Apoptosis and Necrosis in the Liver: A Tale of Two Deaths?

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Death of hepatocytes and other hepatic cell types is a characteristic feature of liver diseases as diverse as cholestasis, viral hepatitis, ischemia/reperfusion, liver preservation for transplantation and drug/toxicant-induced injury. Cell death typically follows one of two patterns: oncotic necrosis and apoptosis. Necrosis is typically the consequence of acute metabolic perturbation with ATP depletion as occurs in ischemia/reperfusion and acute drug-induced hepatotoxicity. Apoptosis, in contrast, represents the execution of an ATP-dependent death program often initiated by death ligand/death receptor interactions, such as Fas ligand with Fas, which leads to a caspase activation cascade. A common event leading to both apoptosis and necrosis is mitochondrial permeabilization and dysfunction, although the mechanistic basis of mitochondrial injury may vary in different settings. Prevention of these modes of cell death is an important target of therapy, but controversies still exist regarding which mode of cell death predominates in various forms of liver disease and injury. Resolution of these controversies may come with the recognition that apoptosis and necrosis frequently represent alternate outcomes of the same cellular pathways to cell death, especially for cell death mediated by mitochondrial permeabilization. An understanding of processes leading to liver cell death will be important for development of effective interventions to prevent hepatocellular death leading to liver failure and to promote cancer and stellate cell death in malignancy and fibrotic disease. (HEPATOLOGY 2006;43:S31-S44.)

The liver is a multifunctional organ that plays essential roles in metabolism, biosynthesis, excretion, secretion and detoxification. These processes require energy, making the liver a highly aerobic, oxygen-dependent tissue. These processes also impart vulnerability of the liver to anoxia, increase susceptibility to noxious insults, and create a demand for cell replacement after tissue loss. Enhanced liver cell death and impaired regeneration are indeed features of most liver disorders. However, unlike almost any other organ, the liver can regenerate from even massive cellular loss by cell proliferation. For example, after 70% hepatectomy, livers regenerate to nearly their original mass in just days. However, when cellular loss exceeds a certain threshold, regeneration fails, and hepatic failure and death ensue. In many chronic liver diseases and after chronic exposure to hepatoxins like alcohol, regeneration may not keep pace with hepatocellular death. Fibrotic scars synthesized largely by hepatic stellate cells gradually replace and displace functional hepatocytes, compromising liver function and eventually leading to hepatic failure. For these reasons, an understanding of how liver cells die and how such cell death can be modulated is of obvious clinical relevance.

Abbreviations: ANT, adenine nucleotide transporter; ATP, adenosine triphophate; CRS, Carolina Rinse Solution; CypD, cyclophilin D; DR, death receptor; EGFR, epidermal growth factor receptor; HCV, hepatitis C virus; JNK, c-jun N-terminal kinase; MPT, mitochondrial permeability transition; NASH, nonalcoholic steatohepatitis; NFκB, nuclear factor-kappaB; RNA; XIAP, X-linked inhibitor of apoptosis protein; siRNA, small interfering TRAIL, tumor necrosis factor–related apoptosis inducing ligand; TNFα, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

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Modes of Cell Death

Although the causes of cell death are disparate, the mode of cell death to liver cells typically follows one of two patterns. The first leads to a pathologic pattern of necrosis, the process of which is called oncosis or oncotic necrosis.[1] Oncotic necrosis is typically the consequence of acute metabolic perturbation as occurs in ischemia/reperfusion or acute drug-induced hepatotoxicity. Apoptosis, in contrast, represents the execution of a death program often initiated by quite specific stimuli.[2,3] Apoptosis leads to the orderly resorption of individual cells that minimizes

inflammatory responses and leakage of cellular components into the extracellular space. Apoptosis and necrosis are usually considered separate entities, but an alternate view is emerging that apoptosis and necrosis are frequently the consequence of the same initiating factors and signaling pathways. Rather than being separate entities, apoptosis and necrosis in their pure form may represent extremes on a continuum of cell death.[4,5]

Oncotic Necrosis. Oncotic necrosis is most often the consequence of metabolic injury leading to ATP depletion. As its name implies, swelling is a prominent feature of oncotic necrosis.[1,6] Early after ATP depletion to hepatocytes, moderate cellular swelling occurs associated with small protrusions of the plasma membrane, called blebs.[7,8] Bleb formation is likely a consequence of ATP depeletion-dependent cytoskeletal alterations.[9,10] After many minutes or even hours, a metastable state develops characterized by mitochondrial depolarization, lysosomal breakdown, bidirectional leakage of anionic (but not cationic) fluorophores, rapid ion changes and accelerated bleb formation and swelling.[11-14] This metastable state, which lasts only a few minutes, culminates in outright rupture of plasma membrane bleb.[13,14] Bleb rupture causes irreversible breakdown of the plasma membrane permeability barrier, collapse of all electrical and ion gradients across the plasma membrane, leakage of cytosolic enzymes and metabolic intermediates, and uptake of dyes like trypan blue and propidium iodide that enter and label the nuclei of non-viable cells. Some work suggests that opening of relatively nonspecific anion death channels in the plasma membrane initiates the metastable state.[15,16] Together with cation channels that open earlier, death channel opening initiates the rapid swelling of the metastable state, which is driven by colloid osmotic (oncotic) forces. Swelling in the metastable state continues until a bleb ruptures, the final event precipitating cell death. The cytoprotective amino acid glycine inhibits this final phase of swelling apparently by blocking the anion death channel. In this way, glycine protects against cell killing without restoring ATP or preventing other metabolic derangements.[17-19]

Apoptosis. Apoptosis represents a distinctly different pattern of cell death from oncotic necrosis.[2,3] Whereas in necrosis large groups of contiguous cells die, in apoptosis individual dying cells separate from their neighbors and shrink rather than swell, a phenomenon of piecemeal necrosis in the older pathologic literature.[20] Distinctive nuclear changes also occur in apoptosis, including chromatin condensation, internucleosomal DNA degradation, and nuclear lobulation and fragmentation. Eventually, cells fragment into apoptotic bodies (Councilman bodies) that are phagocytosed by adjacent cells and macrophages for lysosomal degradation. Many stimuli initiate apoptosis, including death receptor ligands (TNFα, Fas ligand), DNA damage (ionizing radiation, cancer chemotherapeutic agents) and growth factor withdrawal, to name a few.[21-23] Most often these stimuli lead to activation of a cascade of cysteine-aspartate proteases, called caspases, which cleave cellular proteins to impart the classic morphologic phenotype. Leakage of proapoptotic proteins like cytochrome *c*, Smac/Diablo and others, from the mitochondrial intermembrane space into the cytosol also typically occurs.[24] This latter process may represent the final and committed step of apoptosis.

