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Cell Death and Cell Death Responses in Liver Disease: Mechanisms and Clinical Relevance

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Summary

Hepatocellular death is present in almost all types of human liver disease and is used as a sensitive parameter for the detection of acute and chronic liver disease of viral, toxic, metabolic, or autoimmune origin. Clinical data and animal models suggest that hepatocyte death is the key trigger of liver disease progression, manifested by the subsequent development of inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Modes of hepatocellular death differ substantially between liver diseases. Different modes of cell death such as apoptosis, necrosis, and necroptosis trigger specific cell death responses and promote progression of liver disease through distinct mechanisms. In this review, we first discuss molecular mechanisms by which different modes of cell death, damage-associated molecular patterns, and specific cell death responses contribute to the development of liver disease. We then review the clinical relevance of cell death, focusing on biomarkers; the contribution of cell death to drug-induced, viral, and fatty liver disease and liver cancer; and evidence for cell death pathways as therapeutic targets.

I. Introduction

The presence of hepatocyte death, reflected by increased levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), is the most widely used parameter to screen for and monitor patients with liver disease. Moreover, these markers drive therapeutic decisions; have prognostic value for patients with hepatitis B virus (HBV)^{1, 2, 3 and 4} and hepatitis C virus (HCV)^{5, 6, 7 and 8} infections, nonalcoholic steatohepatitis (NASH)^{9, 10 and 11} and autoimmune hepatitis¹²; and correlate with overall and liver-specific mortality in the general population.^{13, 14 and 15} These well-established facts emphasize the importance of cell death as the ultimate driver of liver disease progression and the development of liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).

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In the healthy liver, cell death controls organ homeostasis, with a tight equilibrium between the loss and replacement of hepatocytes.¹⁶ Turnover is low in the normal liver, with approximately 0.05% of hepatocytes at any given time being removed by apoptosis, mostly in zone 3.^{17 and 18} This is reflected by almost undetectable ALT levels in healthy subjects. Despite the fact that most hepatic cell types rest in G₀ phase, the liver is endowed with an astounding ability to regenerate in response to massive hepatocellular death or loss of functional liver mass.¹⁹ This regenerative ability not only reflects essential metabolic functions of the liver but is also directly related to its high vulnerability to insults causing massive hepatic cell death, such as food-derived toxins or infections with hepatotropic viruses, bacteria, and parasites. As such, the wide range of metabolic and detoxifying functions predisposes hepatocytes to xenobiotic- and toxin-induced injury. Rapid regeneration represents an efficient mechanism to avoid the loss of key hepatic functions in this setting. Although acute liver failure caused by foodborne poisons and infections may have posed the biggest threat in former times, the bulk of modern liver diseases result from chronic disease processes such as chronic viral hepatitis, nonalcoholic fatty liver disease (NAFLD), and alcoholic liver disease (ALD). In these settings, the hepatic response to cell death, which is primarily geared toward restoring hepatic architecture and function in response to an acute threat to life (by providing extracellular matrix for mechanical stability and triggering hepatocyte regeneration to restore functional liver mass), becomes maladaptive and promotes the development of tissue fibrosis, cirrhosis, and HCC. The contribution of cell death to liver disease is cell-, stage- and context-specific. Although increased cell death may be a key driver of many chronic disease processes, including fibrogenesis and hepatocarcinogenesis (Table 1), loss or malfunction of programmed cell death (PCD) induction in subsets of epithelial cells contributes to the malignant transformation and constitutes a hallmark of cancer.²⁰ Likewise, whereas increased cell death in hepatocytes contributes to fibrogenesis, cell death in fibrogenic cells is an important mechanism for resolution of liver fibrosis.²¹ Our review focuses on cell death, but it is also likely that cellular injury (not full-blown cell death) triggers stress responses that contribute to disease development. However, these aspects will not be covered in this review.

In view of the fundamental role of cell death in virtually all hepatic diseases, precise knowledge of mechanisms regulating cell death and cell death responses is essential to understand the pathophysiology of liver disease and develop new therapeutic approaches.

II. Regulation of Cell Death in the Liver

Cell death occurs not only as a passive response to physicochemical stress or noxious insults but may also be actively induced by the host via PCD. PCD plays an active role in development and organismal homeostasis.²² Accordingly, inhibition of PCD by genetic ablation of key cell death regulators leads to hepatic hyperplasia.²³ Moreover, PCD is directly involved in the defense against pathogens, including hepatotropic viruses,²⁴ and represents a key mechanism preventing malignant transformation.²⁰ Traditionally, 2 distinct forms of cell death have been recognized: apoptosis as the mediator of PCD, actively induced by specific signaling cascades and occurring in a highly controlled fashion, and necrosis as the accidental form of cell death.^{25 and 26} However, recent evidence indicates that PCD can also trigger a specific form of necrosis, termed necroptosis.²⁷ The regulated nature

of multiple cell death modes not only affects our understanding of the underlying pathophysiology but also suggests the possibility of therapeutically interfering with regulatory mechanisms in diseases in which cell death was classically considered to be nontargetable (Figure 1).

Necrosis

Necrosis is viewed as a largely unregulated consequence of physicochemical stress, characterized by mitochondrial impairment, depletion of adenosine triphosphate (ATP), and subsequent failure of ATP-dependent ion pumps. This results in rapid swelling of cells and cell organelles (“oncosis”) accompanied by the formation of membrane “blebs” and ultimately cellular rupture.^{25, 28 and 29} As a consequence, cellular constituents spill into the extracellular environment and elicit significant inflammatory responses, rendering necrosis an “immunogenic” form of cell death^{29 and 30} (Figure 1). Recent studies have highlighted that necrosis also contains regulated elements involving mitochondrial events. As such, the mitochondrial permeability transition (MPT) leads to the opening of a mitochondrial pore, triggering mitochondrial swelling and uncoupling of oxidative phosphorylation as a result of osmotic forces.³¹ The relevance of pathways regulating necrosis (“regulated necrosis”) is shown by the ability of drugs that target cyclophilin, a key contributor to the MPT,^{32, 33 and 34} or drugs targeting mediators downstream of mitochondria such as c-Jun N-terminal kinase (JNK) to inhibit hepatocyte necrosis.^{35, 36, 37 and 38} Furthermore, lack of ATP may convert apoptotic death into secondary necrosis (also sometimes referred to as “necroptosis” or “aponecrosis,” to be distinguished from “necroptosis”³⁹). Accordingly, multiple forms of cell death, including necrosis, apoptosis, and necroptosis, commonly exist, most likely side by side, in relevant liver diseases such as ALD and NAFLD.^{40, 41, 42, 43 and 44} The therapeutic relevance of this finding has been highlighted by recent studies in renal ischemia-reperfusion in which combinations of regulated necrosis and necroptosis inhibitors exerted additive effects.⁴⁵ Diseases with cell death that used to be considered largely a consequence of unregulated necrosis, such as acetaminophen-induced liver injury and ischemia-reperfusion injury, can be modulated by MPT inhibitor cyclosporin A or JNK inhibitors,^{35, 36, 46 and 47} suggesting an important role of regulated necrosis in these settings. With increasing data on pathways regulating hepatic necrosis, the question arises to what extent pure necrosis contributes to liver disease.

