Apoptosis: Mechanisms and Relevance in Liver Diseases

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Abbreviations: ATF6 activating transcription factor 6, ATF4 activating transcription factor-4, CHOP C/EBP-homologous protein, ER endoplasmic reticulum, eIF2α eukaryotic translation initiation factor-2α, FADD Fas associated death domain, IRE1 inositol-requiring protein-1, JNK c-jun N-terminal kinase, NK natural killer, NFκB nuclear factor kappa B, PERK protein kinase RNA-like ER kinase, TNF-α tumor necrosis factor alpha, TRAIL tumor necrosis factor-related apoptosis inducing ligand, TNFR1 tumor necrosis factor receptor 1, TNFR2 tumor necrosis factor receptor 2, TRAIL-R1 tumor necrosis factor-related apoptosis inducing ligand receptor 1, TRAIL-R2 tumor necrosis factor-related apoptosis inducing ligand receptor 2, TRAF-2 tumor necrosis factor receptor associated protein, XBP1 X-box binding protein-1,

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Apoptosis is a ubiquitous form of cell death occurring in human liver diseases. Apoptosis has historically been defined morphologically by the presence of cytoplasmic shrinkage (pyknosis), chromatin condensation, nuclear fragmentation (karyorhexis), the presence of plasma membrane blebbing and the maintenance of an intact plasma membrane which retains its integrity as the cell fragments into apoptotic bodies(1). Indeed, apoptotic bodies were first described in the liver in patients with Yellow Fever where they were referred to as councilman bodies(2). Apoptosis is classically defined morphologically, and therefore the detection and confirmation of apoptosis has previously required tissue or cellular analysis. Caspases are a group of intracellular enzymes that mediate the cellular demolition characterized phenotypically as apoptosis. Circulating markers of caspase activity can now be measured in serum and serve as surrogate markers of caspase-mediated cell death. In particular, serum levels of specific caspase-mediated cleavage products of cytokeratin 18 are indicators of epithelial cell apoptosis, including hepatocyte apoptosis. Apoptosis is a highly regulated form of cell death, with multiple check points and molecular mediators. Also, apoptosis occurring during development and ageing is genetically regulated and therefore the term programmed cell death is used to describe apoptosis in this context.

Hepatocyte apoptosis can be initiated via the death receptor or extrinsic pathway of apoptosis, or by cellular perturbations that together comprise the intrinsic pathway of apoptosis(3) (Figure 1).
intracellular domain of ligated homotrimerized receptors in conjunction with adaptor proteins, leading to caspase 8 activation, Bid cleavage, and activation of Bax and Bak. TNF-α signaling pathway can promote apoptosis by Bid induced lysosomal permeabilization. Intracellular perturbations such as ER stress, lysosomal permeabilization, or JNK activate the intrinsic pathway of cell death. ER stress induced apoptosis is partly mediated by the transcription factor CHOP, which can upregulate TRAIL-R2 or Bim expression. JNK activation can be induced by TNF-α, ER stress, or reactive oxygen species. These pathways are regulated by the proapoptotic and antiapoptotic proteins of the Bcl-2 family.

In hepatocytes, both pathways converge on mitochondria. Multiple intracellular molecules both mediate and regulate the apoptotic signalling cascades, upstream and downstream of mitochondria(4). Mitochondrial permeabilization is not only requisite but also sufficient for hepatocyte apoptosis; therefore, regulators downstream of mitochondrial permeabilization cannot prevent cell death. Unlike developmental apoptosis which is carefully regulated in a spacio-temporal pattern and does not involve secondary events, pathologic apoptosis is unregulated and can be massive. This pathologic apoptosis can evoke tissue injury, inflammation and fibrosis. Thus, in acute liver injury apoptosis is massive and correlates with outcome, i.e. liver transplantation or death(5). In chronic liver injury apoptosis is continuous, modulates the inflammatory response and promotes fibrogenesis, resulting in cirrhosis(6). Hepatocyte apoptosis is evident in liver injury related to viral hepatitis, metabolic diseases, alcoholic steatohepatitis, autoimmune hepatitis and drug induced liver injury, (7-11), emphasizing the shared pathogenic role of hepatocyte cell death in liver injury from multiple, varied, acute and chronic insults. Apoptosis of other cellular compartments, such as sinusoidal endothelial cells and stellate cells, also plays a role in liver injury. Apoptotic signalling concepts, mediators and regulators of apoptosis are discussed further, with information from both hepatocyte and select non-hepatocyte cellular paradigms, with inclusion of injury stimulus-specific information within each mechanism.

