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Apoptosis as a Mechanism for Liver Disease Progression

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Abstract

Hepatocyte injury is ubiquitous in clinical practice, and the mode of cell death associated with this injury is often apoptosis, especially by death receptors. Information from experimental systems demonstrates that hepatocyte apoptosis is sufficient to cause liver hepatic fibrogenesis. The mechanisms linking hepatocyte apoptosis to hepatic fibrosis remain incompletely understood, but likely relate to engulfment of apoptotic bodies by professional phagocytic cells and stellate cells, and release of mediators by cells undergoing apoptosis. Inhibition of apoptosis with caspase inhibitors has demonstrated beneficial effects in murine models of hepatic fibrosis. Recent studies implicating Toll-like receptor 9 (TLR9) in liver injury and fibrosis are also of particular interest. Engulfment of apoptotic bodies is one mechanism by which the TLR9 ligand (CpG DNA motifs) could be delivered to this intracellular receptor. These concepts suggest therapy focused on interrupting the cellular mechanisms linking apoptosis to fibrosis would be useful in human liver diseases.

Keywords

Bcl-2 proteins; caspase inhibitors; death receptors; stellate cells; Toll-like receptor 9

Liver injury is quite common in human disease. Indeed, biomarkers of hepatocyte injury such as alanine transaminase (ALT) values are universally present in human sera. What constitutes the normal range for the serum ALT, and therefore, a ‘healthy liver’ is an unresolved topic [1]. However, the mere presence of circulating ALT in man implies low-levels of hepatocyte injury. It is now widely appreciated that liver injury is accompanied by liver cell death, often by apoptosis. Indeed, hepatocyte apoptosis is ubiquitous in human liver diseases (Table 1)[2]. Given the remarkable regenerative capacity of the liver, loosing a few liver cells would appear to be inconsequential to health of the organ and organism. Unfortunately, prolonged hepatocellular injury results in an exuberant wound healing response, causing hepatic fibrosis, and, in its extreme form, liver cirrhosis. It is hepatic cirrhosis with its sequela of portal hypertension, end-stage liver disease and hepatocellular carcinoma that eventually compromises human life in most chronic liver diseases. One of the pressing unmet needs in clinical medicine is how to prevent, retard, or even reverse hepatic fibrosis. Therefore, important biomedical questions emanating from these observations are how does liver injury promote hepatic fibrosis, and is cell death by apoptosis a pro-fibrogenic process. This contribution to Seminars in Liver Diseases will address this issue. We will review current information linking apoptosis to fibrosis and highlight evolving mechanisms which merit further detailed study. This review will be an update since our past review on this subject [3]. Current information continues to imply a

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direct link between hepatocyte apoptosis and liver fibrosis, thereby suggesting that anti-apoptotic therapies should also be anti-fibrogenic.

Overview of Apoptosis

Apoptosis is a form of cell death characterized by membrane blebbing, shrinkage of the cell, chromatin condensation, and nuclear fragmentation [4]. Fragmentation of the cell into membrane defined bodies termed apoptotic bodies is a hallmark of apoptosis; in the liver, these apoptotic bodies were termed councilman bodies for decades until their true pathogenesis could be easily verified [5]. Apoptosis is mediated biochemically by activation of intracellular zymogens termed caspases, cysteine-dependent aspartate specific protease [6]. These zymogens are themselves activated by proteolytic cleavage at aspartate moieties, either within special protein complexes (initiator caspases) or by other active caspases (down stream or effector caspases). The initiator caspase 8 is activated by induced proximity and dimerization of the proform within a death receptor complex [7], whereas procaspase 9 is activated by recruitment to and assembly within a large protein complex termed the apoptosome [6]. The activating cleavage of effector caspases 3 and 7 by initiator caspases (i.e., caspase 8 or 9) generates a neoepitope which can be identified by immunohistochemical techniques in liver tissue specimens [8]. Likewise caspase cleavage of cytokeratin 18 also generates a neoepitope which is released into the serum and can be measured by enzyme-linked immunosorbent assay (ELISA, M30 assay) as an index of hepatocyte apoptosis [9]. Effector caspases are responsible for activating CAD (caspase-activated DNase) by cleaving ICAD (inhibitor of caspase-activated DNase)[10]. CAD activation results in DNA cleavage at internucleosomal linker regions causing the classic ladder pattern of DNA fragments (multiples of the 180 base pair nucleosomal regions). DNA cleavage during apoptosis is the basis for the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. Thus, apoptosis can be readily identified by its biochemical biomarkers, and this information can be used to explore the relationship between hepatocyte apoptosis and liver fibrosis.