Mechanisms of Cytochrome* c *Release. The mechanisms of cytochrome *c* release remain controversial, and multiple mechanisms may exist (Fig. 1). In one mechanism, proapoptotic Bcl2 family members, such as *t*Bid, Bax and Bak, promote formation of specific cytochrome *c* release channels in the mitochondrial outer membrane.[21,25-27] The precise molecular composition of these channels remains unclear. A second mechanism proposes that pores form in the inner membrane that nonspecifically conduct solutes up to 1500 Da. Opening of these pores, the mitochondrial permeability transition (MPT) pores, leads to mitochondrial swelling. Consequently, the outer membrane ruptures to release intermembrane proteins.[28-30] The composition of the MPT pore, like that of the cytochrome *c* release channel of the outer membrane, is incompletely understood. In one model, the MPT pore is comprised of the voltage dependent anion channel (VDAC) from the outer membrane, the adenine nucleotide transporter (ANT) from the inner membrane and cyclophilin D (CypD).[31-33] CypD is a binding protein for cyclosporin A, which inhibits the MPT in a fashion seemingly unrelated to its immunosuppressive properties.[34,35] This model, which has been widely accepted, has been called into question from findings that the MPT still occurs in ANT deficient liver mitochondria from a conditional double ANT knockout mouse.[36] Another model proposes that MPT pores form by aggregation of damaged, misfolded membrane proteins.[37] Chaperones, including CypD, close nascent pores, but pore opening occurs when mitochondria accumulate Ca^{2+}. When the number of misfolded aggregates exceeds the number of available chaperones, an unregulated and Ca^{2+}-independent MPT occurs that is not inhibited by cyclosporin A. A wide range of injurious stresses relevant to liver disease lead to MPT pore opening.[38] Onset of the MPT causes either necrotic cell death from ATP depletion or caspase-dependent apoptotic signaling initiated by cytochrome *c* released after mitochondrial swelling (Fig. 1).[39,40]

Necrapoptosis/Aponecrosis. Whatever they are, the events leading to cytochrome *c* release typically cause

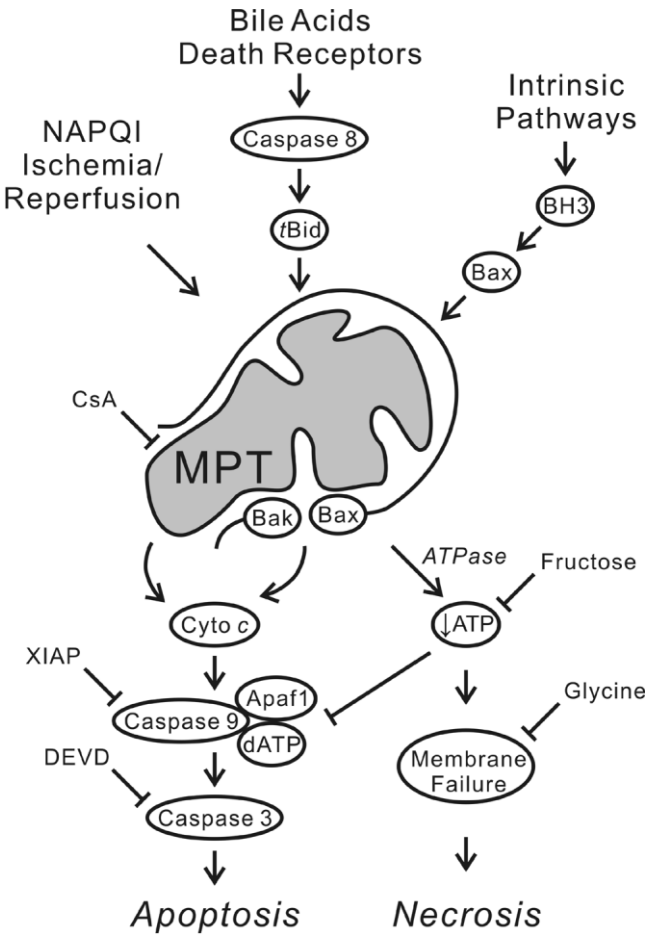

Fig. 1. **Necrosis and apoptosis as alternate outcomes of mitochondrial permeabilization.** Ischemia/reperfusion, hepatotoxins including NAPQI (*N*-acetyl-*p*-benzoquinone imine, the toxic metabolite of acetaminophen), bile acids, death receptor activation and other stresses (*e.g.*, activation of intrinsic pathways) converge on mitochondria to induce membrane permeabilization. BH3 only Bcl2 family members, including *t*Bid formed by death receptor-linked caspase 8 activation, cause Bax/Bak-dependent permeabilization of the outer membrane. Permeabilization may involve formation of channels in the outer membrane or induction of a cyclosporin (CsA) sensitive MPT followed by mitochondrial swelling and outer membrane rupture. Other stresses act directly to cause the MPT. After membrane permeabilization, cytochrome *c* is released to the cytosol and activates in sequence caspase 9 and caspase 3 in a reaction requiring Apaf1 and dATP (or ATP). XIAP blocks caspase 9/3 activation, whereas DEVDcho is a substrate analog inhibitor that also blocks caspase 3 activity. With severe and pervasive mitochondrial dysfunction, ATP decreases in part due to activation of the mitochondrial uncoupler-stimulated ATPase. With ATP depletion, caspase activation is blocked, and necrosis occurs instead. Fructose, an ATP-generating glycolytic substrate, and glycine, a cytoprotective amino acid, prevent membrane failure and necrotic cell death.

global mitochondrial dysfunction with mitochondrial membrane depolarization and uncoupling of oxidative phosphorylation. For this reason, the same mechanisms that promote cytochrome *c* release and apoptosis can cause ATP depletion-dependent necrotic cell killing. Although apoptosis and oncotic necrosis have been considered distinct and independent phenomena, the two modes of cell death frequently coexist in liver pathology, which has led to spirited debates as to what mode of cell death is actually occurring in a given circumstance.[41] An alternative view is that necrosis and apoptosis are interdependent phenomena resulting from activation of shared pathways and signals.[4] Thus, an admixture of necrosis and apoptosis may be expected to occur in many pathophysiological settings (Fig. 1).