Apoptosis

In contrast to necrosis, apoptosis is a highly controlled biochemical process in which the cell and many of its components are virtually “chopped” into pieces,⁴⁸ mediated by the concerted action of aspartate-specific proteases, known as caspases⁴⁹ (Figure 1). Accordingly, apoptosis is morphologically distinct from necrosis, with characteristic features such as cellular shrinkage, nuclear condensation, and fragmentation. Apoptosis is a common feature of viral, cholestatic, fatty, and alcoholic liver disease.^{44, 50, 51, 52 and 53} In viral hepatitis, apoptosis is readily seen in liver sections by the presence of characteristic Councilman bodies. Apoptosis is considered noninflammatory or low inflammatory due to the rapid removal of apoptotic cells, triggered “eat-me” signals such as phosphatidyl serine expressed on apoptotic bodies, which promotes engulfment by phagocytotic cells and thereby prevents leakage of cellular contents.⁵⁴ Whereas low-grade leakage of specific

proinflammatory mediators from apoptotic cells, including chemokines, ATP, uridine triphosphate, and sphingosine-1-phosphate mediators,^{55, 56 and 57} may be required for efficient detection of apoptotic cells by phagocytes (“find me” signals), activated caspases may also process intracellular contents in a way that renders them less inflammatory.⁵⁸ Accordingly, patients with chronic HCV infection, a disease in which apoptosis is the dominant form of cell death,^{51 and 52} can display normal or minimally elevated serum ALT levels despite ongoing hepatitis.^{59 and 60} On the other hand, serum ALT levels can increase with higher numbers of apoptotic cells, supporting the hypothesis that either apoptotic hepatocytes leak some of their intracellular contents, possibly at higher levels when engulfment of apoptotic hepatocytes is saturated or not rapid enough, or some hepatocytes do not die from pure apoptosis. Thus, hepatocellular apoptosis may not be as inert as often assumed and might contribute to hepatic inflammation, fibrosis, and HCC (discussed in the following text).

Depending on whether the triggering event is cell intrinsic or cell extrinsic, apoptosis is distinguished into mechanistically largely separate intrinsic and extrinsic pathways (Figure 1). The intrinsic pathway commonly triggers apoptosis via members of the B-cell lymphoma 2 (Bcl-2) family, which control mitochondrial outer membrane permeabilization, cytochrome *c* release, and subsequently caspase activation.^{61, 62, 63 and 64} Several intracellular triggers of apoptosis activate this pathway, including endoplasmic reticulum (ER) stress and p53 activation. ER stress is typically the result of accumulating unfolded or misfolded proteins in the ER, leading to the so-called unfolded protein response. Whereas mild ER stress is cytoprotective, profound or prolonged ER stress promotes cell death by activation of JNK and CHOP.⁶⁵ As such, increased ER stress induces cell death and disease progression in α_1 -antitrypsin deficiency.⁶⁶ Viral infections also commonly induce ER stress, and HCV infection triggers ER stress in culture and in infected patients.^{67, 68 and 69} Exposure of hepatocytes to free fatty acids induces ER stress and an intrinsic cell death pathway termed lipoapoptosis in vitro and in vivo,⁷⁰ but it appears that lipoapoptosis is predominantly mediated by JNK activation in an ER stress-independent manner.^{34, 71 and 72} p53 is another important regulator of the intrinsic death pathway and the central component of a continuously operative cell fate program that determines whether a cell should initiate DNA damage repair or die by apoptosis. In response to oncogene activation, DNA damage, and senescence, p53 becomes activated and, through mostly transcriptional regulation of specific target genes such as Bax, induces apoptosis. When damage is less severe, p53 induces cell cycle arrest through targets such as p21, allowing for cellular repair. Hence, p53 functions as the “guardian of the genome,” preventing malignant transformation of hepatocytes. Accordingly, HCCs commonly escape from this control mechanism by acquiring p53 mutations.⁷³

The extrinsic cell death pathway is typically triggered by members of the tumor necrosis factor (TNF) family of death receptor ligands, comprising TNF itself, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL).⁷⁴ Hepatocytes express high levels of death receptors, possibly allowing efficient eradication of hepatocytes infected by hepatotropic viruses.⁷⁵ Death receptor-mediated apoptosis is a key feature of many types of liver diseases.⁷⁵ The high susceptibility of hepatocytes to death receptor-induced cell death is

highlighted by the fact that Fas agonistic antibodies trigger rapid apoptosis in murine and human hepatocytes as well as acute liver failure and death in mice.^{76 and 77} In the liver, the extrinsic and intrinsic pathways are linked, because hepatocytes as “type 2 cells” require mitochondrial amplification via cytochrome *c* release-mediated activation of caspase-3 for cell death execution⁷⁸ (Figure 2).

TNF- and Fas ligand-induced cell death share many components of signal transduction such as formation of a death-inducing signaling complex after receptor oligomerization-induced recruitment of caspase-8, resulting in the activation of executioner caspases (reviewed in Muddipati et al,⁷⁴ Wajant,⁷⁹ and Reinehr and Haussinger⁸⁰) (Figure 2). However, whereas the outcome of Fas activation is hepatocyte death, TNF receptor activation affects multiple cellular responses that besides cell death also include survival, inflammation, and proliferation.⁸¹ Transcription factor nuclear factor κ B (NF- κ B) represents a key cytoprotective pathway that up-regulates antiapoptotic genes such as Bcl-xl and c-FLIP and blocks prolonged activation of JNK,⁸² a key pathway through which TNF induces cell death. Prolonged JNK activation is inhibited by NF- κ B-dependent up-regulation of antioxidant proteins such as ferritin and SOD2. Inhibition of NF- κ B unmasks a TNF-induced self-amplifying pathway of mitochondrial oxidative stress that is mediated through the interaction of activated JNK and a protein in the outer membrane of mitochondria known as Sab (SH3BP5). This pathway sustains JNK activation, which then leads to degradation of cFLIP, inhibition of antiapoptotic members of the Bcl2 family, and activation of proapoptotic Bcl2 family members (Figure 2).⁸³ Accordingly, TNF-mediated cell death requires inhibition of NF- κ B or NF- κ B target genes^{84 and 85} and can be blocked by JNK inhibition.^{82 and 86} JNK also promotes protective responses such as hepatocyte proliferation and liver regeneration.⁸⁷

The dichotomous nature of TNF signaling and the dominance of antiapoptotic and proinflammatory signals over cell death induction have been shown in multiple disease models. Injection of TNF or lipopolysaccharide alone does not cause significant liver injury. However, blockage of NF- κ B, through conditional deletion of *Nemo*, *Tak1*, NF- κ B target genes like the caspase-8 inhibitor c-Flip, or both *Ikk α* and *Ikk β* in hepatocytes or hepatic inhibition of transcription with D-galactosamine, sensitizes hepatocytes to TNF-induced apoptosis and liver failure.^{88, 89, 90, 91 and 92}

Necroptosis

The concept of apoptosis and its prevention by NF- κ B seemed to sufficiently explain how TNF mediates cell death in the liver. However, this concept was recently challenged by a paradigmatic shift in our understanding of PCD based on evidence for a third form of cell death, necroptosis, which incorporates features of necrosis and apoptosis. Necroptosis uses the same upstream molecular machinery as apoptosis⁹³ (Figure 2), supporting the hypothesis that it functions as a backup pathway to enable cell death in settings where apoptosis is inhibited (eg, by viruses expressing antiapoptotic genes).⁹⁴ Despite sharing upstream mediators with apoptosis, the final outcome of necroptosis is cellular leakage as a result of organelle and cellular swelling (Figure 1). Necroptosis is best characterized in the setting of

TNF-induced cell death, which has high relevance for many types of liver diseases but may also occur in other conditions, including ischemia-reperfusion injury.²⁷

The decision whether death receptor activation induces apoptosis or necroptosis depends on 2 kinases: receptor-interacting protein (RIP) 1 and RIP3.^{95, 96, 97 and 98} Activation of caspase-8 shifts the balance toward apoptosis by cleaving RIP1 and RIP3, while inhibition of caspase-8 leads to assembly of RIP1/RIP3 complexes, forming the “necrosome,” a key transducer of the necroptotic signal.^{27 and 99} The exact mechanism of how necrosome activation executes necroptosis remains a matter of debate. Mixed lineage kinase domain-like protein (MLKL) is a key mediator of necroptosis. It has been suggested that MLKL increases mitochondrial reactive oxygen species (ROS) production through mitochondrial targets.¹⁰⁰ Recent studies have shown that MLKL triggers cytotoxic influx of either calcium or sodium ions and that this requires MLKL to translocate to the plasma membrane.^{101 and 102}

