and GADD45 and simultaneously down-regulate Bcl-xL, leading to several-fold enhanced apoptosis. However, mice that lack CHOP show the opposite gene expression regulation, decreased cathepsin B,

decreased GADD45, increased Bcl-xL, and no apoptosis. Inflammation and some other markers of ER stress are observed in both groups of mice. Thus, CHOP is a significant mediator of ER stress-induced apoptosis in liver disease secondary to alcohol. Fibrogenesis in this model and the importance of CHOP in human alcoholic liver disease needs further study. Significant advances have been made in ethanol-mediated hepatocyte apoptosis and pathways of liver injury; however, the characteristics of the subset of individuals who develop progressive liver injury and fibrosis from chronic ethanol ingestion are still not clear. Differences in CHOP regulation could be one potential explanation.

Nonalcoholic Steatohepatitis

Nonalcoholic steatohepatitis shares histologic similarities with alcoholic steatohepatitis⁴; however, the candidate mediators of liver injury are distinct and potentially include alterations in circulating cytokines, adipokines, and free fatty acids. Elevated circulating (serum) free fatty acid levels occur in patients with nonalcoholic steatohepatitis.¹⁰⁵ Liver triglyceride is derived primarily from circulating free fatty acids, derived from adipose tissue lipolysis.¹⁰⁶ In insulin resistance, adipose tissue lipolysis and liver triglyceride deposition are enhanced. Comprehensive lipidomic profiling of human subjects with nonalcoholic fatty liver disease and nonalcoholic steatohepatitis have recently been reported.¹⁰⁷ Several interesting observations can be made on the basis of this profiling. The first confirms that liver triacyl glycerol and diacyl glycerol levels are significantly increased in patients with nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. The second observation pertains to hepatic free fatty acids. These are unchanged across the spectrum from controls to nonalcoholic fatty liver disease to nonalcoholic steatohepatitis, although careful analysis of the triacyl glycerol composition shows increased saturated fatty acid (particularly palmitic acid) and monounsaturated fatty acid levels (oleic acid in both triacyl glycerol and diacyl glycerol); simultaneous serum free fatty acid concentrations were not measured. Elevated circulating palmitic acid and oleic acid levels in previous studies and their subsequent availability for formation of triglyceride are consistent with the observed increase in their esterified forms in the liver. It also confirms the belief that free fatty acid toxicity is mediated by pathways independent of their availability for formation of triglyceride (vide infra) and that triglyceride accumulation is a surrogate marker for the very metabolic abnormalities that result in elevated free fatty acid levels.

Free fatty acid-induced lipooptosis has been shown in many cell types in the metabolic syndrome, including pancreatic beta cells, vascular endothelial cells, cardiac myocytes, and hepatocytes.^{58,108} In hepatocytes, free fatty acids can activate the lysosomal pathway of cell death, sensitizing cells to TNF- α toxicity.²³ Furthermore, free fatty acids induce a JNK-dependent lipooptosis, which is mediated by intracellular Bim levels and Bax activation, leading to mitochondrial permeabilization, cytochrome *c* release, and caspase activation.⁵⁸ Saturated fatty acids induce Bim expression via a FoxO3A-dependent pathway.¹⁰⁹ JNK activation correlates with observed toxicity for each free fatty acid tested, and saturated free fatty acids lead to sustained JNK activation and are more toxic than monounsaturated free fatty acids. Even minimally toxic oleic acid sensitizes hepatocytes to TRAIL-mediated apoptosis.⁵² The ER stress pathway is also activated by saturated free fatty acids in pancreatic beta cells, and its role in steatohepatitis merits further investigation.¹¹⁰

In keeping with the in vitro observations, steatotic livers are sensitized to Fas-mediated and TRAIL-mediated apoptosis.^{50,111} In human biopsy samples, hepatocyte apoptosis correlates with inflammation and fibrosis.⁴ Elevated Fas, TNF receptor, and TRAIL receptor 2 expression is seen in liver biopsy samples. Circulating caspase target M30 is also elevated in patients with nonalcoholic steatohepatitis and serum levels correlate with hepatic levels of the same, again offering a noninvasive means of measuring apoptosis.¹⁴ Sensitization to death ligands may

occur independently of JNK activation in vivo, which is known to mediate insulin resistance and liver injury in models of dietary obesity.⁵⁷