Ischemia/Reperfusion Injury

No-Flow and Low-Flow Hypoxia. Highly aerobic tissues are sensitive to damage from loss of blood supply (ischemia), and the liver is no exception. Hepatic hemodynamic perturbations can lead to no-flow ischemia, in which blood supply is totally blocked, or to low flow-hypoxia, in which blood flow is inadequate to meet oxygen demand.[42] In no-flow ischemia, the entire liver or lobe becomes anoxic, whereas in low-flow hypoxia, pericentral (centrolobular) regions but not periportal regions of individual liver lobules become anoxic and subject to injury.[7,8] Due to the dual blood supply of the liver, the pattern of pericentral necrosis due to low-flow hypoxia is most often encountered and is a frequent finding at autopsy.[43] Ischemia/reperfusion in liver has two phases. The first phase reflects the immediate cellular aftermath of ischemia and subsequent reperfusion, whereas the ensuing second phase involves the innate immune system, specifically activation of Kupffer cells and infiltration of circulating neutrophils and lymphocytes into the post-ischemic liver.[44,45]

pH-Dependent Reperfusion Injury. During ischemia and hypoxia, liver cells will eventually lose viability due lack of ATP production by oxidative phosphorylation. However, the naturally occurring acidosis of ischemia and hypoxia delays onset of necrotic cell death by as much as hours.[11,46] After reperfusion, tissue pH normalizes and the protection of acidosis is lost. Thus, reperfusion paradoxically precipitates necrotic cell killing, a large component of which is due to the recovery of intracellular pH to a normal value.[11,47,48] After warm ischemia and cold ischemia (cold preservation), respectively, hepatocytes and endothelial cells are most susceptible to pH-dependent necrotic cell killing.[42,49] In models of warm ischemia/reperfusion to cultured hepatocytes, restoration of pH after reperfusion induces opening of MPT pores, leading to mitochondrial inner membrane permeabilization, depolarization and large amplitude swelling, as directly visualized by confocal microscopy.[40,50] The ensuing ATP depletion then causes necrotic cell death. Cell death occurring acutely (within about an hour or less of reperfusion) is oncotic necrosis, since caspases are not activated

and caspase inhibitors do not protect. *In vivo*, the acute phase of cell death after ischemia/reperfusion also displays the major features of necrosis, including cell swelling, karyolysis, vacuolization, enzyme release and involvement of large numbers of contiguous cells.[51] Moreover, caspase activation is minimal or absent, and apoptosis assessed by morphological criteria is only about 2% of cells.

The Innate Immune System and the Late Phase of Ischemia/Reperfusion Injury. Ischemia/reperfusion also activates Kupffer cells, the resident macrophages of liver.[41,49,52,53] Activated Kupffer cells release reactive oxygen species, cytokines, chemokines and other factors, which then leads to infiltration and activation of other cells of the innate immune system, including neutrophils and CD4+ T lymphocytes.[54,55] These responses developing as long as 1 or 2 days after reperfusion promote apoptosis as well as necrosis. Since Kupffer cell activation seems to be a primary response to ischemia/reperfusion rather than a reaction to necrosis, activation of the innate immune system may also occur after shorter periods of ischemia that do not lead to necrotic cell killing after reperfusion. Thus, after ischemia/reperfusion to liver *in vivo*, both apoptosis and necrosis can occur. More extreme injury leads to early necrotic killing, whereas milder injury may result in delayed apoptosis. A complex balance between the magnitude and duration of injury determine the eventual mode of cell death. The distinction of necrosis and apoptosis may in fact be artificial, since necrosis in some circumstances can be viewed as aborted apoptosis, due to insufficient ATP to drive the apoptotic program (Fig. 1). In other instances, apoptosis may develop in damaged cells that survive the injurious stresses that would otherwise cause necrotic cell death.

Cellular Targets of Ischemia/Reperfusion Injury. Cell death after hepatic ischemia/reperfusion occurs primarily in hepatocytes and sinusoidal endothelial cells.[42,49,56,57] After cold ischemic liver storage for periods of time associated with graft failure after transplantation, sinusoidal endothelial cells undergo necrotic cell death and Kupffer cells become activated within 15 minutes of warm reperfusion.[49] Death of hepatocytes does not occur acutely but becomes manifest after about 4 h of implantation of livers that will fail from storage/reperfusion injury.[58] Endothelial cell killing and Kupffer cell activation are truly reperfusion injuries, since Carolina Rinse Solution (CRS) infused at the end of storage prevents endothelial cell killing and Kupffer cell activation with marked improvement of graft survival after implantation.[59] Active ingredients in CRS include glycine and acidotic pH, which prevent necrotic killing of endothelial cells, and adenosine and antioxidants (as well as glycine), which suppress Kupffer cell activation.[60,61] Efficacy of CRS is dependent on its combination of ingredients to block both endothelial cell killing and Kupffer cell activation. Thus, individual strategies to decrease Kupffer cell activation (gadolinium chloride, methylpalmitate) or block endothelial cell killing (glycine) ameliorate hepatic injury but to a lesser extent.[60,62-65]