Methods for visualizing necroptosis, allowing determination of its role in human disease, are only starting to be developed, with phosphorylated MLKL representing a potential marker for necroptosis in both animal models and patients with drug-induced liver injury (DILI).¹⁰³ So far, most data on the contribution of necroptosis to liver diseases and mechanistic insights are derived from murine studies. Interestingly, RIP3 is only weakly expressed in healthy murine livers compared with other organs such as lung or spleen¹⁰⁴ but shows up-regulated protein levels in cells that are sensitized to undergoing necroptosis (eg, by ablation of caspase-8).¹⁰⁵ Infection of mice with vaccinia virus induced assembly of RIP1/RIP3 complexes in the liver, suggesting necroptosis as a component of antiviral responses.⁹⁵ RIP3-deficient mice show decreased hepatocyte death after acetaminophen intoxication and long-term ethanol feeding, suggesting an involvement of necroptosis in these diseases.^{40 and 41} Although inhibition of necroptosis by RIP3 deficiency, RIP1 blockade by necrostatin-1, or MLKL deficiency all reduce cell death at early time points,^{40 and 106} RIP3 deficiency was unable to abrogate acetaminophen-induced liver injury at 24 hours.⁴⁰ Hence, additional pathways may contribute to cell death or compensate for the loss of RIP3. Finally, studies in a model of mice with liver-specific Tak1 deletion, a model of chronic liver injury, showed opposing roles of RIP3-mediated necroptosis and caspase-8-mediated apoptosis. In this model, necroptosis in mice with combined ablation of TAK1 and caspase-8 was associated with low proliferation of hepatocytes and cholangiocytes, leading to reduced hepatocarcinogenesis and development of cholestasis.¹⁰⁵ Conversely, apoptosis was associated with high hepatocyte proliferation and hepatocarcinogenesis but absent cholestasis.¹⁰⁵ The mutual relationship between apoptosis and necroptosis in hepatocytes, similar to findings in other organs,^{107 and 108} is further highlighted by RIP3-mediated spontaneous liver injury in mice with *caspase-8* deletion in parenchymal liver cells.¹⁰⁵

Autophagy and Cell Death

Autophagy is the catabolic degradation of cellular components through the lysosome, involving different types of “cargo,” and serves as both a removal mechanism for dysfunctional cell content and an energy source.¹⁰⁹ Autophagy is considered a predominantly cytoprotective pathway that protects from ALD,¹¹⁰ TNF-induced liver

injury,¹¹¹ acetaminophen-induced liver injury,¹¹² ischemia-reperfusion injury,¹¹³ and high-fat diet-induced lipid accumulation.¹¹⁴ In specific circumstances, autophagy may be associated with cell death,¹⁰⁹ but cell death-promoting functions still need to be better understood, particularly in the context of liver disease.¹¹³

III. Specific Considerations of Cell Death Regulation in the Liver

Cholestasis and Bile Acid-Induced Cell Death

Cholestasis is a common feature of acute and chronic liver disease, and accumulation of toxic bile acids contributes to hepatocyte death in this setting. Bile acid-induced cell death in cultured cells is largely apoptotic and mediated by ligand-independent activation of death receptors, including the Fas receptor.^{50 and 115} Cholestatic liver disease also often triggers necrotic cell death, reflected by the appearance of “bile infarcts” after bile duct ligation or in Mdr2ko mice, as well as in cholestatic liver disease in patients. However, at present it is not clear whether this is induced by bile acids or mediated by other mechanisms.

Cell Death in Non-hepatocyte Populations

Cell death in non-hepatocyte populations is also an essential feature of chronic liver disease. Cell death of cholangiocytes is not as well characterized as hepatocyte death, both in terms of mechanisms and its contribution to liver disease. TNF receptor family-mediated apoptosis, mediated by activation of Fas, TRAIL receptor 2, or CD40, appears to be the most common form of cholangiocyte death.^{116, 117, 118, 119 and 120} Cholangiocyte apoptosis occurs in immune-mediated and drug-induced cholangiopathies¹²⁰ and may contribute to disease progression and ductopenia.¹¹⁷ In contrast to hepatocyte death as a common trigger of wound healing and fibrosis, it is believed that cholangiocyte proliferation rather than cholangiocyte death promotes disease, for example, by contributing to the development of peribiliary fibrosis seen in cholangiopathies.¹²⁰ Hepatic stellate cell (HSC) death is a mechanism that achieves removal of activated myofibroblasts and resolution of hepatic fibrosis²¹ and therefore is generally considered beneficial in chronic liver disease. Death of liver sinusoidal endothelial cells occurs after ischemia-reperfusion injury (eg, after liver transplantation), but its relative contribution to organ dysfunction remains controversial.^{121 and 122} Likewise, the regulation and contribution of macrophage death in the liver is not well understood. Finally, the liver also serves as the “graveyard” of specific immune cell subsets such as activated CD8⁺ T cells,¹²³ which may contribute to the high immune tolerance of the liver.

IV. Response Pathways to Cell Death

Although massive liver cell death can impair liver function in acute or acute-on-chronic liver disease, in most chronic liver diseases only a small percentage of hepatocytes die at the same time. In this setting, cell death has no direct significant impact on liver function. Instead, hepatic responses to cell death, often persisting over decades, dictate the development of long-term consequences and clinical outcomes (Figure 3).

Damage-Associated Molecular Patterns as a Link Between Cell Death and Inflammation in Chronic Liver Disease

Pattern recognition is a cornerstone of innate immunity.¹²⁴ Matzinger suggested that abnormal molecular patterns play a role not only in pathogen recognition by the host but also in the recognition of “danger” associated with tissue injury.¹²⁵ Damage-associated molecular patterns (DAMPs), released from dying cells, are believed to trigger sterile inflammation occurring after tissue injury.^{125, 126 and 127} DAMP release is believed to occur mainly after necrosis and necroptosis due to the loss of membrane integrity, thus explaining the inflammatory nature of these cell death modes.^{30, 126, 127, 128 and 129} Some DAMPs such as high-mobility group box 1 (HMGB1) may even be actively retained during apoptosis.¹²⁹ In the liver, several DAMPs and DAMP receptors, including HMGB1 (via the receptor for advanced glycation end products or Toll-like receptor 4), formyl peptides (via FPR1), or ATP (via P2X7), trigger inflammatory cell recruitment^{130, 131 and 132} and contribute to inflammation and exacerbation of injury in acute liver diseases such as hepatic ischemia-reperfusion injury¹³⁰ and acetaminophen intoxication.^{133 and 134} The contribution of DAMP-induced sterile inflammation to chronic disease processes is much less understood. A recent study reported that RAGE, one of the receptors for HMGB1, is required for oval cell proliferation and hepatocarcinogenesis, suggesting that this receptor might provide a mechanistic link between DAMPs and hepatocarcinogenesis in chronic liver disease.¹³⁵ Given that DAMPs represent a novel and potentially highly relevant class of therapeutic targets which may be involved in driving many of the complications of chronic liver injury, further studies are needed to examine if low-grade release of DAMPs from apoptotic cells may contribute to inflammation in relevant chronic liver diseases such as viral hepatitis, NAFLD, and ALD. Moreover, secretion of mediators from stressed cells may additionally mediate hepatic responses to injury. As such, IL-33 is released from stressed hepatocytes to promote fibrogenesis and bile duct proliferation via innate lymphoid cells.^{136 and 137}