Mechanisms of Hepatocyte Apoptosis

Apoptosis is a process required to maintain tissue homeostasis and health by counterbalancing cell proliferation and eliminating damaged and/or aged cells. This is particularly crucial in an organ like the liver which is naturally exposed to toxins and viruses [11]. Any alteration in the balance between proliferation and cell death, by either excessive or insufficient apoptosis, invariably leads to a pathologic condition. In the liver, massive hepatocyte apoptosis results in acute liver failure, whereas persistent hepatocyte apoptosis is often associated with fibrogenesis, chronic liver dysfunction, and even cancer development [12].

Apoptosis can be triggered by a variety of intra- and extra-cellular stimuli. The intracellular stimuli, such as DNA damage, generally result in mitochondrial outer membrane permeabilization (MOMP) and release of pro-apoptotic factors, including cytochrome *c*, second mitochondria-derived activator of caspases (SMAC)/direct IAP-binding protein with low PI (DIABLO), and apoptosis-inducing factor (AIF), from the intermembrane space into the cytosol. This signaling cascade, known as the mitochondrial (or intrinsic) pathway of apoptosis, is initiated by the activation of pro-apoptotic BH3-only (i.e., Bid, Bim, Bad, PUMA, Noxa) and multi-domain (Bax and Bak) members of the Bcl-2 family, which are responsible for the induction of MOMP [13], and antagonized by the anti-apoptotic members of the same family (Bcl-2, Bcl-x_L, Mcl-1) [14]. Released cytochrome *c* associates with apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome, a large multimeric

complex which recruits procaspase 9 and facilitates its autoactivation [6]. Caspase 9 then cleaves and activates caspase 3 and 7, which, in turn, proceed to degrade several cellular substrates, resulting in the morphological changes associated to apoptosis. At the same time, endogenous cellular inhibitors of apoptosis proteins (IAPs), normally inhibiting accidentally activated caspases, are neutralized by SMAC/DIABLO, which is released from the mitochondria together with cytochrome *c* [15].

The extracellular stimuli signal through the engagement of death receptors on the plasma membrane by their cognate ligands, and the formation of a large death-inducing signaling complex (DISC)[16]. This pathway is referred to as the extrinsic pathway of apoptosis. Four of these death receptors, Fas, tumor necrosis factor receptor 1 (TNF-R1) and death receptor 4 and 5 (DR4 and DR5, also know as TNF-related apoptosis-inducing ligand receptor 1 and 2, TRAIL-R1 and TRAIL-R2), as well as their ligands, Fas ligand (FasL), TNF-α and TRAIL, are abundantly expressed in the liver [17], and their signaling cascades have been extensively studied over the years. Despite some differences in adaptors and other proteins recruited to their respective DISC, one common event occurring after the stimulation of all death receptors is the recruitment of the adaptor Fas-associated protein with death domain (FADD) and procaspase 8, which results in its autoactivation [7]. Subsequently, caspase 8 can either directly cleave and activate caspase 3 and 7 (type I cells, such as lymphocytes) similarly to caspase 9, or can engage the mitochondrial pathway by cleaving the BH3-only protein Bid (type II cells, such as hepatocytes), whose truncated fragment translocates to the mitochondrial outer membrane causing MOMP [18,19]. Therefore, the intrinsic and extrinsic pathways are not mutually exclusive, with Bid mediating the crosstalk between the two pathways in type II cells.