Fibrogenesis

Hepatic fibrosis is the hallmark of chronic liver injury. Sustained hepatocyte losses could conceivably be corrected by repopulation were it not for the development of fibrosis. Hepatic stellate cells remain quiescent in health and are activated in response to injury. Activated stellate cells are recognized as fibrogenic and lead to the deposition of collagen type 1. Kupffer cells, resident liver macrophages, are activated by engulfment of apoptotic hepatocytes; this leads to removal of dead cells from the liver. Furthermore, activated Kupffer cells secrete inflammatory cytokines, linking apoptosis in the liver to inflammation. Similarly, apoptotic bodies can be engulfed by stellate cells, leading to their activation and enhanced expression of α -smooth muscle actin, transforming growth factor β , and collagen type I.¹¹² In vivo, inhibition of hepatocyte apoptosis, such as in the Fas-deficient bile duct-ligated mouse, also leads to less fibrosis.⁷⁴ This phenomenon could result from a direct interaction between apoptotic hepatocytes and stellate cells in vivo or an indirect effect of decreased Kupffer cell activation and inflammation. Stellate cells undergo apoptosis during the resolution of liver injury.¹¹³ In conditions of ongoing liver injury, selective induction of stellate cell apoptosis can dissociate hepatocyte apoptosis from fibrogenesis.⁷⁶ Activated stellate cells also express enhanced levels of TRAIL receptor 2 and are susceptible to TRAIL-mediated apoptosis.¹¹⁴ A model of the central role of hepatocyte apoptosis in liver injury and fibrosis is depicted in Figure 5.

Therapeutic Implications

Advances in mechanistic understanding impart clinical significance if therapeutic interventions can be derived from them. In this context, cell death pathways present many potential therapeutic targets.¹¹⁵ Some recent exciting developments are discussed here. The monoclonal M30 antibody that recognizes a caspase-generated cytokeratin 18 neoantigen is an excellent example of a clinical application. This test is reliable, easy, and accurate in many disease models. Studies in larger validation cohorts are needed before it can replace more invasive testing. It is promising because highest levels were found in cirrhotic patients in one study, and in another study it could reliably differentiate nonalcoholic fatty liver disease from nonalcoholic steatohepatitis.^{14,82} Inhibition of the mitochondrial permeability transition utilizing *N*-methyl-4-isoleucine cyclosporine (NIM811) or minocycline led to a decrease in liver injury and improved graft survival in experimental liver transplantation; the former also improved regeneration of small-for-size liver grafts.¹¹⁶ Pan-caspase inhibitor, IDN 6556, showed promise in preclinical studies by inhibiting hepatocyte apoptosis, decreasing inflammation and fibrosis.¹¹⁷ In a small phase 2 clinical trial, it lowered serum aminotransferase activity following oral administration for 2 weeks. Larger and longer studies, especially with incorporation of other measures of injury and fibrosis, are necessary. Ursodeoxycholic acid is commonly used in the treatment of cholestatic liver disorders. Inhibition of apoptosis in hepatocytes partly mediates the salutary effects of ursodeoxycholic acid.^{118,119} TRAIL receptor antibodies are being investigated in cancer therapy; emerging data indicate that in chronic liver disease (HBV, HCV, nonalcoholic fatty liver disease), hepatocytes are sensitized to TRAIL-induced apoptosis, a potential toxicity that may limit their therapeutic potential.⁵⁰ JNK inhibitors are in clinical development and based on preclinical studies may be lifesaving in acetaminophen-induced acute liver failure and beneficial in nonalcoholic fatty liver disease. Of the BH-3-only proteins, Bim has been studied in liver disease; these are also attractive potential targets.

Biographies

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Abbreviations used in this paper

ATF, activating transcription factor
 CHOP, C/EBP-homologous protein
 ER, endoplasmic reticulum
 FADD, Fas-associated death domain
 FasL, Fas ligand
 IRE1, inositol-requiring protein 1
 JNK, c-jun N-terminal kinase
 NAPQI, *N*-acetyl-p-benzoquinoneimine
 NK, natural killer
 PERK, protein kinase RNA-like endoplasmic reticulum kinase
 TNF, tumor necrosis factor
 TNFR, tumor necrosis factor receptor
 TRAIL, tumor necrosis factor-related apoptosis inducing ligand receptor
 UPR, unfolded protein response
 XBP1, X-box binding protein 1

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Figure 1.

Extrinsic and intrinsic pathways of hepatocyte apoptosis. The extrinsic pathway is activated by death receptors. Fas or TRAIL (depicted here) bind to their cognate receptors, leading to the formation of the death-inducing signaling complex (DISC), with caspase 8 activation, Bid cleavage, and subsequent mitochondrial permeabilization. Bim activation can also occur downstream of death receptor signaling, leading to Bax activation and mitochondrial permeabilization. The TNF- α signaling pathway also leads to Bid cleavage with lysosomal permeabilization, leading to release of lysosomal contents and mitochondrial permeabilization. The intrinsic pathway of cell death can be initiated by myriad intracellular stressors that can activate the ER stress pathway, lysosomal permeabilization, or JNK activation. These cascades lead to inhibition of the antiapoptotic proteins (Bcl-xL, Bcl-2) and activation of the proapoptotic proteins (Bax, Bim, Bad, Bid). Mitochondrial permeabilization occurs eventually and is required for hepatocyte apoptosis.