Hepatic Fibrogenesis in Response to Cell Death

Fibrosis is one of the clinically most relevant consequences of chronic liver disease. Development of fibrosis is seen in all animal models with significant hepatocyte injury and correlates with elevated ALT levels in various liver diseases^{1, 7, 8 and 138} as well as elevated levels of cleaved keratin 18 (K18), a marker of caspase activation.¹³⁹ HSCs are the dominant contributors to fibrosis in the liver,¹⁴⁰ but the molecular links between hepatocyte death and HSC activation remain poorly understood. Although the hypothesis that DAMPs provide a direct link between hepatocyte death and HSC activation is attractive, it has not been rigorously tested and there is no convincing evidence for DAMPs that directly activate HSCs. Alternatively, DAMPs could act on other cell types that in turn release fibrogenic mediators such as transforming growth factor β or platelet-derived growth factor to activate HSCs. Among these, hepatic macrophages are best known to interact with HSCs and to promote HSC fibrogenesis.^{141, 142 and 143} Moreover, it needs to be considered that cell death in many noncholestatic liver diseases (eg, viral hepatitis) is predominantly apoptotic and that DAMP release may be low in these conditions. There is ample evidence that apoptosis may trigger fibrogenesis. Mouse models with selective increases of hepatocyte apoptosis by hepatocyte-specific deletion of Nemo, Mcl-1, or Bcl-xl develop liver fibrosis,^{62, 63 and 90} thus providing a link between apoptosis and fibrogenesis. Whether this is pure apoptosis or

also involves necrotic cell death remains uncertain because these models are typically accompanied by elevated ALT levels. Whereas the pan-caspase inhibitor IDN-6556 reduced the development of bile duct ligation-induced liver fibrosis,¹⁴⁴ the pan-caspase inhibitor VX166 exerted only little effect on NASH-induced liver fibrosis despite improved injury and inflammation.¹⁴⁵ In contrast, we recently showed that inhibition of necroptosis through ablation of RIP3 protects mice from NASH-induced liver fibrosis,¹⁴⁶ highlighting the hypothesis that necroptosis rather than apoptosis controls liver fibrosis on metabolic injury. The mechanistic link between apoptosis or necroptosis and HSC activation is not fully understood. Several studies have proposed that phagocytosis of apoptotic bodies by HSCs links cell death to HSC activation, with several in vitro studies showing increased HSC activation, migration, and survival after phagocytosis of apoptotic bodies.^{147, 148 and 149} Phagocytosis of apoptotic hepatocytes by hepatic macrophages results in increased secretion of profibrogenic mediators, suggesting that apoptosis may promote HSC activation and fibrosis through indirect mechanisms involving professional phagocytic cells.¹⁵⁰

Cell Death and Compensatory Proliferation

Partial hepatectomy is classically used to investigate liver regeneration and model regeneration after surgery in patients. In contrast, regeneration in human disease may be direct response to acute hepatocyte death (eg, after acetaminophen intoxication) or persistent cell death in chronic liver diseases rather than a direct consequence of loss of anatomical or functional liver mass. Hence, it is not clear whether compensatory regeneration after cytotoxic loss of hepatocytes differs mechanistically from liver regeneration after partial hepatectomy. The relevance of compensatory proliferation for hepatocarcinogenesis is well established across animal models.^{105 and 151} In particular, the aforementioned Tak1 and Nemo liver-specific knockout mice show massive compensatory proliferation, which is believed to contribute to the spontaneous development of HCC.^{90 and 152} In both models, apoptosis appears to be the driving force triggering proliferation,¹⁰⁵ but it is presently unclear which mediators and cell types mechanistically link these processes.

Cell Death and Hepatocarcinogenesis

Hepatocarcinogenesis is tightly linked to cell death. However, one needs to carefully distinguish between cell death occurring in nontransformed hepatocytes and cell death occurring in transformed hepatocytes because these have opposite functional consequences. Cell death in nontransformed hepatocytes represents a tumor-promoting mechanism, mediated by increased compensatory regeneration, fibrogenesis, and inflammation. The tumor-promoting effects of hepatocyte apoptosis have been clearly shown by hepatocyte-specific deletion of the antiapoptotic proteins Mcl-1 or Bcl-xl, which not only increased the rate of hepatocyte apoptosis^{62, 63 and 64} but also resulted in spontaneous development of HCC.^{64 and 153} In the Bcl-xl liver knockout model, hepatocarcinogenesis could be suppressed by additional knockout of Bak, thus providing a direct link between hepatocyte apoptosis and HCC.¹⁵³ Similar evidence comes from models in which NF- κ B inhibition through conditional deletion of *Nemo* in hepatocytes leads to massively increased cell death and spontaneous development of HCC.¹⁵¹ Increasing carcinogen-induced apoptosis and compensatory proliferation through hepatocyte-specific IKK β deletion promotes formation of HCC,¹⁵¹ whereas decreasing it through knockout of Bcl-2 family member p53 up-

regulated modulator of apoptosis (PUMA) decreases it.¹⁵⁴ The key role of apoptosis is highlighted by the fact that development of HCC in these latter models can be prevented by codeletion of the proapoptotic genes *Fadd*⁹⁰ or caspase-8.¹⁰⁵ and ¹⁵⁵ Likewise, antibody-mediated neutralization of Fas ligand prevented not only hepatocyte apoptosis but also hepatocarcinogenesis in a transgenic hepatitis B surface antigen HCC mouse model.¹⁵⁶

In contrast to the tumor-promoting effect of cell death in non-transformed hepatocytes, cell death in transformed hepatocytes limits development of HCC. Accordingly, tumor cells often undergo a selection process that allows them to successfully evade apoptosis, such as mutations of p53.⁷³ and ¹⁵⁷ Down-regulation of proapoptotic Bax and Bcl-X_S³⁰ and ¹⁵⁸ and up-regulation of antiapoptotic proteins such as Bcl-X_L, Mcl-1, survivin, and X-linked inhibitor of apoptosis protein (XIAP) endows transformed hepatocytes with increased survival properties.^{27, 40, 41, 159, 160} and ¹⁶¹ Development of genetic resistance to cell death is favored in settings of chronic cell death and high cell turnover by selection of clones that are fittest to survive and proliferate. In addition to the previously described mechanisms, infection with hepatotropic viruses may also lead to increased resistance to apoptosis.¹⁶² Additional survival signals may derive from cell-extrinsic sources, such as the altered hepatic microenvironment,¹⁶³ or a leaky gut that increases Toll-like receptor signaling in the liver.¹⁶⁴ NF-κB represents a key antiapoptotic pathway that promotes survival of transformed hepatocytes,¹⁶⁵ as shown by reduced development of cancer in Mdr2-knockout mice in which NF-κB is inhibited by expression of an IκB superrepressor.¹⁶⁶

Distinct modes of cell death differentially affect carcinogenesis in the liver. Despite the widely held belief that apoptosis represents an “unreactive” form of cell death, aforementioned evidence from mouse models with increased hepatocyte apoptosis^{64, 90, 152, 153} and ¹⁶⁷ and interventions that reduce apoptosis^{105, 153, 155} and ¹⁵⁶ strongly suggest that apoptosis is the primary driver of HCC development. Accordingly, co-deletion experiments in TAK1-knockout mice showed that apoptosis but not necroptosis promoted carcinogenesis in this model.¹⁰⁵ Further studies are required to confirm these findings in additional hepatocarcinogenesis models. We currently do not understand how this classically “areactive” form of cell death promotes cancer. It is possible that the engulfment of apoptotic bodies triggers HCC-promoting compensatory proliferation or fibrosis more efficiently than necrosis or that secondary necrosis associated with apoptosis promotes HCC. Alternatively, it could be envisioned that proapoptotic executioner enzymes, including DNases, induce collateral damage in neighboring cells or that some cells survive the “apoptotic attack” but show genetic alterations that ultimately lead to cancer.

V. Biomarkers of Cell Death

ALT and AST

Serum ALT is the most common and best-established biomarker for diagnosis and monitoring of acute and chronic liver disease¹⁶⁸ (Table 2). As a reflection of its nearly hepatocyte-specific expression, serum ALT levels in the general population are associated with overall and liver-specific mortality^{13, 14} and ¹⁵ but not with mortality from other causes.¹⁵ Elevations of other markers of liver disease, such as γ-glutamyl transpeptidase and AST, are associated with overall mortality but do not specifically reflect liver-related

mortality.^{13 and 15} Elevated ALT levels also correlate with clinical progression to fibrosis and cirrhosis in patients with HBV infection,^{1, 2, 3 and 4} HCV infection,^{5, 6, 7 and 8} and NASH¹³⁸ (Table 2). Whereas ALT seems to be the best-established marker for predicting disease progression in chronic liver disease, there appear to be more sensitive markers that outperform ALT in acute liver disease, such as microRNA (miR)-122, HMGB1, and K18. In contrast to ALT, AST is expressed in a wider range of tissues, including cardiac and skeletal muscle, kidney, and blood.¹⁶⁸ Hence, elevated serum AST levels are less specific than elevated serum ALT levels. Higher AST levels and in particular an AST/ALT ratio >2 are indicative but not specific to severe ALD.¹⁶⁹ However, AST has only low sensitivity for detecting alcohol intake and ALD.¹⁷⁰ An increased AST/ALT ratio is also associated with increased risk of development of fibrosis in NASH.^{9, 10 and 11}