Because of the ubiquitous expression of death receptors and ligands in liver cells, apoptosis in the liver is generally mediated by the extrinsic pathway. In particular, activation of Fas and TNF-R1 is associated with hepatocyte apoptosis in a wide variety of liver diseases, including viral hepatitis, fulminant hepatic failure, cholestatic liver disease, alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), Wilsons' disease and ischemia-reperfusion injury [20]. For example, during viral infection, the liver damage is only marginally caused by a cytopathic effect of the virus itself, but rather due to the infiltrating FasL-expressing cytotoxic T lymphocytes (CTL) which eliminate the infected hepatocytes by engaging Fas on the hepatocyte surface. CTL also induce hepatocyte apoptosis via the TNF-TNF-R1 system, and secretion of the cytotoxins perforin and granzyme [21]. In cholestasis, elevated intracellular concentrations of toxic bile salts result in increased Fas density on the plasma membrane and ligand-independent activation of the receptor [22,23], as demonstrated by the absence of liver injury in Fas-deficient mice, but not FasL-deficient mice, after bile duct ligation (a model of extrahepatic cholestasis). Toxic bile salts are also known to up-regulate DR5 expression, therefore increasing sensitivity to TRAIL-mediated apoptosis [24]. Elevated Fas and FasL are also features of alcoholic liver injury [25]. Moreover, alcohol promotes Kuppfer cells activation and TNF-α production, and increases the sensitivity of hepatocytes to TNF-α-mediated apoptosis [26]. Fas-mediated hepatocyte apoptosis is also increased in patients with NASH, and correlates with the progression of the disease from simple steatosis to steatohepatitis [27]. However, liver injury during NASH is not only due to activated death receptors. Free fatty acids (FFAs) accumulating in the liver as a consequence of insulin resistance also induce cell death, a process referred to as lipoapoptosis. In particular, excessive FFAs accumulate in the endoplasmic reticulum (ER), resulting in ER stress. Saturated FFAs induce Bim expression and phosphorylation [28], JNK-mediated PUMA expression and Bax activation [29,30], resulting in MOMP as well as lysosomal permeabilization [31]. Moreover, concentrations of FFAs too low to induce lipoapoptosis have

been shown to up-regulate DR5 expression and increase sensitivity of hepatocytes to TRAIL-mediated apoptosis [32].

Apoptosis and Liver Fibrosis

Several human studies have linked apoptosis to fibrosis. For example, the magnitude of apoptosis correlates with stage of fibrotic disease in non-alcoholic steatohepatitis (NASH)[27,33], and in fibrosis progression in recurrent hepatitis C (HCV) following liver transplantation[33]. Although these human studies are correlative, experimental models more mechanistically link hepatocyte apoptosis to fibrosis. Mice deficient in Fas display reduced hepatic fibrosis following bile duct ligation [34]. Reduction of hepatocyte apoptosis with a caspase inhibitor also repressed hepatic fibrosis in this rodent model of cholestatic liver injury [8]. Although these observations could be explained by the potential pro-inflammatory effects of Fas-mediated liver injury, which require caspases [35,36], other data suggest hepatocyte apoptosis alone is sufficient to elicit a pro-fibrogenic response in the liver. The anti-apoptotic members of the Bcl-2 family of proteins are the guardians of the mitochondrial pathway of cell death [37]. The hepatocyte depends upon two such proteins for survival, Bcl-x_L and Mcl-1 [38]. Hepatocyte-specific deletion of either protein results in hepatocyte apoptosis, elevation of the serum ALT values and hepatic fibrosis [39,40]. These data are quite remarkable because they appear to be pure models of hepatocyte apoptosis yet they recapitulate many facets of human liver disease. Moreover, these findings also suggest the hepatocyte is constitutively undergoing pro-apoptotic stress, even in the absence of disease, an observation consistent with the presence of ALT in the serum of even healthy human subjects. Thus, the question is not if apoptosis contribute to progressive liver injury, but how? There are two broad mechanisms by which apoptotic hepatocytes beget hepatocyte fibrosis: 1) engulfment of apoptotic bodies is pro-fibrogenic; 2) apoptotic cells release pro-fibrogenic mediators. These concepts are not mutually exclusive and likely both are operational in human liver disease.