THE EXTRINSIC PATHWAY

Death receptors are cell surface transmembrane proteins that belong to the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily, and are defined on the basis of ligand specificity, i.e., their affinity for tumor necrosis factor alpha (TNF-α), Fas ligand (FasL), or tumor necrosis factor-related apoptosis inducing ligand (TRAIL)(12). The extracellular N-terminal domain binds their respective ligands; there is a membrane spanning region and then the intracellular C-terminal domain, which contains a conserved sequence known as the death domain (DD). The ligand-bound trimerized receptor complex brings together the DD allowing recruitment of other adaptor proteins. For death signaling, Fas-associated protein with death domain (FADD), must be recruited to the receptor stimulated protein complex(13). FADD contains a death effector domain (DED) through which it binds inactive initiator caspases 8 and 10, in their procaspase form. The procaspases form homodimers and undergo autoproteolytic cleavage with formation of active caspase 8 or 10(14). The
complex consisting of trimerized receptor death domains, adaptor proteins and procaspases 8 or 10 is referred to as the death-inducing signaling complex (DISC).

In hepatocytes, mitochondrial permeabilization with amplification of the apoptotic cascade occurs in death receptor initiated apoptosis. This involves release of mitochondrial mediators of apoptosis, eventual activation of caspase 3 and 7, with positive feedback amplification of caspase 8 activation. This requirement for mitochondrial amplification categorizes hepatocytes as type-II cells, in distinction to type-I cells in which caspase 8 or 10 can directly activate caspase 3 and 7, without mitochondrial involvement(15). Caspase 8 proteolytically cleaves the proapoptotic BH3 only protein of the Bcl-2 family Bid to tBid (truncated Bid), which leads to activation of Bax and Bak (proapoptotic multidomain members of the Bcl-2 family), and pore formation in the outer mitochondrial membrane(16). Multiple levels of signal transduction and amplification present opportunities for regulation of death receptor mediated apoptosis at many levels. Availability of cell surface receptor and ligand is one level, e.g., the hepatocyte growth factor (HGF) receptor met, associates with and regulates the availability of Fas for binding its ligand(17). Cellular caspase-8 (FLICE)- like inhibitory protein (cFLIP) can inhibit cytotoxic signaling by death receptors(18). cFLIP is an enzymatically inactive homolog of caspase 8 with conserved structural homology in the DED that allows binding to FADD. This binding precludes maximal cellular activation of caspase 8. Pro- and antiapoptotic members of the Bcl-2 family regulate the extrinsic pathway, by modulating the ability of tBid to activate Bax and Bak (vide infra)(19).

**Tumor necrosis factor-α:** TNF-α is a circulating cytokine, primarily produced by the macrophage component of the innate immune system, represented by Kupffer cells in the liver, and can also be produced by other cell types, such as hepatocytes. Hepatocytes express both tumor necrosis factor receptor 1 (TNFR1), a 55 kDa protein and tumor necrosis factor receptor 2 (TNFR2), a 75 kDa protein, though their functional significance differs(20). TNFR1 is thought to mediate most of the biologic effects of TNF-α; it expresses a cytoplasmic death domain and executes the apoptotic program by interacting with adaptor proteins(21, 22) (Figure 2, below).
Figure 2 Complex I and complex II of Tumor necrosis factor-alpha: Tumor necrosis factor receptor 1 (TNFR1), upon binding TNF-α on its extracellular domain activates complex I and complex II. Complex I is formed by the adaptor proteins, TNFR1-associated death domain protein (TRADD) and receptor interacting protein (RIP), which recognize and bind via their death domains (DD) and TNF receptor associated factor (TRAF2) via its kinase domain or an intermediate domain. Complex I mediates the activation of nuclear factor κ B (NF-κB) and transient c-jun N-terminal kinase (JNK) activation. NF-κB translocates to the nucleus transcriptionally activating antiapoptotic and inflammatory genes, such as, cellular FLICE like inhibitory protein (cFLIP), Bcl-xL, Mcl-1, A1 and XIAP, which regulate apoptosis at multiple levels. Sustained JNK activation requires the adaptor protein RIP and is mediated in part by oxidative stress. Complex II is formed by receptor dissociation of TRADD, RIP and TRAF2 and ligand independent recruitment of Fas associated death domain (FADD) via its DD. FADD contains a death effector domain (DED) leading to recruitment and activation of procaspase 8.

On binding TNF-α, TNFR1 recruits the adaptor protein tumor necrosis factor receptor-associated death domain (TRADD)(23). Signaling then proceeds in two steps, the first step or complex I involves recruitment of tumor necrosis factor receptor associated protein (TRAF-2) and receptor interacting protein 1 (RIP1) leading to activation of nuclear factor κB (NFκB) (24, 25). TRADD then dissociates from the ligated receptor, recruits FADD and procaspase 8 to initiate apoptotic signaling; this signaling pathway is referred to as complex-II. TRADD does not interact with TNFR2, nor does FADD directly interact with TNFR1. Therefore, TNF-α-TNFR1 signaling first leads to NFκB mediated transcriptional activation of prosurvival (e.g. Bcl-xL, A1, XIAP and cFLIP) and
proinflammatory genes (e.g. interleukin 6). In cells resistant to NFκB, or in the presence of a transcriptional inhibitor (actinomycin D), the apoptotic effect of TNF-α is unmasked.