Storage/reperfusion may also lead to apoptosis of endothelial cells and such apoptosis correlates with impaired graft function.[66-68] In some studies, the extent of apoptosis may be overestimated due to use of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) to identify apoptosis from DNA strand breaks. Although TUNEL reliably identifies the internucleosomal DNA cleavage associated with apoptosis, DNA degradation also occurs during necrosis, especially *in vivo* because of release of nucleases from infiltrating inflammatory cells.[41,51,69,70] Thus after liver transplantation, necrotic endothelial cells labeled with trypan blue soon develop TUNEL reactivity within just a few minutes.[41] Nonetheless, pharmacologic inhibition of caspases, overexpression of the anti-apoptotic protein, Bcl2, and inhibition of calpains provide a degree of protection against endothelial cell killing and graft failure from storage/reperfusion injury.[71-73] Additionally, Fas increases and small interfering RNA (siRNA) to knock down expression of caspase 8 and caspase 3 attenuates inflammation, decreases injury, and improves survival in a mouse model of ischemia/reperfusion.[74] Caspase 3 but not caspase 8 inhibition also prevents apoptosis in a cellular model of acute warm ischemia/reperfusion injury to hepatocytes.[40] However, Bcl2 overexpression also inhibits necrotic cell death, possibly by inhibiting the MPT, and inhibitors of caspases and calpains may exert anti-inflammatory as well as other effects that suppress the late phase of hepatic ischemia/reperfusion injury mediated by the innate immune system.[75-78]

Switches From Necrosis to Apoptosis After Ischemia/Reperfusion. In cultured hepatocytes subjected to conditions of ischemia/reperfusion, glycine and fructose given at reperfusion prevent MPT-dependent necrotic cell death.[40] Fructose is a glycolytic substrate that protects by promoting ATP regeneration, whereas glycine prevents plasma ATP depletion-dependent membrane failure. When necrosis is blocked by fructose plus glycine, caspase-dependent apoptosis occurs instead 8 to 12 h later. This apoptosis requires ATP for cytochrome *c*- and apoptosome-dependent activation of caspase 3 (Fig. 1). In the absence of ATP, apoptotic signaling through caspases is blocked. Thus, ATP depletion after reperfusion promotes necrotic cell killing while simultaneously suppressing apoptotic signaling. By contrast, if ATP levels are partially restored after reperfusion, necrosis is prevented.

Instead, caspase activation occurs due to cytochrome *c* release from mitochondria swollen after onset of the MPT. Inhibitors of the MPT, such as cyclosporin A and its analogs, prevent both necrosis and apoptosis when administered at the time of reperfusion. Thus, the MPT is a common pathway leading to both oncotic necrosis and apoptosis. A similar ATP-dependent switch from MPT-dependent necrotic cell killing to MPT-dependent apoptosis occurs in calcium ionophore and acetaminophen toxicity to hepatocytes.[39,79] Another blocker of the MPT after reperfusion is nitric oxide (NO), which acts through a guanylyl cyclase/cGMP/protein kinase pathway.[80] In different models, NO inhibits both necrotic and apoptotic killing of hepatocytes.[80,81]

After ischemia, hepatocytes seem to become sensitized to stresses that would not otherwise cause cell death, especially in the late phase of injury mediated by the innate immune system. During ischemia, the anti-apoptotic X-linked inhibitor of apoptosis protein (XIAP) decreases progressively in hepatocytes.[82] XIAP antagonizes cytochrome *c*-dependent caspase 9/3 activation,[83,84] and XIAP depletion during ischemia is associated with increased caspase activation and apoptosis after reperfusion, an effect augmented by postischemic exposure to TNFα.[82,85] Hepatocytes from XIAP deficient mice show a 10-fold enhancement of apoptosis after short periods of ischemia, which is reverted by treatment with an XIAP-expressing adenovirus. Thus, decreases of XIAP during ischemia sensitize hepatocytes to apoptosis after reperfusion.

Death Receptor Injury

Death Receptors and Their Ligands. Death receptor-ligand interactions are important initiators of apoptosis by the so-called extrinsic pathway (the intrinsic pathway being typically activated by DNA damage, p53 activation and PUMA expression).[86-89] Death receptors belong to the tumor necrosis factor/nerve growth factor receptor superfamily and are transmembrane proteins with three domains: an extracellular ligand-interacting domain, a transmembrane domain, and an intracellular death domain.[89] Death receptors of importance in liver include Fas (CD95/Apo-1), tumor necrosis factor receptor 1 (TNFR1), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor 1 (TRAIL-R1/Death receptor 4 [DR 4]); TRAIL receptor 2 (TRAIL-R2/DR5/Killer/TRICK2), death receptor 3 (DR3/Apo-3/TRAMP/WSL-1/LARD), and death receptor 6 (DR6). Death receptor engagement by their corresponding ligands (Fas ligand [FasL], TNFα, and TRAIL) causes receptor oligomerization and activation, which triggers

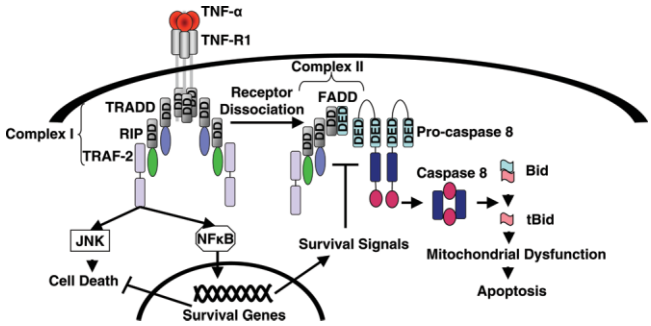

Fig. 2. **TNFα receptor mediated signaling.** Upon binding of TNFα to TNFR1, Complex I forms, shown on the left, comprised of TRADD (TNFR-associated protein with death domain), RIP (receptor-interacting protein), TRAF-2 (TNF-associated factor-2), which activates NFκB (nuclear factor κB) and JNK (c-jun N-terminal kinase). NFκB activates the transcription of several survival genes, including antiapoptotic proteins c-FLIP, IAPs, Bcl-XL, and A1. Complex I then undergoes modification and ligand-dissociated internalization with formation of Complex II, also known as the DISC (death-inducing signaling complex), shown on the right. Complex II recruits FADD (Fas-associated death domain) via interactions between conserved death domains (DD) and activates procaspase 8 via interaction between death effector domains (DED). Active caspase 8 cleaves Bid to *t*Bid, which translocates to mitochondria leading to mitochondrial permeabilization, dysfunction and apoptosis.

