Defying death: the hepatocyte’s survival kit

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ABSTRACT

Acute liver injury can develop as a consequence of viral hepatitis, drug- or toxin-induced toxicity or rejection after liver transplantation, whereas chronic liver injury can be due to long-term exposure to alcohol, chemicals, chronic viral hepatitis, metabolic or cholestatic disorders. During liver injury, liver cells are exposed to increased levels of cytokines, bile acids and oxidative stress. This results in death of hepatocytes. In contrast, stellate cells become active and are resistant against cell death. Eventually, acute and chronic liver injury is followed by loss of liver function for which no effective therapies are available. Hepatocytes are well equipped with protective mechanisms to prevent cell death. As long as these protective mechanisms can be activated, the balance will be in favour of cell survival. However, the balance between cell survival and cell death is delicate and can be easily tipped towards cell death during liver injury. Therefore understanding the cellular mechanisms controlling death of liver cells is of clinical and scientific importance and can lead to the identification of novel intervention targets. This review describes some of the mechanisms that determine the balance between cell death and cell survival during liver diseases.

ACUTE AND CHRONIC LIVER INJURY

The liver is exposed to many potential harmful agents that, in a normal situation, do not damage the liver cells due to protective mechanisms and a large repair capacity of these cells. However, acute or chronic exposure to insults such as cytokines, ROS (reactive oxygen species) and bile acids results in disturbed liver function.

Acute liver failure develops in a short period of time as a consequence of viral hepatitis, drug-induced (e.g. paracetamol overdose) or toxin-induced (e.g. mushroom-derived Amanitin) toxicity, or due to rejection after
Figure 1  Schematic representation of events during acute and chronic liver injury that lead to cell death-induced loss of hepatocytes, proliferation of stellate cells and liver fibrosis

Cell death can be divided into two different processes: necrosis and apoptosis. However, features characteristic of both necrotic and apoptotic cell death can occur in the same tissue and even in the same cell simultaneously [4]. Necrosis results from metabolic disruption with energy depletion (loss of ATP), mitochondrial and cellular swelling and activation of degradative enzymes. This leads to cell lysis, followed by loss of cell constituents into its surroundings. Therefore necrosis is accompanied by inflammation. In contrast, apoptotic cell death is ATP-dependent and develops more orderly (programmed cell death) following a cascade of events. Apoptosis is characterized by DNA condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage. Eventually, the apoptotic cell breaks into small membrane-surrounded fragments (apoptotic bodies) that are cleared by surrounding cells [5,6]. All these events are tightly controlled and well organized.

Both apoptosis and necrosis almost always occur together in intact organisms; however, the relative contribution of the different modes of cell death may vary [4]. Apoptotic cell death has been reported in liver diseases, in particular acute liver injury [7,8], but also in chronic liver diseases, although the true significance of apoptotic cell death in chronic liver diseases remains to be established [9–11].

DEATH-RECEPTOR-MEDIATED APOPTOSIS

Apoptotic cell death can be initiated by activation of death receptors on the cell membrane that belong to the TNF/NGF (nerve growth factor) receptor superfamily. Hepatocytes express Fas (CD95), TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1), TRAIL-R2 (TRAIL-receptor 2) and TNF-R1 (TNF-receptor type 1) [12]. Death receptors are type 1 transmembrane proteins with an extracellular ligand-binding N-terminal region, a membrane spanning region and a C-terminal intracellular tail. The extracellular region contains cysteine-rich domains, whereas the intracellular region contains the death domain essential for signalling apoptosis.

Unlike Fas or TRAIL-R1 and -R2 signalling, TNF-R1-mediated intracellular signalling is more complex as it activates both apoptotic and survival signals. Since TNF-α is an important cytokine in liver injury, understanding of the TNF signalling pathways in hepatocytes is important in developing new therapeutic strategies for liver diseases.

TNF-R1-mediated signalling

In the liver, inflammatory cells, cholangiocytes and Kupffer cells are the main sources of TNF-α [13,14]. Upon activation by TNF-α, trimerization of TNF-R1 is followed by recruitment of the adaptor protein TRADD (TNF-R-associated protein with death domain), as
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Figure 2  Schematic overview of receptor-mediated and mitochondria-mediated signal transduction pathways

TNF-R1-mediated intracellular signalling activates both apoptotic and survival signals. See text for details.

shown in Figure 2. TRADD recruits signalling proteins such as FADD (Fas-associated death domain), TRAF-2 (TNF-R-associated factor-2) and RIP (receptor-interacting protein). FADD contains a death effector domain, which mediates the recruitment of cysteine aspartyl-specific proteases (caspases), such as caspase 8 and caspase 10 that activate a death signalling cascade (Figure 2). Binding of RIP to TRAF-2 initiates the activation of survival pathways like NF-κB (nuclear factor κB) and MAPKs (mitogen-activated protein kinases) [15].

Caspase 8 contains its prodomain a death effector domain that binds FADD. Local clustering of the inactive pro-form of caspase 8 to the TNF-α-activated TNF-R1 results in a DISC (death-inducing signalling complex) and auto-activation of caspase 8. In fact, internalization of TNF-Rs may be required for this process [16].