TNF-α has pleiotropic effects in vivo, including hepatocyte proliferation, liver inflammation and modulation of hepatocyte apoptosis. In a murine model of TNF-α induced liver injury (TNF-α + D-galactosamine), liver injury is Bax-dependent(26). TNF-α associated caspase 8 activation can also cause lysosomal permeabilization with release of intralysosomal cathepsin B into the cytosol which causes mitochondrial dysfunction(27). Mice deficient in cathepsin B are protected from the injurious effects of TNF-α(28). C-jun N terminal kinase (JNK), a stress activated kinase, is activated by TNF-α. Sustained activation of JNK can lead to apoptosis by modulation of the Bcl-2 family of proteins. JNK can also transcriptionally activate death receptor expression, i.e. TRAIL-receptor 2/death receptor 5. Furthermore, JNK can promote TNF-α induced apoptotic signaling at complex-II by facilitating degradation of cFLIP, thus antagonizing an antiapoptotic TNF-α induced NFκB target gene(29). Similarly, loss of cellular inhibitors of apoptosis proteins 1 and 2, also antiapoptotic NFκB target genes, sensitizes carcinoma cells to TNF-α mediated cytotoxicity(30). TNF-α can lead to superoxide formation and caspase-independent cell death, by TRADD and RIP1 mediated activation of Nox1 NADPH oxidase leading to reactive oxygen species formation(31). This process is independent of FADD, and caspase 8 activation. Thus, a multitude of complex processes contributes to TNF-α cytotoxicity.

In experimental models of liver injury, a role for TNF-α cell death has been elucidated. Following partial hepatectomy, massive hepatocyte cell death occurs after completion of cell cycle progression, due to sustained TNF-α signaling, in mice lacking tissue inhibitor of metalloproteinase 3 (Timp3), a model characterized by abnormal chronically elevated TNF-α activity(32). In ethanol fed mice, TNFR1 deficiency results in decreased hepatocyte apoptosis, serum alanine aminotransferase levels (ALT) and inflammatory foci as compared to wild type ethanol fed mice(33); TNFR2 deficient mice developed liver injury and apoptosis comparable to wild type controls(34). In ischemia reperfusion injury mice lacking TNFR1 and treated with a pentoxyfylline, a pharmacologic TNF-α inhibitor, liver injury and apoptosis are significantly reduced(35) Liver samples from patients with alcoholic steatohepatitis or nonalcoholic steatohepatitis demonstrate enhanced TNFR1 expression(36). Serum levels of TNFR1 in patients with alcoholic hepatitis are predictive of 3month survival(37). Thus, the TNF-α cascade is activated in patients with many liver diseases, including fulminant hepatic failure, alcoholic steatohepatitis, nonalcoholic steatohepatitis, chronic hepatitis C, and chronic hepatitis B(36, 38-40); it is indeed a hallmark of inflammatory changes in these conditions and likely contributes to hepatocyte apoptosis in vivo. Our understanding of why the TNF-R1 initiated NFκB cell survival pathways fail in these diseases remain rudimentary.

**Fas:** Fas (also known as Apo-1, CD95) is ubiquitously expressed in the liver(41- 43). Hepatocytes are exquisitely sensitive to Fas induced apoptosis, and exogenously administered Fas agonistic antibody results in fulminant hepatic failure in mice (44). Fas signaling usually results in
hepatocytes apoptosis; although there are reports of Fas induced proliferation of T cells and fibroblasts and a report describing Fas-mediated acceleration of liver regeneration after partial hepatectomy in mice (45, 46). Fas-Fas ligand (FasL) binding leads to receptor oligomerization, bringing together the intracellular DD, recruitment of FADD and procaspase 8 or 10 at the DISC (Figure 3, below).

Figure 3  Fas and TRAIL receptor signaling: Fas and TRAIL receptors are activated by ligand binding, which leads to receptor oligomerization, bringing together their conserved death domains (DD). The adaptor protein Fas associated death domain (FADD) binds to the trimerized intracellular death domain (DD) and via its death effector domain (DED) leads to activation of procaspase 8. Active caspase 8 leads to proteolytic cleavage of Bid to tBid and downstream mitochondrial permeabilization via activation of Bax and Bak. Mitochondrial permeabilization leads to release of the contents of the intermembrane space including cytochrome c, smac/DIABLO, Apaf 1 and endonuclease G culminating in the activation of caspase 3/7 and cleavage of cellular proteins.