intracellular signaling pathways (Figs. 2 and 3). After ligand engagement, intracellular death domains interact with a number of adaptor proteins leading to the apoptotic cascade. TNFR1 is distinct from Fas and TRAIL receptors in that activated TNFR1 first activates NFκB and c-jun N-terminal kinase (JNK) and then upon internalization activates the apoptotic cascade[90] (Fig. 2). TRAIL receptors are distinct in that only TRAIL-R1 and TRAIL-R2 initiate apoptotic signaling. TRAIL-R3 and TRAIL-R4, which do not contain death domains, function as decoy receptors.[86] All three classes of receptors initiate cleavage of procaspase 8 to active caspase 8. With sufficiently strong activation, caspase 8 directly activates caspase 3, an executioner caspase whose activity promotes the final phase of outright apoptotic cell death. This is the Type 1 pathway.[91,92] In hepatocytes, however, a mitochondrial pathway amplifies and accelerates caspase 8-dependent apoptotic signaling — the Type 2 pathway.[30,93] In Type 2 signaling, caspase 8 cleaves Bid, a BH3 only proapoptotic Bcl2 family member, to a truncated form, *t*Bid. *t*Bid translocates to mitochondria, causing mitochondrial permeabilization and release of mitochondrial effectors of apoptosis, such as cytochrome *c*. Cytochrome *c* associates with Apaf-1 to form haptomeric apoptosomes, which proteolytically activate caspase 9 in a dATP (or ATP)-requiring reaction. Caspase 9, in turn, activates caspase 3 and the final stages of apoptosis[21] (Fig 1).

Fas-Induced Apoptosis in Liver. Hepatocytes, cholangiocytes, sinusoidal endothelial cells, stellate cells, and Kupffer cells all express Fas.[94-98] In hepatocytes, Fas

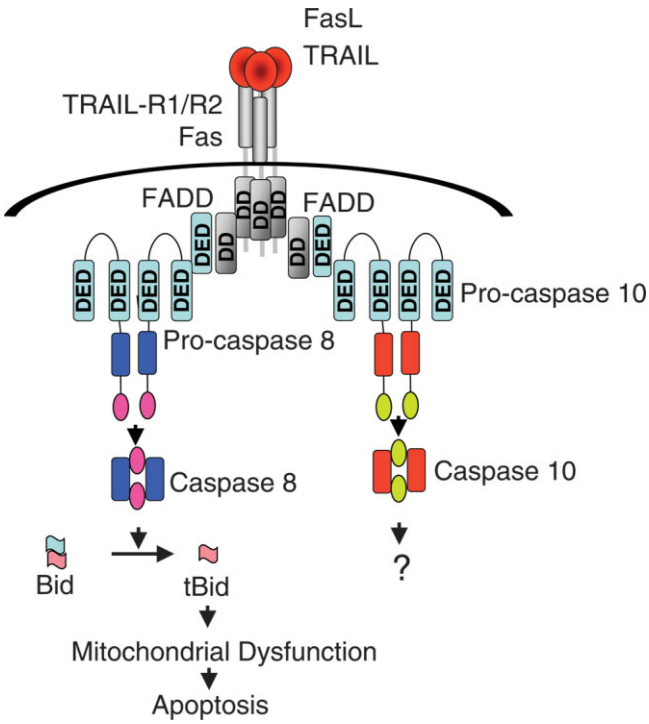

Fig. 3. **Fas and TRAIL receptor signaling.** Fas and TRAIL death-inducing signaling complexes (DISC) are similar. Engagement of TRAIL-R1/TRAIL-R2/Fas with their ligands, TRAIL or FasL, leads to recruitment of the adaptor protein FADD via homotypic interactions of the death domain (DD). FADD contains a death effector domain (DED) that recruits and cleaves procaspase 8 and 10, resulting in their homodimerization and activation. Caspase 8 mediated cleavage of Bid to *t*Bid leads to mitochondrial permeabilization, dysfunction and apoptosis. Cellular substrates of caspase 10 are not well defined.

localizes predominantly to the Golgi complex and trans-Golgi network with smaller amounts in the plasma membrane.[99] Noxious stimuli, such as bile acids, cause translocation of Fas to the plasma membrane.[100] Fas activation can occur in two ways. The common mode of activation involves binding of FasL to trimerized Fas leading to intracellular signal transduction (Fig. 3). Additionally, ligand-independent Fas activation can occur if a high enough membrane Fas density is achieved. FasL itself promotes translocation of Fas to the plasma membrane via a multistep mechanism involving sphingomyelinase activation, ceramide-dependent activation and protein kinase C-zeta leading to activation of NADPH oxidase. Superoxide then activates the Src family tyrosine kinase, yes, leading to EGFR phosphorylation and EGFR interaction with intracellular Fas. The EGFR kinase phosphorylates Fas, and Fas translocates to the plasma membrane.[101,102] Toxic hydrophobic bile acids initiate a similar signaling sequence leading to Fas translocation and activation.[103]

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells secrete FasL. Apoptosis stimulated by FasL provides an efficient means to remove unwanted hepatocytes in various hepatic disorders, for example the removal of virus-infected hepatocytes and cancer cells by T lymphocytes.[104,105] Viral hepatitis from hepatitis B virus (HBV) and hepatitis C virus (HCV) increases soluble Fas and Fas expression whose levels correlate with disease activity and response to therapy.[106,107] When HCV specific CD8(+) cytotoxic Fas-expressing T lymphocytes are transplanted in mice stably expressing HCV structural proteins, liver damage, hepatocyte killing, and increased serum ALT occur.[108] HBV specific cytotoxic T lymphocytes also cause acute liver damage and failure in transgenic mice expressing HBsAg, an effect that is antagonized by soluble Fas.[109] Similarly in alcoholic hepatitis, soluble Fas is increased in serum, and hepatic Fas and FasL correlate with liver injury. Alcohol consumption in hepatitis C also increases hepatocyte apoptosis, which correlates with increased Fas expression. Thus, convergence of apoptotic stimuli on the Fas signaling pathway likely promotes synergistic liver damage from alcohol and HCV.[110]