Apoptotic Bodies and Their Engulfment

Apoptotic bodies are eliminated from the body by cellular engulfment [41]. If not eliminated, the cellular membrane defining the apoptotic body becomes permeant, releasing cellular constituents into the extracellular space eliciting an inflammatory response, a phenomenon termed secondary necrosis [41]. In massive liver injury, the ability of phagocytic cells to identify and clean up all the apoptotic bodies is likely overwhelmed, and this process could explain liver failure and inflammation despite an initial apoptotic stimulus, such as liver injury by Fas stimulation [42]. In order to undergo engulfment, the apoptotic cell must generate two cells signals, a “find me” signal and an “eat me” signal. One recognized “eat me” signal is the translocation of phosphatidylserine from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet, which results in engulfment of the apoptotic bodies by phagocytic cells [10]. In the liver, both hepatic stellate cells (HSC), the pericytes of the sinusoids, and Kupffer cells, the resident macrophages of the liver, can phagocytose apoptotic bodies (Fig. 1) [43-45].

Apoptotic Bodies and The “Find Me” Signal

Recent information suggests that apoptotic cells release the nucleotides ATP and UTP into the extracellular space [46]. These chemical mediators have been termed the scent of death [47]. ATP and UTP bind to purinergic receptors on macrophages and HSC, especially the $P2Y_2$ receptor [46]. The binding to macrophages serve as a chemoattractant recruiting these professional phagocytic cells to the site of the dying cell. As $P2Y_2$ receptors are also present on HSC [48], release of nucleotides by apoptotic hepatocytes is likely an additional mechanism by which apoptosis promotes hepatic fibrosis. Indeed, ATP and UTP released

from apoptotic cells may function to recruit HSC to sites of liver injury and promote their activation to myofibroblasts (Fig. 1). Other chemoattractants released from apoptotic cells include fractalkine, adenosine and the lipid lysophosphatidylcholine[41,49]. Both compounds also serve as ligands for G-protein coupled receptors and also likely contribute to HSC activation in liver injury. Although a role for lysophosphatidyl choline has not been explored in liver fibrosis, fractalkine has been linked to hepatic inflammation and fibrosis and merits further attention as a chemokine promoting liver fibrosis by apoptotic cells [50,51].

Apoptotic Bodies and Liver Indigestion

Apoptosis in development is a non-inflammatory process responsible for removing excess cells in a spatial-temporal sequence. In contrast, apoptosis in pathologic conditions is not controlled and can be deleterious to the organ and the entire organism [52]. Apoptotic bodies and their engulfment are a major source of this hepatic “indigestion”. For example, hepatocyte inflammation is a major driving force for hepatic fibrogenesis [53]. Apoptosis contributes to inflammation by promoting Kupffer cell activation. Following engulfment of apoptotic bodies, Kupffer cells express the death ligands TNF-α, TRAIL and FasL [43,54] (Fig. 1) which may exert a pro-inflammatory effect in this context [55]. All these death ligands have cognate receptors on hepatocytes and may induce death receptor-mediated apoptosis further aggravating liver injury. Thus, engulfment of apoptotic bodies by Kupffer cells is likely an important pro-fibrogenic mechanism in liver disease.