This leads to activation and autoproteolytic activation of procaspase 8 or 10, generation of tBid, activation of Bax and Bak, mitochondrial permeabilization with eventual activation of caspase 3 and 7. Fas can be activated by soluble or circulating as well as membrane bound FasL. Fas ligand is expressed by cells of the immune system, such as cytotoxic T lymphocytes (CTL) and Natural Killer (NK) cells (47). The liver is enriched in both these cell populations, therefore under constant “Fas-
attack”. However Fas induced signaling is regulated at many levels. Cell surface expression of Fas, levels of FasL, and cFLIP inhibition of caspase 8 activation at the DISC are potential regulatory sites. Of interest in hepatocytes is the sequestration of Fas by the hepatocyte growth factor receptor (HGF), Met(17). Met-Fas complexes prevent binding of FasL to Fas; however, Fas does not affect HGF binding to its receptor Met. Pretreatment of cells with HGF releases Fas from this complex, and enhances FasL binding and toxicity at lower concentrations of FasL; High concentrations of FasL are maximally toxic even in the absence of HGF. Thus, the Met-Fas complex fine tunes and regulates the biologic availability of Fas in hepatocytes. In embryonic hepatocytes, Met prevents Fas induced cFLIP degradation, thus preventing apoptosis(48).

In adult mice, genetic deficiency of Fas leads to hepatic hyperplasia, in addition to enlargement of lymph nodes and spleen (49). The induction of fulminant hepatic failure in mice by exogenous administration of Fas agonistic antibody is further regulated by the Bcl-2 family of proteins. It can be abrogated by overexpression of Bcl-2 and enhanced by genetic inhibition of Bcl-xL (50, 51). Genetic inhibition of Fas itself or Bid mitigates liver injury by Fas agonists(51, 52).

Circulating levels of serum Fas are elevated in patients with fulminant hepatic failure(5, 53). Levels of serum Fas vary by etiology, and the highest levels occur in patients with drug induced liver injury. Fas expression and apoptosis are enhanced in liver samples from patients with chronic hepatitis C(54). Circulating levels of soluble Fas correlate with histologic activity, and along with levels of caspase 3 activity, are predictive of response to therapy(55-57). Similarly in patients with chronic hepatitis B hepatocyte Fas levels and circulating levels of sFas are elevated(54, 58, 59). Fas expression is enhanced in liver samples from patients with nonalcoholic fatty liver disease(7). In experimental models of dietary and genetic fatty liver, steatotic livers are sensitized to exogenous Fas administration. Indeed, in patients with nonalcoholic fatty liver disease, the inhibition of Fas by Met is diminished, providing another mechanism to explain the enhanced sensitivity to Fas induced hepatocyte apoptosis(60). Furthermore, free fatty acid treatment can increase Fas expression in vitro, in cell culture models of hepatocyte steatosis, sensitizing cells to Fas-induced apoptosis. In the bile duct ligated mouse model of cholestatic liver injury hepatocyte apoptosis is mediated by Fas, and Fas induced apoptosis promotes hepatic fibrosis(61, 62). Toxic bile acids promote cell surface expression of Fas, and can lead to ligand-independent Fas oligomerization and induction of hepatocyte apoptosis(63, 64). In bile salt mediated ligand-independent hepatocyte apoptosis Fas phosphorylation is required for its translocation to the cell surface; this can occur in a Yes kinase, epidermal growth factor receptor-dependent, and JNK-dependent manner(65, 66).

**Tumor necrosis factor-related apoptosis inducing ligand (TRAIL):** The role of tumor necrosis factor-related apoptosis inducing ligand (TRAIL, also known as Apo-2 Ligand) and its receptors in liver disease is an area with remarkable recent advances. TRAIL binds with several receptors(67). TRAIL receptor 1 (TRAIL-R1/ Death receptor (DR) 4) and TRAIL receptor 2 (TRAIL-R2/ DR 5/ Killer/ TRICK2) are complete receptors and can induce apoptosis via caspase activation,
similar to Fas(68). This occurs via the adaptor protein FADD, recruitment of procaspase 8 and 10 to the TRAIL receptor DISC, in a cFLIP-regulated manner (Figure 3). TRAIL receptor 3 (TRAIL-R3/ Apo-3/ TRAMP/ WSL-1/LARD, Decoy receptor 1(DcR1)) and TRAIL receptor 4 (TRAIL-R4, DR6, Decoy receptor 2(DcR2)) are incomplete cell surface receptors and cannot stimulate apoptotic signaling. Normal human hepatocytes, in situ and in vivo, are considered resistant to TRAIL-induced apoptosis, though there are occasional reports of in vitro TRAIL-induced hepatocyte apoptosis (69-71). This resistance to cell death may be secondary to cFLIP induced inhibition of caspase 8 activation at the DISC or cell surface expression/availability of TRAIL-R1 or TRAIL-R2. However, diseased hepatocytes are sensitized to TRAIL-induced apoptosis(72-75). TRAIL also sensitizes to Fas induced hepatocyte apoptosis by activating JNK and the proapoptotic BH3 only protein Bim(76).