K18

K18 is a 48-kilodalton intermediate filament protein highly expressed in epithelial cells. When released into the extracellular space, K18 can be used as a serum marker for epithelial cell death. K18 is cleaved sequentially by caspases to first generate 44-kilodalton and 4-kilodalton fragments, with a second caspase digestion occurring within the 44-kilodalton fragment to generate 29-kilodalton and 23-kilodalton fragments.¹⁷¹ The M30 antibody, which has been used in numerous clinical studies, recognizes exposed neoepitopes on the C-terminus of the 44-kilodalton and 23-kilodalton fragments; additional antibodies, such as the M65 antibody, are used in some studies as a necrosis readout even though it recognizes both uncleaved and caspase (or other protease) cleaved K18. Although there is an ongoing debate whether antibody-based assays reliably reflect caspase-cleaved K18 and apoptosis, numerous studies have shown the usefulness of K18 in quantifying cell death and predicting clinical outcomes.¹⁷² The strongest evidence for K18 as a biomarker comes from studies in NAFLD, where it has high sensitivity and specificity in diagnosing NASH among patients with NAFLD¹⁷³ and positively correlates with clinical parameters and the histological activity score (Table 2).^{174 and 175} Studies in HCV infection showed that 50% of patients with normal aminotransferase levels exhibited elevated serum K18 levels and, among these, 30% showed advanced stages of fibrosis.¹³⁹ Mallory–Denk bodies, a hallmark of ALD, largely consist of ubiquitinated K8 and K18 aggregates. Accordingly, full-length K18 and K18 fragments correlate with the presence of Mallory–Denk bodies, hepatocyte ballooning, and liver fibrosis in patients with ALD.¹⁷⁶ Despite these encouraging data on K18 as a biomarker for several hepatic diseases, additional data are required to recommend its use for routine clinical practice.

HMGB1

HMGB1 is a nonhistone DNA binding protein that is present in virtually all eukaryotic cells.¹⁷⁷ HMGB1 is passively released from necrotic cells and may, after hyperacetylation, also be actively secreted from inflammatory cells.¹⁷⁷ Apoptotic cells only release little HMGB1 due to retention by cruciform DNA.¹²⁹ HMGB1 has largely been studied in liver disease with necrotic cell death. Total and hyperacetylated HMGB1 are increased in patients with acetaminophen intoxication.¹⁷⁸ Total HMGB1 concentrations were found to be superior to serum ALT levels in identifying acute liver injury within 8 hours of acetaminophen overdose. Increases in total and acetylated HMGB1 were associated with

worse prognosis after acetaminophen intoxication, a finding not reflected by serum ALT levels.¹⁷⁸

microRNAs

microRNAs are small noncoding RNAs with important roles in the regulation of gene expression. miR-122 is the most abundant microRNA in hepatocytes and is released into serum after liver injury. After acetaminophen intoxication, miR-122 levels are increased and detect liver injury earlier than serum ALT levels¹⁷⁹ and outperform serum ALT for the prediction of subsequent acute liver injury.¹⁸⁰ In chronic liver disease, miR-122 levels correlate inversely with the severity of liver fibrosis, probably reflecting the loss of functional hepatocyte mass rather than the rate of hepatocyte cell death.¹⁸¹ HSC activation and fibrosis, as responses to hepatic cell death, are reflected by increased serum levels of miR-29, miR-133, miR-571, and miR-652.^{182, 183 and 184}

Carbamoyl Phosphate Synthetase 1

Carbamoyl phosphate synthetase 1 is the most abundant protein in liver mitochondria and more liver specific than ALT.¹⁸⁵ A recent study showed carbamoyl phosphate synthetase 1 in acute liver injury triggered by acetaminophen intoxication, Wilson disease, and ischemic liver injury.¹⁸⁵ Because of its very short half-life, carbamoyl phosphate synthetase 1 may more accurately predict when liver injury terminates than ALT.

VI. Cell Death in Clinical Liver Disease

Considerable evidence for a role for cell death in promotion of liver disease in animals (Table 1) and in numerous clinical studies has provided the basis to move cell death-based therapies for acute and chronic liver disease toward clinical studies (Table 3). In the following section we review the contribution of cell death to specific liver diseases and approaches to therapeutically target cell death in patients.

DILI

DILI is the major cause of acute liver failure in Western countries and an important cause of acute hepatitis or cholestasis in clinical practice. The most common cause of DILI is acetaminophen toxicity, which induces dose-related necrosis of hepatocytes through a mechanism involving conversion of a small fraction of the dose to a reactive metabolite that depletes glutathione and covalently binds to proteins, with the mitochondria being the critical target organelle, resulting in cell death promotion by sustained JNK activation.^{35, 83 and 186} *N*-acetylcysteine (NAC) is a highly effective antidote for early acetaminophen overdose and successfully prevents toxicity by replenishing glutathione, thereby preventing covalent binding of the reactive metabolite.^{187 and 188} However, after acetaminophen is metabolized and glutathione is depleted, the role of NAC is limited.¹⁸⁹ Anecdotal reports suggest that late administration of NAC may have some efficacy in dampening toxicity in humans and it is widely used in clinical practice, even up to 48 hours after overdose or when subacute unintentional poisoning is identified. In this setting, additional treatments that target downstream pathways such as JNK may be beneficial. Although delayed JNK inhibition works well in animal models, the effectiveness of NAC in

early stages, theoretical concerns about interference with the late protective role of JNK-dependent regenerative signaling, and the inability to identify the critical window in patients with acetaminophen intoxication make it unlikely that direct JNK inhibitors will be used in clinical settings. Although necroptosis is important in the early phase of acetaminophen toxicity, the failure of RIP3 inhibition to block late liver injury makes the necrosome an unlikely therapeutic target for acetaminophen intoxication.

Aside from acetaminophen, most DILI is idiosyncratic (IDILI), meaning that only a small proportion of patients treated with an IDILI drug develop liver injury, and the cell death appears to be mediated by the adaptive immune system, as evidenced by striking recently described HLA associations that imply a genetic risk.¹⁹⁰ Current concepts suggest that IDILI drugs induce hepatocellular stress, which may provide a danger signal to the immune system of genetically susceptible individuals. At present, there is no specific treatment for IDILI. Drugs that inhibit immune-mediated cell death mechanisms might have benefit in early stages of overt or in prolonged IDILI but have not been formally studied. Once acute liver failure develops, such an approach has been unsuccessful.¹⁹¹

Viral Hepatitis

Most acute and chronic infections with HBV or HCV elicit an active antiviral immune response, resulting in active killing of infected hepatocytes.^{192 and 193} Elimination of infected hepatocytes occurs largely through CD8⁺ effector T cells and natural killer cells.^{192 and 193} In patients who do not clear the HBV or HCV virus, there is persistent T cell- and natural killer cell-mediated hepatocyte apoptosis. Although apoptosis of infected hepatocytes is primarily a protective response, it is often not sufficient to eliminate the virus in patients with chronic viral hepatitis and then becomes a key driver of liver disease development. T cell- and natural killer cell-induced hepatocyte apoptosis is largely mediated by members of the TNF receptor family (including Fas and TRAIL receptor 2) and, to a lesser extent, by granzyme B and perforin.^{52 and 194} Based on aforementioned studies in mice, in which apoptosis is sufficient to trigger fibrosis and HCC,^{62, 63, 64, 90, 152, 153 and 167} it is likely that apoptosis, induced by persistent elimination of infected hepatocytes in chronic HBV and HCV infection, constitutes the main contributor to disease progression. Based on this principle, several studies have tested caspase inhibitors IDN-6556 and GS-9450 in HCV-infected patients (Table 3), but no positive results have yet been reported. In view of the advent of direct antivirals with cure rates of more than 90% in HCV-infected patients¹⁹⁵ and high rates for hepatitis B e antigen seroconversion and/or suppression of viral replication in HBV-infected patients,¹⁹⁶ strategies to target cell death (eg, by caspase inhibitors) are unlikely to represent a clinically relevant avenue in viral hepatitis in the future. Glycyrrhizin, an ingredient of licorice that inhibits HMGB1,¹⁹⁷ improved necroinflammation and fibrosis in HCV-infected patients without changing serum HCV RNA,¹⁹⁸ suggesting that glycyrrhizin may be used to suppress HMGB1-mediated cell death responses in viral hepatitis and potentially other chronic liver diseases.