In the liver, as in other organs, activated myofibroblasts generate collagen and are responsible for organ scarring. Myofibroblasts are derived from portal fibroblasts and hepatic stellate cells [56,57]. Whatever their lineage, activated myofibroblasts are phagocytic and capable of engulfing apoptotic bodies [44,45,58,59]. When activated myofibroblast cell lines engulf apoptotic bodies derived from hepatocytes, they produce profibrogenic cytokines (such as TGF-β) and type I collagen [44]. The engulfment of apoptotic bodies also results in up-regulation of NADPH oxidase, an enzyme which generates oxygen free radicals [59]. These data help link apoptosis to oxidative stress, which is frequently implicated as a mechanism contributing to hepatic injury.

If engulfment of apoptotic bodies is pro-fibrogenic, what in the apoptotic bodies is responsible for this phenomenon? Apoptotic bodies contain micronuclei, a source of DNA. Unmethylated cytosine-phosphate guanosine (CpG)-DNA motifs are recognized by Toll-like receptor 9 (TLR 9) (Fig. 2). Indeed, denatured DNA from apoptotic cells is a ligand for TLR 9 [60,61] and TLR 9-deficient mice display reduced hepatic injury and fibrosis following liver injury by carbon tetrachloride, bile duct ligation, acetaminophen, and a model of fatty liver disease [60-62]. The engulfment of the apoptotic DNA would permit access of the CpG-DNA ligand to the intracellular TLR9 where it would, in turn, activate a variety of signaling cascades resulting in myofibroblast generation of collagen 1 and the pro-fibrogenic cytokine TGF-β [63] (Fig. 2). Although TLR9 is thought to be restricted to dendritic cells in humans, Watanabe et al. demonstrated that, in human stellate cells, CpG oligonucleotides induced up-regulation of TGF-β and collagen 1 mRNA, and that these effects were blocked by TLR9 antagonists [60]. These very important data link apoptosis to activation of the innate immune response within myofibroblasts.

Apoptosis and Anti-Fibrogenic Strategies for Human Liver Disease

The most direct therapeutic strategy for repressing liver fibrosis is to eliminate the cause for the liver injury. This has proven clinically possible for viral hepatitis where direct and indirect anti-viral therapies are quite effective. However, effective treatments do not exist for many liver diseases such as primary sclerosing cholangitis, NASH, alcohol-mediated hepatitis, and patients with HCV or HBV unresponsive to antiviral therapies. For such

patients, anti-apoptotic and anti-fibrogenic strategies have a hepatoprotective role. Two therapeutic strategies emanating from this review include caspase inhibitors and TLR9 receptor antagonists (Fig. 3).

Small molecule caspase inhibitors are attractive for the treatment of human liver diseases. Indeed, caspase inhibitors attenuate hepatic injury and fibrosis in pre-clinical models of cholestasis and non-alcoholic liver disease [8,64]. More importantly, in a human trial a pan-caspase inhibitor reduced serum ALT values in patients with HCV without affecting viral replication [65,66]. This agent also attenuates liver ischemia/reperfusion injury in rodents and man during organ transplantation [67,68]. Although there is a concern that apoptosis inhibition by caspase inhibitors would promote carcinogenesis, we view this as unlikely. There are two major apoptotic pathways, an extrinsic or death receptor-mediated pathway and an intrinsic or mitochondrial pathway, as described above. Although the mitochondrial pathway is characterized by caspase 9 activation, caspase 9 deletion does not prevent cell death [69]. Indeed, once a sufficient number of mitochondria have displayed MOMP, cell death is caspase-independent [70]. Since most oncogenic processes elicit a DNA damage response which engages the mitochondrial pathway of cell death [71], cell death and elimination of the potentially oncogenic cell should ensue whether or not caspases are inhibited. In contrast, the extrinsic pathway is truly dependent on caspase 8 and, perhaps, caspase 10 [72]. For example, the genetic absence of caspase 8 (mice do not express caspase 10) attenuates liver injury in experimental murine models mediated by death ligands [72,73]. Thus, caspase inhibition should only reliably inhibit death receptor-mediated apoptosis, a common mechanism of cell death in human liver diseases [17,74]. As knockout murine models of various death receptors do not develop spontaneous cancers, caspase inhibitors should prove to be safe in man. However, more animal data in this regard would be reassuring.