TRAIL-induced hepatocyte apoptosis has been demonstrated in cholestatic, viral and metabolic liver diseases. Toxic bile acids transcriptionally regulate hepatocyte cell surface TRAIL-R2 expression in Fas deficient cells, and inactivate cFLIP by phosphorylation, thus dually sensitizing cells to TRAIL-induced apoptosis(77, 78). In the bile duct ligated mouse model of cholestasis, hepatocyte TRAIL-R2 expression is enhanced and hepatocytes are sensitized to exogenously administered TRAIL(79). By corollary, liver injury and hepatocyte apoptosis are significantly reduced in TRAIL deficient mice following bile duct ligation(80). Steatosis is also associated with increased hepatocyte expression of TRAIL-R2 and TRAIL-R1 which imparts sensitivity to TRAIL toxicity(69). Free fatty acids, which are elevated in the metabolic syndrome, transcriptionally enhance TRAIL-R2 expression in cell culture and render steatotic cells sensitive to TRAIL toxicity(75). In acute hepatitis B-induced liver failure in humans and experimental adenoviral acute hepatitis in mice, TRAIL-R2 expression is enhanced, as is sensitivity to TRAIL. This occurs independently of Kupffer cells and NK cells, suggesting a hepatocyte generated paracrine loop for elimination of virally infected cells(72). Circulating soluble TRAIL levels are elevated in patients with chronic viral hepatitis B. Hepatitis B x antigen increases TRAIL-R1 expression in cell culture experiments, conferring sensitivity to TRAIL(81). In liver samples from patients with chronic hepatitis C, TRAIL-R1 and TRAIL-R2 expression and TRAIL induced apoptosis were enhanced(69). Hepatitis C virus core protein also selectively modulates cellular responsiveness to TRAIL by promoting TRAIL induced Bid cleavage(82).

**THE INTRINSIC PATHWAY**

Intracellular stress leads to the activation of the intrinsic pathway of apoptosis. Stress can be perceived and transduced by any membrane defined organelle in the cell. For example, lysosomes can mediate steatotic liver cell death, as can the endoplasmic reticulum. DNA damage and steatosis can activate c-jun N terminal kinase, also a mediator of the intrinsic pathway of apoptosis. These processes converge on mitochondria and are transduced by the Bcl-2 family of proteins, therefore, are usually referred to as the Bcl-2-regulated or mitochondrial pathway of apoptosis. The Bcl-2 family consists of proapoptotic and antiapoptotic proteins. The proapoptotic proteins are structurally divided
based on the number of shared Bcl-2 homology (BH) domains, into multidomain (Bak and Bax, display BH1,2, and 3 domains) and BH3 only proteins (Bid, Noxa, Puma, Bim, Bmf, Bik, Hrk and Bad). The antiapoptotic proteins include Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1 and Boo, share 3 (Mcl-1) or 4 BH domains. The liver expresses Bcl-xL and Mcl-1; Bcl-2 is not expressed by hepatocytes. Bax and Bak are both expressed by hepatocytes. The large number of BH-3 domain only proteins, while may impart redundancy, primarily imparts stimulus specificity. For example free fatty acids activate Bim(83); Puma and Noxa are target genes of the tumor suppressor p53(84). The antiapoptotic members of this family are located on the cytoplasmic aspect of membrane bound organelles. They protect cells from death, and may be necessary for survival of certain cell types. Bax and Bak are required from mitochondrial permeabilization, while Bax is located in the cytosol and translocates to mitochondria upon activation; Bak is a resident mitochondrial membrane protein. The activation of Bax and Bak is regulated by interactions between the antiapoptotic Bcl-2 proteins and the BH-3 domain only proapoptotic proteins. Several models have been proposed to explain the biochemical activation of Bax or Bak by proapoptotic BH-3 only proteins. Using Bim as an example, upon activation, Bim is released from the dynein motor complex, and can directly engage and activate Bax and Bak. Alternatively, Bim can bind and negate the inhibitory effect of Bcl-2 or Bcl-xL, releasing Bax and Bak from inhibition by these proteins (the derepression model).

**Mitochondria:** In addition to the metabolic functions of mitochondria, hepatocytes require mitochondria to die. The mitochondrial intermembrane space sequesters a number of proapoptotic proteins including cytochrome c, SMAC/DIABLO (second mitochondrial activator of caspase/direct IAP binding protein with low pI), HtrA2/Omi, AIF (apoptosis inducing factor), and endonuclease G(4, 19). Active Bax or Bak form pores in the outer mitochondrial membrane leading to mitochondrial outer membrane permeabilization (MOMP) and release of these mediators into the cytosol. MOMP can also occur secondary to the permeability transition pore, a complex of adenine nucleotide transporter (ANT) on the inner mitochondrial membrane, voltage dependent anion channel (VDAC) on the outer mitochondrial membrane, and cyclophilin D located within the mitochondrial matrix. Opening of the permeability transition pore leads to rapid fluxes of ions and water, dissipation of the mitochondrial inner transmembrane potential, swelling of the mitochondria, outer mitochondrial membrane rupture leading to the release of the contents on the intermembrane space. Recent studies have demonstrated that stimuli leading to mitochondrial permeability transition require cyclophilin D and that this can occur independently of ANT(85, 86). However, in mice and isolated liver mitochondria lacking cyclophilin D, stimulus-specific MOMP occurs via engagement and activation of Bax or tBid(86), which could also be the case in intact hepatocytes, given the richness of death receptor expression and sensitivity to death ligands.