Figure 2.

The ER stress pathway. The ER membrane contains 3 stress transducers: IRE1, ATF6, and PERK. Their activation is controlled by the ER molecular chaperone immunoglobulin-binding protein of B cells (BiP/GRP78). Release from BiP binding activates the ER stress transducers. IRE1 contains a cytoplasmic kinase domain and an endoribonuclease domain. The latter cleaves XBP1 to spliced XBP1 (sXBP1), leading to transcriptional activation of adaptive molecules to overcome ER stress, such as ER chaperones, lipid synthesis, and increasing ER-associated degradation (ERAD). PERK activation leads to phosphorylation of eukaryotic translation initiation factor 2 α , leading to global translation attenuation. ATF6 also transcriptionally activates UPR target genes. These processes collectively function to overcome and correct ER stress. In the face of sustained ER stress, apoptotic machinery becomes activated. PERK leads to selective translation of ATF4, transcription of CHOP, and activation of the apoptotic machinery. CHOP can affect Bim levels and death receptor 5 levels. IRE1 can also activate JNK. Active Bax/Bak can permeabilize the ER membrane, leading to calcium release, activation of calpains, and apoptosis. Growth arrest and DNA damage protein 34 (GADD34) associates with protein phosphatase 1 (PP1), leading to dephosphorylation of eukaryotic translation initiation factor 2 α and attenuation of the UPR.

Figure 3.

Fas and TRAIL receptor signaling. Fas and TRAIL receptors activate conserved signaling pathways on receptor ligand interactions. FADD binds to the intracellular death domain (DD) containing trimerized receptor. FADD also contains a death effector domain (DED) that leads to activation of caspase 8 by cleavage and homodimerization of procaspase 8. Caspase 8 leads to cleavage of Bid to tBid and downstream mitochondrial permeabilization. Mitochondrial contents, including cytochrome *c*, SMAC/DIABLO, APAF-1, and endonuclease G, are released, leading to the activation of caspase 3/7. Caspase 3/7 leads to cleavage of cellular proteins and the characteristic apoptotic morphology.

Figure 4.

TNF- α signaling. TNF- α binds to the extracellular domain of its cognate receptor, TNFR1. The intracellular portion of TNFR1 contains a death domain (DD). This interacts with the DD of TNFR1-associated death domain protein (TRADD) and recruits receptor interacting protein also via its DD, and TNF receptor-associated factor (TRAF2) via its kinase domain or an intermediate domain. This signal transduction complex is referred to as complex 1. Nuclear factor κ B (NF- κ B) activation and transient JNK activation occur downstream of complex 1. Active NF- κ B translocates to the nucleus, leading to the transcription of antiapoptotic genes. Among its known target genes are cellular FLICE-like inhibitory protein (cFLIP), Bcl-xL, Mcl-1, A1, and XIAP, which regulate apoptosis at multiple levels. The early and transient activation of JNK also promotes survival. Sustained JNK activation requires receptor interacting protein; although the exact pathways are not fully known, oxidative stress is recognized as one mediator of sustained JNK activation. TRADD, receptor interacting protein, and TRAF2 then undergo receptor dissociation, recruit FADD via its DD. FADD also contains a death effector domain (DED) that leads to activation of caspase 8, cleavage of Bid to tBid, and downstream mitochondrial permeabilization.

Figure 5.

The central role of hepatocyte apoptosis in liver injury. Vulnerable hepatocytes undergo apoptosis when stressed. Apoptosis can be initiated via Kupffer cell release of TNF- α , leading to activation of JNK. Activated NK and NK T cells release Fas or TRAIL, and interferon gamma, which up-regulates Fas or TRAIL release, leading to death receptor-mediated hepatocyte apoptosis. Hepatocyte apoptosis can also occur via activation of the intrinsic pathway (not shown here). Apoptotic hepatocytes are engulfed Kupffer cells, leading in turn to their activation. Activated Kupffer cells secrete TNF- α , interleukins, and interferon to promote the inflammatory response. They also secrete transforming growth factor β , leading to activation of stellate cells. Stellate cells can also be directly activated by apoptotic bodies. Activated stellate cells secrete collagen type I, leading to liver fibrosis. Attenuation of hepatocyte apoptosis, or forced apoptosis of activated stellate cells, such as with proteasomal inhibitors or TRAIL, can lead to resolution of fibrosis.

Table 1

The Bcl-2 Family Proteins

Bcl-2 Family Proteins		
Antiapoptotic	Proapoptotic	
	BH3 only	Multidomain
Bcl-xL	Bid	Bax
Mcl-1	Bim	Bak
Bcl-w	Puma	Bok
Bcl-2	Noxa	Bcl-x _s
A1	Bad	
Boo	Bik	
	Bmf	
	Hrk	