There is considerable interest in developing short oligonucleotides rich in guanine residues as inhibitors of TLR9 antagonists as immunomodulatory agents in immune-mediated diseases such as systemic lupus erythematosis [75,76]. Many of these compounds are salutary in murine models of this disease. They have yet to be applied broadly to animal models of fibrosis, and we await these studies with anticipation.

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Abbreviations Used

AIF	Apoptosis-inducing factor
ALT	Alanine transaminase
ATP	Adenine triphosphate
Bcl-2	B-cell lymphoma-2
Bcl-x_L	B-cell lymphoma-2 like protein
CAD	Caspase-activated DNase
Caspase	Cysteine-dependent aspartate specific protease
CpG	Cytosine-phosphate guanosine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
HBV	Hepatitis B
HCV	Hepatitis C
ICAD	Inhibitor of caspase-activated DNase
Mcl-1	Myeloid cell leukemia-1
NASH	Non-alcoholic steatohepatitis
$P2Y_2$	Purinergic 2 Y_2
SMAC/DIABLO	Second mitochondria-derived activator of caspases/Direct IAP-binding protein with low PI (DIABLO)
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTP	Uridine triphosphate

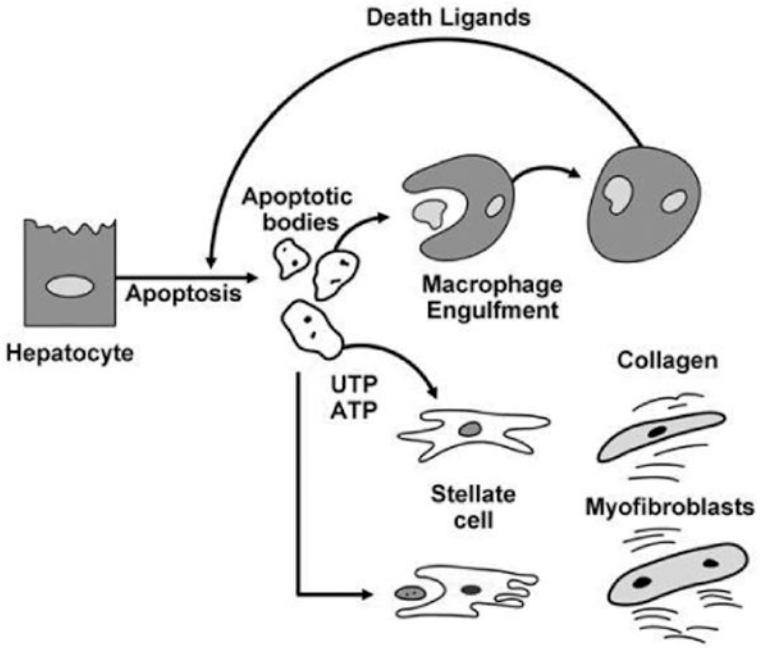

Figure 1. Cellular mechanisms linking hepatocyte apoptosis to liver fibrogenesis

Engulfment of apoptotic bodies by Kupffer cells enhances their expression of death ligands which in a feed-forward loop further promote hepatocyte apoptosis and generation of apoptotic bodies. Stellate cell or myofibroblast engulfment of apoptotic bodies enhances their expression of collagen type 1 and TGFβ1, promoting development of fibrosis and cirrhosis. Apoptotic cells also release the nucleotides UTP and ATP, which bind $P2Y_2$ purinergic receptors on myofibroblasts further promoting collagen generation.