MOMP releases intermembrane contents into the cytosol and commits the cell to apoptosis. SMAC inactivates post-mitochondrial inhibitors of apoptosis proteins (IAP). Cytosolic cytochrome c, apoptotic protease activating factor-1 (Apaf) and ATP form a complex called the apoptosome, leading
to activation of procaspase 9 and effector caspases 3 and 7(87). These effector caspases cleave over 500 substrates resulting in cellular demolition. Cytokeratin 18 is a structural protein expressed in most epithelial cells that is cleaved by caspase 3 at aspartate positions 238 and 396. The fragment generated by this cleavage, cytokeratin 18-aspartate 396 (CK18-asp396) forms a neoepitope that is recognized by the M30 antibody. This neoepitope can be detected in apoptotic tissues as well as serum by a commercially available ELISA. Indeed circulating levels of CK18-asp396 are elevated in patients with liver injury and can correlate with outcome(5). Thus this biomarker presents a noninvasive, simple and mechanistic tool to monitor progress, and response to therapy in liver injury.

**Lysosomes:** Lysosomes are intracellular organelles with acid intravesicular pH that contain lysosomal proteases, known as cathepsins(88). Cathepsin B and D, two of 11 known human cathepsins, are stable and active at neutral pH. Methodical dissection of pathways that mediate intracellular death signals, demonstrates that lysosomes can be involved in the intrinsic pathway of cell death. Typically lysosomal permeabilization, when it mediates apoptosis, is selective and partial and is observed upstream of mitochondrial permeabilization. Cathepsin B induced mitochondrial permeabilization can occur via caspase 2 (in mice) and via proteolytic cleavage of Bid similar to death receptor induced activation of Bid(89, 90). Indeed Bid also links death receptors to lysosomal permeabilization; providing cross talk between death receptors and their engagement of the lysosomal and mitochondrial pathways(89, 91). Bax activation by intracellular stress can also result in lysosomal permeabilization(92). Cathepsin D levels were elevated in serum from patients with fulminant hepatic failure as well as chronic hepatitis(93, 94). Cathepsin B deficient mice are resistant to TNF-α induced hepatocyte apoptosis(28). In models of cellular steatosis, cathepsin B inhibition prevents mitochondrial permeabilization and apoptosis(95). In cathepsin B deficient mice liver apoptosis, injury and fibrosis are diminished following bile duct ligation(6); liver apoptosis and injury are abrogated in ischemia reperfusion injury as well(96).

**Endoplasmic Reticulum:** The endoplasmic reticulum (ER) has an inbuilt mechanism to cope with excess or altered unfolded proteins that serves to correct the inciting imbalance. This process is termed the unfolded protein response (UPR). The UPR can also be activated by stimuli that affect the function of the ER, such as calcium depletion, glycosylation inhibition (tunicamycin), ultraviolet radiation and insulin resistance. The ER stress response consists of a series of compensatory processes to correct both the excess and the stress of the unfolded proteins. Global translation is attenuated to reduce the functional protein load of the ER. There is also selective translation of UPR target genes aimed at protecting the ER(97, 98). The transducers of ER stress are membrane proteins that have an ER lumenal domain and a cytosolic domain. Inositol-requiring protein-1 (IRE1) and protein kinase RNA-like ER kinase (PERK) auto-transphosphorylate, when released from the ER chaperone BiP/Grp78. IRE1 possesses endoribonucleaseolytic activity leading to excision of an intron within Xbox binding protein-1 (XBP1) mRNA to generate spliced XBP1 (sXBP1), a transcription factor that activates a subset of UPR target genes. IRE1 also recruits TRAF2 leading to JNK activation. PERK
phosphorylates and inactivates the eukaryotic translation initiation factor-2α (eIF2α), resulting in global translation attenuation with selective translation of activating transcription factor-4 (ATF4) which leads to transcription of C/EBP-homologous protein (CHOP), and the ER chaperone BiP/Grp78. Activating transcription factor-6 (ATF6) is cleaved within the ER membrane, generating an ATF6 fragment that translocates to the nucleus and activates a subset of UPR target genes. It is not known if ATF6 also regulates apoptotic signaling.

ER stress also activates a negative feedback regulatory loop that terminates the UPR; however in the setting of sustained ER stress pro-apoptotic signaling occurs(99). Bax and Bak, both bind to the cytoplasmic domain of IRE1, and in cells lacking Bax and Bak, IRE1 stress generated JNK activation and XBP1 splicing are reduced(100), thus linking the core apoptotic machinery to ER stress response. Bax and Bak localize on the ER membrane, in addition to mitochondrial membranes. In cells lacking both Bax and Bak, the ER is depleted of calcium and unable to respond to certain death stimuli(101). The proapoptotic transcription factor CHOP can increase Bim expression, transcriptionally and by inhibiting its proteasomal degradation, leading to Bim-dependent ER stress induced apoptosis(102). CHOP can also upregulate TRAIL-R2 expression, sensitizing cancer cells to TRAIL induced apoptosis(103).