NAFLD

NAFLD represents the most common chronic liver disease in the Western world.^{199 and 200} NASH is a more aggressive disease entity within the spectrum of NAFLD that is

distinguished from simple steatosis by the presence of hepatocyte death and subsequent cell death responses, visible as hepatocyte ballooning, an inflammatory infiltrate, and/or collagen deposition, and promotes the development of fibrosis, cirrhosis, and HCC.²⁰¹ Despite clinical trials of multiple molecular targets in patients with NASH,²⁰² no effective pharmacological strategy against NASH-induced liver fibrosis and HCC has yet been established in clinical practice, highlighting the need to identify signaling pathways regulating the transition from NAFLD to NASH. This transition is typically accompanied by elevated ALT levels,^{173 and 203} indicating the pivotal role of cell death in this process. Of all cell death pathways, apoptosis is the best characterized form in this context.^{145, 204 and 205} Accordingly, plasma K18 fragment levels correlated with the magnitude of hepatocyte apoptosis and independently predicted the presence of NASH in several large trials.^{206 and 207} Beyond the putative role of apoptosis, we recently showed that human NASH livers express high levels of RIP3,¹⁴⁶ suggesting that necroptosis might represent an alternative target for clinical studies in NASH.

Multiple cell-intrinsic mechanisms have been suggested to trigger cell death and the progression to NASH. As such, saturated fatty acids are more hepatotoxic than unsaturated fatty acids.¹¹⁵ Fatty acid accumulation stimulates ROS generation in the liver, presumably due to enhanced β -oxidation and the subsequent electron overflow in the mitochondrial electron transfer chain.¹²⁰ Increased ROS directly injure DNA, proteins, and lipids and promote cell death through activation of stress-related signaling pathways such as JNK or p38 mitogen-activated protein kinase.⁸¹ JNK induction by fatty acid has been suggested to be a key factor in this setting,^{71 and 72} whereas the role of the ER stress response remains controversial because ablation of CHOP worsens NASH.^{208 and 209} In contrast to these intrinsic apoptosis pathways, the function of extrinsic death receptor-mediated apoptosis pathways in NASH is less clear. Although adipose tissue expansion in patients with NASH is associated with the release of proinflammatory cytokines such as TNF,¹¹⁶ their contribution to cell death and clinical relevance remain unknown. In a murine NASH model, TNF receptor knockout mice were protected from hepatic steatosis and fibrosis.²¹⁰ Moreover, TRAIL receptor 2 has a role in hepatocyte lipoapoptosis, but its contribution to NASH remain untested.²¹¹

Bariatric surgery successfully treats the underlying cause and achieves sustained amelioration of insulin resistance, hepatic steatosis and inflammation, serum ALT and K18 levels, and hepatic fibrosis.^{212, 213 and 214} Pharmacological strategies have focused on insulin resistance as the most relevant underlying pathophysiological mechanism and have found beneficial effects of the thiazolidinediones pioglitazone and rosiglitazone on serum ALT levels and histology during the first year of therapy.^{215 and 216} However, these treatments are not considered first-line therapies by current guidelines for various reasons.²¹⁷ In contrast, targeting cellular stress and cell death pathways has led to promising findings and changed clinical standards. Based on the prominent role of ROS in cell death and NASH pathophysiology, the PIVENS trial tested the effect of the antioxidant vitamin E in nondiabetic patients with NASH.^{218 and 219} The observed decrease in inflammation and serum ALT and AST levels, but not fibrosis, in nondiabetic patients taking vitamin E in this trial²¹⁹ provided the basis for the recent NAFLD guideline recommending vitamin E as a first-line treatment for nondiabetic patients with biopsy-proven NASH.²¹⁷ Investigating

farnesoid X receptor as yet another target, a recent study reported improved insulin sensitivity, weight loss, ALT level, γ -glutamyl transpeptidase level, and liver fibrosis markers in patients with type 2 diabetes mellitus and NASH treated with the FXR receptor agonist obeticholic acid.²²⁰ However, some effects, such as improved ALT levels and fibrosis markers, were only observed at the lower dose of obeticholic acid in this trial. A recent phase 2 study reported normalization of ALT levels in 35% of patients with NASH treated with the selective caspase inhibitor GS-9450,²¹⁶ the effects on liver histology are unknown, and reduction of another relevant biomarker, K18, was not significant. In summary, there is increasing evidence that targeting cell death and oxidative stress may be beneficial in NASH, but larger trials with longer observation intervals will be needed to determine effects on relevant clinical outcomes.

ALD

ALD represents one of the most common causes of liver-related morbidity and mortality worldwide.²²¹ Moreover, its relative contribution to liver disease is expected to further increase due to a worldwide increase of alcohol abuse¹²¹ and the declining prevalence of hepatitis B²²² and ²²³ and hepatitis C.¹²² Alcoholic steatohepatitis is the severe form of ALD and may progress to fibrosis, cirrhosis, and HCC.¹³² Similar to NASH, alcoholic steatohepatitis is characterized by hepatic steatosis, cell injury, and inflammation.⁶³ In ALD, there is evidence for both apoptosis and necrosis.^{42, 44 and 224} Cytotoxic effects of alcohol are at least in part caused by its metabolite acetaldehyde, which causes excess ROS production leading to lipid peroxidation, mitochondrial damage, and cell death.^{221 and 225} Accordingly, treatment with antioxidants ameliorates experimental alcoholic liver injury.⁶² An increased gut permeability, resulting in increased levels of portal vein lipopolysaccharide, contributes to the production of inflammatory cytokines TNF and IL-6, ROS, and liver injury, as evidenced by improved liver injury in Toll-like receptor 4-deficient mice or mice receiving nonabsorbable antibiotics for gut sterilization.^{226, 227 and 228} TNF induces liver injury on alcohol consumption,¹⁴⁷ and anti-TNF treatment prevented alcohol-induced hepatic cell death in rats.¹⁴⁹ A recent study using RIP3-deficient mice suggested that necroptosis is involved in TNF-dependent cell death in alcoholic liver injury.⁴¹ However, the clinical relevance of these pathways for the treatment of ALD and/or prevention of progression to HCC remains to be established.

As continued alcohol ingestion is the single most important risk factor for disease progression, and abstinence represents the most effective measure.^{221 and 225} Pharmacological treatments for patients with ALD are largely restricted to the setting of severe acute hepatitis, a life-threatening disease with high mortality.²¹⁹ However, it is not clear whether hepatocellular death, presumably triggered by ROS, TNF, and bacterial pathogen-associated molecular patterns, is a main contributor to acute hepatitis. Corticosteroids improve short-term survival in acute hepatitis in the majority of studies,¹⁵³ making this the first-line treatment in responders as defined by the Lille score.²²⁹ The prominent role of TNF in alcoholic steatohepatitis-dependent cell death in murine studies provided the basis for clinical trials assessing TNF inhibitors such as etanercept and pentoxifylline, an inhibitor of TNF synthesis. However, a recent large trial did not show a benefit of combined pentoxifylline and corticosteroids versus corticosteroids alone.¹⁵⁶

Moreover, etanercept increased mortality and infections in patients with acute hepatitis.²³⁰ Likewise, the effect of antioxidants such as NAC remains uncertain because the combination of NAC and corticosteroids increased 1-month but not 6-month survival in comparison to corticosteroids alone.²³¹