In nonalcoholic steatohepatitis (NASH), Fas expression and apoptosis also increase.[111] In a murine model of diet-induced steatosis, increased hepatocellular Fas expression leads to increased apoptosis from Jo2, a Fas agonistic antibody. Fas-induced apoptosis is implicated in patients with fulminant hepatic failure, in which hepatocyte apoptosis, Fas expression, infiltration with FasL-expressing cytotoxic T lymphocytes and increased soluble Fas occur.[112] Bax expression is enhanced in non-apoptotic hepatocytes during human fulminant hepatic failure and correlates overall with TUNEL positive apoptosis.[113] Serum cytochrome *c* is also elevated, which correlates with increased amino transferases,[114] and interruption of apoptotic signaling may be beneficial (see below). In animal models, anti-Fas antibody causes fulminant hepatic failure secondary to massive apoptosis, which can be prevented by the anti-apoptotic protein Bcl2.[115]

TNFα Signaling. TNFα signaling in the liver shares many features with Fas signaling, specifically the activation of caspase 8, Bid cleavage and mitochondrial signaling to apoptosis, and TNFα signaling overlaps with Fas signaling in the diseases mentioned above (Fig. 2). Oligomerized TNFR1 internalizes and via the adaptor protein FADD activates caspase 8.[116] In normal liver TNFR1 expression is low, but high TNR1 expression occurs in hepatocytes, cholangiocytes, sinusoidal epithelium and inflammatory cells in various acute and chronic disease states,[117] TNFα also stimulates survival pathways via NFκB-dependent gene expression (see below). By such alternative signaling pathways, TNFR1 is essential for liver regeneration after partial hepatectomy. Nonetheless, TNFR overexpression can lead to liver injury and failure

of liver regeneration.[118,119] In liver transplantation, graft and recipient TNFR1 has opposing effects, as shown by transplantation of livers from TNFR1 knockout and wildtype mice in all four possible donor and recipient combinations.[120] Graft TNFR1 deficiency increases graft injury, whereas recipient TNFR1 deficiency moderates injury and decreases neutrophil infiltration.

In patients with alcoholic hepatitis, serum TNFα and TNFR1 are elevated and correlate with mortality.[121,122] In viral hepatitis, TNFα levels negatively correlate with response to therapy. In other forms of liver injury, including ischemia/reperfusion and fulminant hepatic failure, TNFα signaling also appears to play a role in augmenting injury.[123-126]

Death Receptor-Induced NFκB Signaling. Additionally, other signals emanate from death receptor activation. Activated death receptors recruit adaptor proteins, such as tumor necrosis factor receptor associated protein 2 (TRAF2) and receptor interacting protein (RIP), that stimulate IκB kinase (IKK) and proteosomal IκB degradation, leading to activation and nuclear translocation of NFκB (90) (Fig. 2). NFκB induces increased expression of survival genes, including Bcl_{XL}, A1, XIAP, cFLIP and iNOS.[127,128] Thus, mouse hepatocytes in culture do not undergo apoptosis after exposure to TNFα or Fas unless NFκB-dependent gene expression is blocked, as for example with cycloheximide to block all protein synthesis or introduction of an IκB superrepressor that is resistant to degradation.[30,94] Resistance to Fas-induced apoptosis *in vitro* by mouse hepatocytes may be due to NFκB activation during cell culture, since Fas ligation *in vivo* induces massive hepatocyte apoptosis.[129] The response to Fas ligation is also species-dependent with rat hepatocytes relatively insensitive and cultured human hepatocytes more sensitive even in the absence of NFκB inhibition.[130,131] The ability of TNFα to stimulate both pro- and anti-apoptotic pathways accounts, at least in part, for its pleiomorphic and seemingly opposing effects. Indeed, in addition to stimulating apoptotic signaling, TNFα acts to promote hepatocellular proliferation after partial hepatectomy.[132,133] Sensitization to the proapoptotic effects of TNFα occurs when toxic and metabolic stresses suppress NFκB signaling, for example by inhibiting protein synthesis. Thus, galactosamine, which depletes dUTP and blocks protein synthesis, sensitizes hepatocytes to apoptosis *in vivo* after TNFα and lipopolysaccharide, the latter a bacterial cell wall product that stimulates TNFα formation by macrophages.[134,135] The role of NFκB is also determined by its cellular location.[136] In contrast to NFκB activation in hepatocytes which mitigates liver injury, NFκB activation in Kupffer cells promotes macrophage activation, secretion of cytokines, expression of death ligands and production of superoxide anion, which augment liver injury. Some reports suggest NFκB-dependent pathways inhibit cell death by inactivating JNK pathways, and JNK inhibition attenuates liver injury with preservation of hepatic architecture following warm ischemia/reperfusion and cold storage/reperfusion injury.[137,138]

TRAIL and TRAIL Receptors. TRAIL-mediated apoptosis in normal hepatocytes has not been unequivocally demonstrated. TRAIL receptor agonists are reported to induce apoptosis to cultured human hepatocytes,[139] but TRAIL agonists do not cause hepatic cell death in mice *in vivo*.[140] In experimental murine models involving activation of mononuclear cells, TRAIL signaling leads to hepatocyte apoptosis, and transfer of TRAIL expressing mononuclear cells to TRAIL deficient mice restores sensitivity to hepatitis.[141] TRAIL is increased in the serum of patients with viral hepatitis,[142] and viral infection of murine hepatocytes induces TRAIL expression.[143] TRAIL expression in this way may create a paracrine loop leading to TRAIL and TRAIL-R-dependent apoptosis of infected hepatocytes. Thus, viral infection not only upregulates TRAIL expression, but also makes previously refractory cells sensitive to its effects. TRAIL resistance in normal hepatocytes coupled with TRAIL sensitivity in tumors and viral infection offers an opportunity to modulate apoptotic signaling selectively in diseased or transformed hepatocytes.[144,145]