The involvement of the ER stress-induced apoptotic pathway in liver diseases is an area of emerging research. In the bile duct ligated mouse model of cholestasis, an early and transient induction of CHOP expression is observed(104). Mice deficient in CHOP are protected from hepatocyte apoptosis, liver injury and liver fibrosis. In cell culture, the toxic bile acid, glycochenodeoxycholic acid, also induces ER stress and CHOP expression in isolated rat hepatocytes(105). In transgenic mice expressing hepatitis C viral core and E2 proteins hepatocyte apoptosis is associated with CHOP expression(106). Cycloheximide, an inhibitor of protein synthesis, induces ER stress, induction of CHOP expression and apoptotic hepatocyte cell death, in rat livers(107). In nonalcoholic fatty liver disease, markers of ER stress were variably activated(108). Toxic saturated fatty acids also induce ER stress and apoptosis in liver cell lines(109, 110) In a mouse model of alcohol induced liver injury CHOP deficient mice are protected from hepatocyte apoptosis, though able to mount an ER stress response(111).

**C-jun N-terminal Kinase:** Given the role of c-jun N-terminal kinase (JNK) in multiple models of cell death, it warrants a separate discussion as a final common cell death mediator. JNK 1 and 2 are ubiquitously expressed, including liver, whereas JNK 3 is not expressed in the liver(112). JNK activation occurs downstream of kinase cascades that can be activated by multiple stimuli including TNF-α, IRE1, reactive oxygen species, free fatty acids, bile acids(113-115). JNK involvement in apoptosis is temporally regulated and stimulus specific(116). The same inciting stimulus, e.g. TNF-α, can induce biphasic JNK activation mediated by distinct intracellular pathways. Transient and early JNK activation promotes survival; and sustained and late activation of JNK promotes apoptosis(117). In the case of TNF-α, production of reactive oxygen species mediates the
delayed and sustained activation of JNK. Other stimuli, e.g. toxic free fatty acids, result in early and sustained JNK activation, culminating in apoptotic signaling(118). JNK stimulated proapoptotic signaling converges on mitochondria via the activation of Bax and Bak. In the absence of Bax and Bak, JNK induced cell death is mitigated(119). Furthermore, mitochondrial permeabilization and release of cytochrome c are abolished in cells derived from mice lacking JNK 1 and 2 genes, in response to stimuli that cause intracellular stress(116). JNK mediated phosphorylation of pro- and anti-apoptotic proteins upstream of mitochondria also regulates apoptotic sensitivity. JNK can phosphorylate and activate the BH-3 only proteins, e.g. Bim phosphorylation releases it from binding to the dynein motor complex and promotes apoptosis(76, 120). Sustained JNK activation promotes caspase 8 formation at the DISC by activation of the E3 ubiquitin ligase Itch, which ubiquinates and degrades cFLIP promoting liver cell death(29). JNK can phosphorylate the antiapoptotic proteins, Bcl-2, Bxl-xL and Mcl-1, and the proapoptotic proteins Bmf and Bad(120-123).

JNK 1 or JNK 2 can both mediate liver injury in a stimulus specific manner. In a murine model of steatohepatitis induced by methionine and choline deficient diet JNK1 plays a predominant role(124). In high fat diet-induced obesity and genetic obesity in mice, JNK was activated, and was predominantly JNK 1; though JNK 2 plays a role, that is unmasked in the absence of JNK 1(125, 126). In free fatty acid based cellular models of hepatocyte steatosis JNK2 is the predominant isoform that mediates apoptosis(118). Oleic acid, a minimally toxic free fatty acids also sensitizes steatotic hepatocytes to TRAIL-induced apoptosis by JNK dependent transcriptional upregulation of the death receptor TRAIL-R2(75). This mechanism is shared by toxic bile acids, which too sensitize to TRAIL induced apoptosis by transcriptionally activating TRAIL-R2 expression in a JNK-dependent manner(77, 79). Liver injury induced by ischemia reperfusion is also mediated by JNK, and pharmacologic inhibition of JNK in the donor livers improved graft survival and decreased apoptosis after orthotopic liver transplantation (127, 128). In acetaminophen (APAP) induced acute liver injury JNK activation was robust and sustained, led to Bax translocation to mitochondria and poor animal survival. Pharmacologic inhibition of JNK decreased liver injury, hepatocyte cell death and improved survival; utilizing genetically deficient models of JNK 1 or JNK 2, it was demonstrated that both mediate liver injury, though JNK 2 was predominant(129). JNK activation was observed in hepatocytes in human liver samples from patients with acetaminophen induced acute liver failure(130). JNK inhibition was more effective in decreasing hepatocyte cell death than N-acetylcysteine in a murine model of acetaminophen induced liver injury(130). In a murine model of TNF-α induced liver injury utilizing galactosamine and lipopolysaccharide, JNK 2 mediated caspase 8 activation and mitochondrial permeabilization(131).