HCC

HCC represents the common end stage of chronic liver diseases, including viral, alcoholic, and nonalcoholic fatty liver disease. Hepatocarcinogenesis is multifactorial, and the relative contribution of cell death to the development of HCC depends on the underlying disease. For example, HCC can develop in patients with HBV infection or NAFLD in the absence of chronic liver injury and/or fibrosis,^{232, 233 and 234} suggesting that cell death-independent signals (eg, HBV-induced signals or altered metabolism) may be sufficient to trigger carcinogenesis in disease-specific contexts. Nonetheless, 80% of HCCs develop in fibrotic or cirrhotic livers, which in turn develop in the setting of chronic hepatocellular death. Moreover, aforementioned data from murine models with increased hepatocellular death^{64, 90, 152, 153 and 167} strongly support the notion that the presence of chronic cell death in the liver is sufficient to trigger development of HCC (Table 1). As such, numerous clinical studies have shown correlations between biomarkers of liver cell death such as ALT and the risk of cancer development. For example, HBV- and HCV-infected patients with persistent ALT levels >45 U/L have a 10-fold and 7-fold higher risk, respectively, of developing HCC than patients with persistently normal ALT hepatitis.^{59 and 60} Measurement of ALT levels is thus part of surveillance and treatment guidelines.²³⁵

Interference with pathways that modulate cell death in the liver appears to be a promising strategy for the prevention or treatment of HCC but is likely to require stage-, disease- and compartment-specific approaches. HCC prevention strategies need to inhibit cell death in early stages to stop the HCC-promoting cell death/inflammation/regeneration/fibrosis cascade. In addition to treating the underlying disease, this could possibly be achieved by DAMP inhibitors such as glycyrrhizin,²³⁶ interruption of the gut microbiome-liver axis by nonabsorbable antibiotics such as rifaximin,¹⁶⁴ or other modifiers of cell death, inflammation, or fibrosis.²³⁶

In contrast, treatment of established HCC requires promotion of cell death in cancerous tissue but not healthy liver. The only approved drug for treatment of HCC, sorafenib, affects HCC proliferation and angiogenesis but not cell death.²³⁷ Besides ablative strategies that induce HCC death with low selectivity, such as radiofrequency ablation and transarterial chemoembolization, more HCC-specific approaches are being developed. Oncolytic viruses can induce selective cell death in HCC, and a recent phase 2 trial showed dose-dependent improvement of survival by oncolytic and immunotherapeutic vaccinia virus JX-594.²³⁸ Studies testing combinations of JX-594 and sorafenib are ongoing (Table 3). Another study is targeting cell death in liver cancer using XIAP antisense in combination with sorafenib to lower the apoptosis threshold of cancer cells (Table 3).

VII. Conclusions

Cell death is at the center of virtually every acute and chronic liver disease. The discovery of novel modes of cell death such as necroptosis and specific pathways that regulate cell death and cell death responses has greatly improved our understanding of the pathophysiology of acute and chronic liver disease. Although revolutionary progress in HCV therapy allows prevention of many of the deadly complications of chronic liver disease in this specific cohort of patients, we cannot halt progression in most other relevant liver diseases. Despite new insights into key pathways through which cell death drives liver disease, we are still awaiting their clinical translation. The discovery of necroptosis as a key backup pathway to apoptosis provides a convincing explanation why caspase inhibitors have not achieved broad application in clinical hepatology. Hence, it may be necessary to simultaneously block multiple pathways or those that are considered more reactive or detrimental (ie, possibly necroptosis rather than apoptosis). In view of these dilemmas and the possibility that blocking one cell death pathway may lead to activation of another, future studies should focus on understanding the regulation of cell death response pathways in the liver. These pathways might hold important clues for tailored treatment of acute and chronic liver disease. Activation of beneficial cell death response pathways (eg, those that trigger hepatocyte proliferation) may be beneficial in acute liver disease, whereas blocking profibrogenic, proliferative, and proinflammatory cell death response pathways may inhibit the progression of chronic liver disease. Finally, novel biomarkers of cell death may allow more accurate predictions for outcome and treatment decisions than transaminases, on which we have relied for the past 6 decades.

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Figure 1.

Figure 2.

Figure 3.

Table 1

Evidence from animal model for cell death as a driver of liver disease

	Experimental evidence	Mode of cell death that promotes disease			References
		Apoptosis	Necroptosis	Necrosis	
Acetaminophen	Rip3 knockout protects from early liver injury		×		40
	CsA inhibits acetaminophen hepatocyte toxicity in vitro and liver injury in vivo			×	46,36
Hepatic I/R injury	Cyclophilin D knockout inhibits necrotic cell death in hepatocytes and cardiac I/R injury	×			33
Fibrosis	Spontaneous fibrosis in Mcl1 hepatocyte ko mice	×			54
	Bcl _{xl} hepatocyte knockout develops fibrosis	×			55
	Necrotic injury models (CCl ₄ , APAP) result in fibrosis			×	Common models in the literature
	Tak1- and Nemo-hepatocyte-specific ko mice develop spontaneous liver fibrosis	×			90, 152, 167
	Caspase inhibitor IDN-6556 inhibits fibrosis after bile duct ligation	×			144
NASH	Decreased inflammation and fibrosis in mice with ablation of RIP3 after MCD diet		×		146
	Caspase inhibitor VX-166 inhibits inflammation and fibrosis in the MCD model	×			145, 204
ALD	Reduced steatosis, injury and inflammation in Rip3-deficient mice		×		41
HCC	Spontaneous HCC in Mcl1 hepatocyte ko mice	×			56
	Spontaneous HCC in mice with Mcl1 or Bcl _{xl} hepatocyte ko, and inhibition of hepatocarcinogenesis by additional Bak ko	×			150
	Spontaneous HCC development in mice with hepatocyte-specific Nemo or Tak1 knockout	×			90, 152, 167
	Reduced HCC development by Caspase 8 ablation in Tak1 hepatocyte-specific ko mice, increase HCC development by Rip3 ko in Tak1 hepatocyte-specific ko mice	×			105

Table 2

Biomarkers of cell death and their prognostic value for acute and chronic liver disease

Biomarker	Cell type, cellular localization and function	Relevant diseases	Key findings	References
ALT	Almost hepatocyte-specific; cytoplasmic enzyme that catalyzes the transfer of amino groups	All liver diseases	Association with overall and liver-related mortality	13 – 15
		Hepatitis B	Association with progression to fibrosis, HCC and mortality	1 – 4
		Hepatitis C	Association with progression to fibrosis, HCC and mortality	5 – 8
		Autoimmune hepatitis	Abnormal ALT 6 months after diagnosis associated with mortality or need for transplant	12
AST and AST/ALT ratio	Highly expressed in hepatocytes but also in many other cell types; mitochondrial enzyme that catalyzes the transfer of amino groups	All liver diseases	Association with overall mortality	13
		NASH	Increased AST/ALT ratio associated with progression to fibrosis	9 – 11
K18	Highly expressed in epithelial cells, in the liver mainly in hepatocytes; intermediate filament; cleaved by caspases into 44 kd, 29kd and 23kd fragments; full length K18 indicates overall cell death, neoepitopes on fragments are indicative of apoptosis	NAFLD/NASH	Associated with presence of NASH in NAFLD patients; correlates with histological disease activity	173,174, 175
		Hepatitis C	Increased K18 levels can occur in patients with normal ALT, and increase risk for fibrosis in these	139
		Alcoholic liver disease	Association with presence of Mallory-Denk bodies, hepatocyte ballooning and liver fibrosis	176
		Acetaminophen intoxication	Increased K18 correlates with worse prognosis	178
HMGB1	Present in all cells, nuclear DNA binding protein that is released from necrotic cells and functions as inflammatory DAMP	Acetaminophen intoxication	Increased total and acetylated HMGB1 in patients, and correlated with worse prognosis (outperforming ALT)	178
miR-122	MicroRNA with liver specific expression, most abundant microRNA in the liver, expressed in hepatocytes; relevant functions in lipid metabolism and HCV infection	Acetaminophen intoxication	Increased miR-122 detects liver injury earlier than serum ALT and is more accurate in predicting liver injury	179, 180