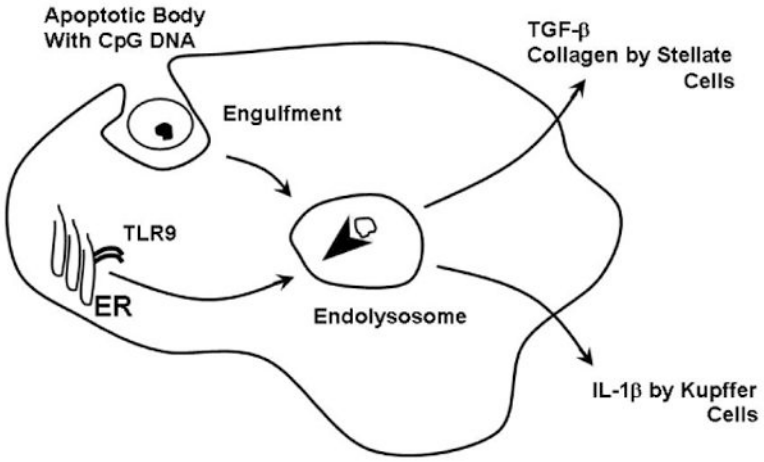

Figure 2. Activation of TLR9 by apoptotic body engulfment

The apoptotic bodies contain micronuclei with CpG DNA motifs. Following engulfment, the apoptotic bodies are translocated into an endolysosome. This process triggers TLR9 trafficking from the endoplasmic reticulum (ER) to the endolysosome. The lower pH of the endolysosome promotes TLR9 activation. The active receptor complex in stellate cells results in transforming growth factor-beta (TGF-β) and collagen I production,[60] whereas in Kupffer cells it promotes interleukin-1β, a proinflammatory cytokine, generation [98]

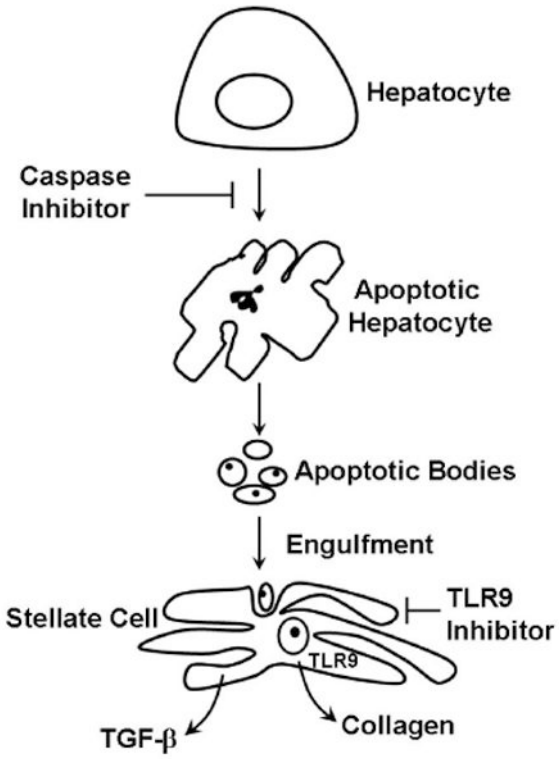

Figure 3. Therapeutic strategies to minimize liver disease progression

Apoptosis can be linked to hepatic fibrosis through engulfment of apoptotic bodies with subsequent activation of TLR9 in stellate cells. Based on these concepts, inhibition of death receptor-mediated apoptosis by caspase inhibitors should attenuate hepatic fibrosis. Likewise, TLR9 inhibitors should prevent stellate cell activation despite engulfment of apoptotic bodies thereby also reducing hepatic fibrogenesis.

Table I

Liver Cell Apoptosis in Human Liver Diseases

Liver Disease	References
Chronic Viral Hepatitis	77-82
Acute Viral Hepatitis	83
Acute/Fulminant Hepatic Failure	84, 85
Alcohol-related Liver Disease	25,86,87
Non-alcoholic Steatohepatitis	27,87
Autoimmune Hepatitis	88
Primary Biliary Cirrhosis	89
Drug-induced Liver Injury	
HCV-related Fibrosis	81
Wilson's Disease	90
Acute Allograft Rejection	91
Hepatocarcinoma	92, 93-97