Cholestatic Liver Injury

In cholestatic liver disease, bile acids accumulate to cause damage.[146] Several cellular responses occur. Transcriptional regulation of bile acid transporters leads to decreased uptake and increased secretion of bile acids to reduce the intrahepatic burden.[147] Survival genes are also activated, but once these compensatory processes are overwhelmed hepatic injury ensues. Bile acid toxicity is not a consequence of detergent action, since bile acid concentrations in the serum of cholestatic patients are much less than required for detergent action but near levels shown to trigger cell death pathways.[148] In cell culture, toxic bile salts cause hepatocyte apoptosis in a Fas- and TRAIL-dependent manner.[146,149] Fas activation in cholestasis occurs by both FasL ligation-dependent and ligand-independent mechanisms. In the absence of FasL, bile acids enhance cellular trafficking of Fas receptor, leading to an increase of plasma membrane density of Fas receptors.[100] As a consequence, spontaneous receptor oligomerization occurs with recruitment of FADD and activation of caspase 8. These signals then converge to produce mitochondrial permeabilization, release of cytochrome *c* and activation of downstream caspases and cathepsin B, the last a lysosomal cysteine protease.

Interruption of these signals, such as in mice genetically deficient in cathepsin B, abrogates hepatocellular apoptosis and subsequent fibrosis after bile duct ligation.[150] Furthermore, in Fas deficient lpr mice, this signaling is interrupted, and hepatocyte apoptosis and consequent fibrosis are diminished after bile duct ligation.[151] Acute cholestasis is associated with oxidative stress. This oxidative stress is the consequence of bile acid induced activation of NADPH oxidase via signaling through sphingomyelinase, ceramide and protein kinase zeta.[152] As described above, superoxide initiates activation of the Yes tyrosine kinase, EGFR phosphorylation and Fas translocation to the plasma membrane. Finally, in the absence of Fas, GCDC can activate TRAIL-R2 to initiate apoptotic signaling.[149]

Bile acids also activate the mitochondrial apoptotic machinery, a characteristic of death receptor-mediated apoptosis in hepatocytes. Thus after bile duct ligation in lpr mice, delayed hepatocyte apoptosis occurs mediated by induction and translocation of Bax, a pro-apoptotic Bcl2 family protein, to mitochondria.[153] This activation and translocation of Bax to mitochondria may be mediated by *t*Bid.[154] Urosdeoxycholic acid (UDCA) is effective treatment against cholestatic liver injury. UDCA prevents the mitochondrial permeability transition and prevents toxic bile salt-induced apoptosis.[155] Caspase inhibition also protects against cholestatic hepatocyte injury and decreases stellate cell activation and fibrosis.[156]

Despite evidence of Fas and TRAIL-dependent apoptotic signaling in cholestatic liver injury, recent studies have suggested that oncotic necrosis is the predominant form of cell death after bile duct ligation in rodents, as judged by morphological and other criteria.[157,158] However, these studies have focused on the cell death occurring in bile infarcts, a late occurrence in the evolution of liver injury. This time point may be too late to classify the mode of cell death by morphologic criteria. Indeed, caspase inhibitors decrease the occurrence of bile infarcts in the bile duct ligated mouse.[156] Nonetheless, pancaspase inhibitors also inhibit inflammation-related caspases (caspase 1, 4 and 5), which block cytokine signaling (*e.g.*, interleukin-1 activation by caspase 1).[159] Thus, pancaspase inhibitors may inhibit tissue injury by mechanisms other than suppression of apoptosis.

In acute cholestasis, Fas signaling and other alterations may also lead to severe mitochondrial dysfunction and ATP depletion. Such ATP depletion blocks activation of downstream caspases while simultaneously promoting necrotic cell killing. Thus, death receptor-mediated cell death may be mixed as both apoptosis and necrosis. Experimental bile duct ligation produces sudden and complete blockage of bile flow, whereas in human disease cholestasis is typically slower in onset. With slower onset, death receptor-induced mitochondrial dysfunction is likely less severe. Because ATP is at least partially preserved, outright necrosis is prevented and caspase activation can proceed to an apoptotic phenotype of cell killing. Thus, a common pathway initiates both modes of cell death: necrosis after acute cholestasis, apoptosis in chronic disease, and perhaps both necrosis and apoptosis in intermediate disease (Fig. 1). Other factors, such as species differences, effects of different bile acids and cholestasis-induced changes of gene expression, will undoubtedly emerge that regulate which mode of cell death ultimately occurs in various forms of cholestatic liver disease.

Lipotoxicity. A subset of patients with nonalcoholic fatty liver disease (NAFLD) develop progressive steatohepatitis (NASH) in which disease activity correlates with hepatocyte apoptosis.[111] Fas and TNFR1 expression increases in livers of patients with NASH.[160] Fas and TNFR1 also increase in experimental models of NASH, and Fas ligand and TNFα promote hepatocyte apoptosis and inflammation in these fatty liver models.[161] In cellular models, free fatty acids activate cell death pathways by at least 2 types of mechanisms. The first is lysosomal permeabilization with release of cathepsin B, activation of a lysosomal pathway of apoptosis, and cathepsin B-dependent TNFα expression.[162] Lysosomal permeabilization is also present in livers of NASH patients and correlates with disease severity. The second involves free fatty acid-induced activation of JNK, resulting in mitochondrial permeabilization, release of cytochrome *c* and cell death.[163] Other mitochondrial abnormalities occur in patients with NASH, including megamitochondria, decreased hepatic mitochondrial DNA content and decreased respiratory chain function.[164] Oxidative stress promoted by increased β-oxidation of free fatty acids and upregulation of cytochrome P450 2E1 may also promote mitochondrial dysfunction in patients with NASH.[165]