Table 3

Clinical studies with cell death based interventions in liver disease

Treatment	Cell death Intervention	Study Design	Results	Year of completion	Phase	Patient number	NCT Identifier or reference
Acute liver failure							
N-acetylcysteine in acetaminophen-induced liver failure	Replenishment of hepatic GSH and reduction of oxidative stress	Non-randomized	Reduction of mortality, early treatment more effective than late treatment	1985		2540	187
Late N-acetylcysteine in acetaminophen-induced liver failure (10–36h after intoxication)	Replenishment of hepatic GSH and reduction of oxidative stress	Randomized	Improved survival, and less edema in patients receiving N-acetylcysteine	Not described.		50	189
N-acetylcysteine in acute liver failure (not acetaminophen induced)	Replenishment of hepatic GSH and reduction of oxidative stress	Randomized	Improved transplant-free survival	2009	3	173	NCT00004467 239
N-acetylcysteine in acute liver failure (not acetaminophen induced) in pediatric patients	Replenishment of hepatic GSH and reduction of oxidative stress	Randomized	No improvement in 1-year survival, 1-year transplant-free survival significantly lower than placebo (!)	2011	3	184	NCT00248625 240.
Emricasan (PF-03491390 / IDN-6556) in patients with acute on chronic liver failure	Caspase-Inhibition, anti-apoptotic	Randomized	pending	2014	2	60	NCT01937130
ALF-5755 in patients with severe acute hepatitis and early acute liver failure (not acetaminophen)	Prevents apoptosis and oxidative stress, promotes regeneration	Randomized	Not fully published	2013	2	60	NCT01318525
Viral Hepatitis							
Combined oral and i.v. antioxidants in chronic HCV patients after failure of interferon	Reduction of oxidative stress	Randomized	Decline in ALT levels	2007	2	100	241
Vitamin E for children with chronic Hepatitis B	Reduction of oxidative stress	Randomized	Non-significant trend to higher HBeAg-seroconversion	2008	2	76	242
Mitoquinone mesylate in patients with chronic HCV	Caspase-inhibition, mitochondria-targeted antioxidant, anti-apoptotic	Randomized	Reduction in ALT and AST levels	2010	2	30	NCT00433108 243
GS-9450 in patients with chronic HCV	Caspase-inhibition, anti-apoptotic	Randomized	Reduction in ALT levels	2010**	2	32	NCT00725803 244
Emricasan (PF-03491390 / IDN-6556) in chronic HCV	Caspase-Inhibition, anti-apoptotic	Randomized	Reduction in ALT and AST levels	2010	2	204	NCT00088140 245
Pentoxifylline added to PEG-Interferon and Ribavirin in chronic HCV	Inhibition of TNF, protection of hepatocytes	Randomized	Increased SVR rates	2013	2	72	246
Peginterferon Alfa-2b (SCH 054031) vs Glycyrrhizin in Interferon (IFN)-Treated Patients With Chronic Hepatitis C	Glycyrrhizin is a natural compound of licorice with HMGB1 inhibitory activity	Randomized	Significantly higher ALT reduction by glycyrrhizin compared to placebo after 12 weeks and improved necroinflammation and fibrosis after 52-weeks				NCT00686881 247
Alcoholic liver disease							
Pentoxifylline in severe alcoholic hepatitis	Inhibition of tumor necrosis factor (TNF)	Randomized	Improved 1-month-survival, reduced fraction of patients with hepatorenal syndrome	2000		101	248
Pentoxifylline vs. prednisolone in severe alcoholic hepatitis	Inhibition of TNF	Randomized	Improved 3-month-survival and lower 1-month-MELD-score	2009	2	68	249
Pentoxifylline plus Anakinra plus zink (vs.	Inhibition of TNF and Interleukin-receptor signaling	Randomized	pending	2015	2/3	130	NCT01809132

Treatment	Cell death Intervention	Study Design	Results	Year of completion	Phase	Patient number	NCT Identifier or reference
methylprednisolone) in alcoholic hepatitis							
Combined antioxidant treatment (alone or with corticosteroids)	Reduction of oxidative stress	Randomized	No improvement in 6-month-survival	2007	2	70	250
Eternacept in moderate to severe alcoholic hepatitis	TNF inhibition	Randomized	Increased mortality and increased infections in eternacept group	2007		48	230
N-acetylcysteine in severe alcoholic hepatitis	Replenishment of hepatic GSH and reduction of oxidative stress	Randomized	No benefits in survival and biological parameters	2010	3	52	NCT00962442 251
N-acetylcysteine plus (plus corticoids) in severe alcoholic hepatitis	Replenishment of hepatic GSH and reduction of oxidative stress (anti-inflammatory)	Randomized	Combination did not improve 6 months-survival compared to corticoids alone (primary endpoint), improved 1-months-survival	2011	3	174	NCT00863785 231
Emricasan (PF-03491390 / IDN-6556) in patients with alcoholic hepatitis and contraindications to steroids	Caspase-Inhibition, anti-apoptotic	Randomized	pending	2018	2	60	NCT01912404
Pentoxifylline and prednisolone versus prednisolone in severe alcoholic hepatitis		Randomized	No improvement in survival by the combination of pentoxifylline and prednisolone vs. prednisolone alone	2010	3	270	NCT01214226. 252
NAFLD							
Pentoxifylline in NASH	Inhibition of TNF	Randomized	Improved NAFLD activity score	2011	3	55	NCT00590161 253
Vitamin E (or Pioglitazone) in non-diabetic NASH patients (PIVENS)	Reduction of oxidative stress	Randomized	Histological improvement in NASH (not Pioglitazone), Reduction in AST/ALT, Reduced steatosis, no improvement in fibrosis score	2010	3	247	NCT00063622 219
Vitamin E (or Metformin) in children and adolescents with NAFLD (TONIC)	Reduction of oxidative stress	Randomized	No benefit of Vit. E and Metformin on ALT/AST; improved hepatocyte ballooning score, NAFLD activity score and proportion of NASH resolution with Vit. E	2011	3	173	218
Vitamin E in NASH: Effects of Dose reduction (200mg, 400mg, 800mg)	Reduction of oxidative stress	Randomized	pending	2018	2	90	NCT01792115
Obeticholic acid in patients with NASH and type II diabetes	FXR agonism	Randomized	Improved insulin sensitivity, ALT, GGT, and liver fibrosis markers in 20 mg OCA dose group; improved GGT in 50 mg OCA dose group	2009	2	64	220
Phyllanthus urinaria (Hepaguard®) in NASH	Reduction of oxidative stress	Randomized	No significant histological or serological improvement compared to placebo	2013	Not provided	60	NCT01210989 254
Anthocyanin in non-diabetic NAFLD patients	Reduction of oxidative stress	Randomized	pending	2014	0	Not indicated	NCT01940263
Metadoxin (and Metformin) in prediabetic NAFLD patients	Reduction in oxidative stress	Randomized	pending	2015	4	100	NCT02051842
GS-9450 in patients with NASH	Caspase-inhibition, anti-apoptotic	Randomized	Reduction in ALT levels	2012	2	124	NCT00740610 216.
Emricasan (PF-03491390 / IDN-6556) in NAFLD patients with elevated ALT	Caspase-Inhibition, anti-apoptotic	Randomized	pending	2014	2	40	NCT02077374
HCC							
AEG35156 in combination with Sorafenib in HCC	XIAP antisense to promote apoptosis of HCC cells	randomized	PFS 4.0 months (combination) vs. 2.4 months (Sorafenib alone) Higher benefit for patients needing dose reductions	2012	2	51	NCT00882869 255

Treatment	Cell death Intervention	Study Design	Results	Year of completion	Phase	Patient number	NCT Identifier or reference
Resminostat (4SC-201) alone or in combination with Sorafenib in 2 nd Line HCC Treatment	Histone -Deacetylase inhibition to induce apoptosis and growth arrest	Open label, two arms	Median OS 8.1 Months in patients treated with Resminostat and Sorafenib in 2 nd line	2013	2	45	NCT00943449 256
Panobinostat (LBH589) in combination with Sorafenib in HCC	Histone-Deacetylase inhibition to induce apoptosis	Non-randomized	pending	Not reported	1	18	NCT00823290
JX-594 in low or high dose	Oncolytic and immunotherapeutic vaccinia virus	Randomized	Median survival of 14.1 months compared to 6.7 months on the high and low dose	2013	2	30	238
JX-594 in combination with sorafenib	Oncolytic and immunotherapeutic vaccinia virus followed by treatment with multikinase	Non-randomized	Pending	2014	2	25	NCT01171651