The consequences of hepatocyte apoptosis: Apoptosis, inflammation and injury are in some ways inseparable, and it is difficult sometimes to dissect the primary event. However, based on the inciting stimulus apoptosis or inflammatory signaling may be the primary event; each stimulating the
other (Figure 4, below).

**Figure 4** Apoptosis and its consequences: Hepatocyte apoptosis and liver inflammation are interconnceted. Apoptosis of vulnerable hepatocytes results in apoptotic bodies that can be engulfed by Kupffer cells and stellate cells. This engulfment leads to Kupffer cell activation and secretion of TNF-α, interleukins and interferon, all of which promote the inflammatory response. With ongoing hepatocyte apoptosis, activated Kupffer cells also facilitate the activation of stellate cells by secreting transforming growth factor beta (TGFβ). Activated stellate cells lead to liver fibrosis by secreting collagen type 1. Inhibition of hepatocyte apoptosis or Kupffer cell depletion, both mitigate liver injury, inflammation and fibrosis. Activated stellate cells are sensitized to apoptosis, such as with TRAIL, and this leads to resolution of fibrosis.

The liver has a large population of Kupffer cells, NK cells and NK T cells(132). These cells are a ready source of TNF-α and other cytokines that mediate inflammation, Fas, TRAIL and TNF-α that mediate hepatocyte apoptosis, and transforming growth factor-beta (TGF β) that activates stellate cells. Apoptotic hepatocytes can be engulfed by Kupffer cells leading to generation of cytokines; pharmacologic inhibition of apoptosis prevents Kupffer cell activation. Also, in the bile duct ligated mouse, Kupffer cell depletion decreases hepatocyte apoptosis, liver injury and liver inflammation(133). In addition, stressed hepatocytes increase expression of NKG2D ligands; thus inviting NK and NKT cell mediated destruction(134).

Fibrosis is the hallmark of ongoing liver injury. Hepatic stellate cells mediate hepatic fibrosis. In the normal liver, stellate cells maintain a quiescent phenotype. On activation, they undergo a metamorphosis, to become myofibroblasts, secreting collagen which leads to liver fibrosis. Stellate
cells in vitro can engulf apoptotic hepatocytes, leading to their activation, and increased expression of TGF β, alpha smooth muscle actin and collagen alpha1(135). Similarly, in vivo hepatocyte apoptosis is a fibrogenic stimulus. Several experimental studies have demonstrated that the inhibition of hepatocyte apoptosis abrogates liver fibrosis(6, 62, 136). By corollary, apoptosis of activated stellate cells should decrease liver fibrosis and dissociate ongoing hepatocyte apoptosis from the ensuing fibrogenic response. Indeed, activated stellate cells are sensitized to apoptotic signaling. This can be achieved by inhibition of NFκB, TRAIL mediated stellate cell apoptosis, and NK cell mediated stellate cell apoptosis(137-139). Indeed, the resolution phase of fibrosis requires apoptosis of activated hepatic stellate cells(140).

The clinical applications of apoptosis are discussed in the conclusion of this chapter. The cytokeratin 18 derived M30 neoantigen reflects epithelial cell apoptosis, is abundant in hepatocytes, can easily be measured in serum by a commercially available ELISA and correlates with hepatocyte apoptosis in diverse liver diseases(141). In a study with a small number of patients with chronic hepatitis C, pre-treatment M30 levels were predictive of response to therapy(57), inferring from this that patients with an apoptotic response to virally infected hepatocytes are more likely to have a treatment response. In another study with chronic hepatitis C patients with normal transaminases, serum M30 levels correlated with fibrosis(57). In patients with nonalcoholic fatty liver disease, serum M30 levels offer reliable discrimination of patients with steatohepatitis from simple steatosis, and increasing levels are predictive of a higher likelihood if inflammation(142). Caspase inhibitors have demonstrated efficacy in preventing hepatocyte apoptosis and injury in experimental models of liver injury(136, 143). In patients with chronic hepatitis C, orally administered caspase inhibitor was found to be safe, and lowered transaminases(144).

In conclusion, hepatocyte apoptosis is a key mediator of liver injury and inflammation in most forms of liver disease. Multiple apoptotic pathways are activated by a given injurious stimulus in a vulnerable hepatocyte. The predominant signalling pathway that results in mitochondrial dysfunction in a given cell is difficult to discern; however, multiple pathways could potentially cooperate or oppose each other, to eventually result in mitochondrial permeabilization. Once mitochondrial permeabilization occurs, the hepatocyte is committed to cell death. Evidence of hepatocyte apoptosis can be demonstrated by serum markers and early studies demonstrate prognostic significance of apoptosis markers. Lastly, therapeutic manipulation of apoptosis is of benefit, by preventing liver injury and fibrosis.

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