Acetaminophen Hepatotoxicity

The hepatotoxicity of acetaminophen serves as another example of the interrelationship of necrotic and apoptotic cell killing and their common origin in mitochondrial dysfunction. Although safe at therapeutic levels, acetaminophen overdose is the most frequent cause of acute drug-induced liver failure in the United States.[166] Cytochromes P450s metabolize acetaminophen to the reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which reacts with and depletes glutathione, forms covalent adducts and initiates mitochondrial oxidative stress.[167,168] These events lead to onset of the MPT, and cyclosporin A protects against acetaminophen toxicity

both *in vitro* and *in vivo*.[79,169] As in other forms of liver injury, the roles played by oncotic necrosis and apoptosis in acetaminophen-induced liver damage have been controversial. Some studies show apoptosis via TUNEL labeling and DNA laddering after exposure of mice and mouse hepatocytes to acetaminophen,[170,171] whereas other reports indicate that apoptosis is very low and that necrosis is the principal mode of acetaminophen-induced liver cell killing.[172] Studies in mouse hepatocytes show that both modes of cell killing can predominate after acetaminophen exposure.[79] When acetaminophen causes profound ATP depletion, ATP depletion-dependent necrotic cell killing ensues. However, when fructose and glycine are used to prevent ATP depletion, necrosis is blocked, whereas caspase-dependent apoptosis increases. Both with and without fructose plus glycine, mitochondrial inner membrane permeabilization (MPT) occurs, and cyclosporin A decreases both the necrotic and the apoptotic modes of cell killing. Thus, acetaminophen toxicity is one more example of necrapoptosis in which necrosis and apoptosis represent alternate outcomes of the same mitochondrial death pathway (Fig. 1).

Can Death Be Fooled?

Hepatocytes exhibit death receptor-dependent and independent cell death, both of which have an obligate need for mitochondrial dysfunction. Bcl2 family proteins, consisting of pro- and anti-apoptotic members, are important mediators of both processes. Of the pro-apoptotic Bcl2 proteins, the BH3 domain only family members represent early sentinels of death-inducing stimuli.[173] Bid, a BH-3 protein, functions as an important death receptor transducer in hepatocytes. Cleavage of Bid by caspase 8 and 10 generates *t*Bid, which translocates to mitochondria to release Bax/Bak from inhibition by Bcl2 or Bcl_{XL} to cause mitochondrial cytochrome *c* release. In cholestatic injury, Bid is essential for hepatocyte apoptosis *in vivo*.[154] Attenuation of Bid-mediated apoptosis also decreases long-term hepatic fibrosis. In the intrinsic pathway to apoptosis, other BH-3 proteins are important mediators. Bim, for example, interacts with VDAC to regulate mitochondrial permeabilization.[174,175] The role in apoptosis of the 10 known mammalian BH3 only proteins is both cell and stimulus specific. The upstream position of BH3 proteins in apoptotic signaling well before mitochondrial dysfunction makes manipulation of these proteins, such as with pharmacologic inhibitors of Bax and Bak binding sites and small interfering RNA, an attractive target to diminish or enhance apoptosis for a therapeutic advantage.

Mitochondria deserve closer scrutiny as well. Manipulation of subunits that form MPT pore complexes might prevent downstream events even in the face of injurious stimuli. For example, recent reports show that cyclophilin D is essential for cell death due to some but not all noxious stimuli.[176,177] MPT-blocking non-immunosuppressive analogs of cyclosporin A, such as NIM811 and sanglifehrin A, also show therapeutic promise.[35,178,179] Unrelated cellular pathways converge on mitochondria in death signaling but utilize distinct subsets of mitochondrial proteins to activate death pathways. A better understanding of stimulus specific pathways may also lead to new opportunities for targeted therapeutics. Caspases, too, are an attractive chemotherapeutic target. The involvement of different caspases as initiators (caspases 8 and 10) or executioners (caspases 3 and 7) of death signaling offers a dual opportunity for intervention in cell death. Suppression of caspase 8/10 by pharmacologic and genetic means ameliorates hepatic cell death in several models.[71,74,156,180] Inhibition of executioner caspases 3/7 also prevents apoptotic cell death in many cell models. However, these caspases are activated after mitochondrial injury. Consequently, apoptosis prevented by caspase 3/7 inhibition may simply be replaced by another phenotype of cell death. A double hit is attained with so-called pancaspase inhibitors, which prevent activation of all caspases. Some of these, such as IDN-6556 are in clinical trials.

Other strategies can target events upstream of caspase activation. For example, neutralization of soluble Fas and TNFα *in vivo* prevents Fas-mediated hepatocyte apoptosis.[181] siRNA silencing of Fas gene expression protects mice from both fulminant hepatic failure in acute injury and ameliorates liver fibrosis after chronic injury.[182] The same paradigm promotes survival of allogeneic hepatocytes in mice.[183] Caspase gene silencing also protects livers from ischemia/reperfusion- and Fas-induced injury.[74,180] Since siRNAs are non-immunogenic and highly specific, their therapeutic potential will surely be studied further.

Induction of Apoptosis as a Therapeutic Strategy

Conversely, sensitizing to cell death also represents therapeutic opportunity. For example, selective sensitization to TRAIL-induced apoptosis leads to death of hepatocarcinoma cells with sparing of normal hepatocytes.[144,145] In addition, targeted apoptosis of stellate cells by pharmacologic and genetic interventions might suppress and even reverse hepatic fibrosis, since activated stellate cells are the source of collagen formation in fibrotic liver disease.[184-188] Stellate cell activation may be the consequence of apoptosis by other hepatic cell types, since engulfment of apoptotic bodies activates quiescent stellate cells in culture, and inhibition of apoptosis of hepatocytes mitigates the fibrotic responses in murine models of liver in-

jury.[189-191] Thus, whereas hepatocyte apoptosis is profibrotic, targeted apoptosis of stellate cells diminishes hepatic fibrosis.[192-195]

Conclusion

Apoptosis and necrosis are prominent features of many (if not most) liver diseases, and prevention of these modes of cell death is a rational target of therapy. As discussed above, controversies still exist regarding which modes of cell death predominate in various forms of liver disease and injury. Resolution of these controversies may come with the recognition that apoptosis and necrosis frequently represent alternate outcomes of the same cellular pathways to cell death, especially for cell death mediated by mitochondrial permeabilization. Thus, an understanding of the processes leading to such necrapoptosis is most important for development of effective interventions to prevent hepatocellular death in acute and chronic liver diseases and to promote cell death in malignancy and fibrotic disease.

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