The active form of a caspase is a heterotetrameric enzyme and consists of two large and two small subunits with two active sites per molecule. Caspases cleave their substrates at aspartic acid residues in the context of tetrapeptide motifs. Active caspase 8 is involved in the cleavage and activation of effector caspase 3 (Figure 2). Caspase 3 is regarded as one of the central executioner molecules and is responsible for cleaving various proteins thereby disabling important cellular structural and repair processes. The apoptotic proteolytic cascade eventually results in the cleavage of nuclear lamins, actins, cyto-keratins, DNA repair proteins, RNA splicing proteins, protein kinases, PARP (poly (ADP-ribose) polymerase) and ICAD (inhibitor of caspase-activated deoxyribonuclease), allowing specific DNA degradation [17].

MITOCHONDRIA-MEDIATED APOPTOSIS

Dependent on the cell type and stimulus, the mitochondria play either a minor or crucial role in controlling apoptotic cell death. Hepatocytes are type II cells in which only a small amount of active caspase 8 is formed at the DISC. Therefore a mitochondrial amplification loop is essential to induce apoptotic cell death in hepatocytes [18]. Thus mitochondria play an essential role in regulating hepatocyte cell death [4,19,20].

Since the mechanisms of mitochondria-mediated apoptosis may differ between different types of apoptotic stimuli, knowledge of mitochondria-controlled apoptosis is needed to identify suitable intervention targets for acute and chronic liver diseases.

In normal conditions, the inner membrane of the mitochondria contains the protein complexes of the respiratory chain and ATP synthase [21]. In response to an apoptotic stimulus, the permeability of the mitochondrial membrane is disrupted, which is called the MPT (mitochondrial permeability transition). The MPT is characterized by rapid permeability of the mitochondrial membrane and release of apoptotic factors from the intermembrane space into the cytosol (Figure 3). Furthermore, there is a rapid decrease in the mitochondrial
membrane potential due to disruption of the electron transport chain [22]. Consequently, ATP production is abolished and electrons escape from the respiratory chain to form ROS [23]. Another consequence of the MPT can be swelling of the matrix and rupture of the outer membrane, allowing release of apoptotic proteins from the mitochondria.

**Oxidative stress and mitochondria**

Oxidative stress is the inappropriate exposure to ROS such as superoxide anions, hydroxyl radicals and H$_2$O$_2$. During acute and chronic liver injury, ROS are generated in increased amounts in hepatocytes, but also derive from Kupffer cells and inflammatory cells such as neutrophils [24–26]. The main sources of ROS in hepatocytes are the mitochondria and cytochrome P450 enzymes.

In normal conditions, the electron transfer in the respiratory chain in the inner membrane of mitochondria mediates the generation of minor quantities of superoxide anions. Superoxide anions are very reactive radicals and can form H$_2$O$_2$ or, in the presence of NO (nitric oxide), peroxynitrite. Normally, these toxic derivatives are scavenged through protective enzymes, such as SOD (superoxide dismutase), catalase and peroxidase, and antioxidants, such as glutathione. Nonetheless, the production of ROS in mitochondria can be enhanced by a variety of stimuli (e.g. TNF-α, bile acids and toxic compounds) due to inhibition of the respiratory chain. This results in the accumulation of reduced intermediates, which auto-oxidize [27]. Moreover, cytochrome P450 enzymes generate high levels of ROS during catalysis of substrates, such as alcohol and (therapeutic) drugs including paracetamol.

If the detoxification mechanisms are impaired, oxidative stress will occur. As a consequence, the MPT can be induced leading to more ROS generation and dysfunction of mitochondria. Furthermore, lipid peroxidation (formation of cytotoxic 4-hydroxynonenal), alterations in cell signalling and gene expression and DNA damage occur, leading to cell death.

Thus, in mild-to-moderate oxidative stress, apoptotic cell death may be induced, whereas severe oxidative stress leads to necrosis. The decision between necrotic and apoptotic cell death may depend on the cellular concentration of ATP. Therefore the MPT mediates both necrosis and apoptosis. When the bulk of mitochondria undergo MPT, a marked depletion of ATP will develop leading to necrotic cell death. When the MPT occurs without severe ATP depletion, apoptosis develops, which may be followed by secondary necrosis when ATP is eventually depleted during apoptosis [28].

**Mitochondria-mediated release of pro-apoptotic proteins**

The MPT followed by the release of mitochondrial proteins is initiated by the opening of pores in the mitochondrial membrane. Different models have been developed to explain pore-mediated release of proteins [23,29,30]. Both direct pore formation by some pro-apoptotic Bcl-2 family members and opening of the PTP

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**Figure 3** Schematic summary of mitochondria-controlled apoptosis

See text for details. Arrows represent induction, whereas inhibition is represented as a dot-arrow.
(permeability transition pore) complex releases apoptotic proteins from the mitochondria. Depending on the apoptotic stimulus either pathway may predominate, [31,32] (Figure 3) but, because active effector caspases can also induce opening of the PTP complex, feedback loops may exist. The importance of Bcl-2 family members in regulating mitochondria-mediated apoptosis is demonstrated using knockout animals. The Bcl-2 family consists of pro- and anti-apoptotic members that can interact through homo- and hetero-dimerization and regulate mitochondrial-mediated apoptosis (Figure 3) [33,34]. Bax and Bak double knockout cells are completely resistant to mitochondrial cytochrome c release during apoptosis [35]. Moreover, Bid-deficient hepatocytes are more resistant to TNF-α-induced and Fas-mediated apoptosis compared with wild-type mice hepatocytes [36].

Anti-apoptotic members such as Bcl-2, Bcl-XL and A1/Bfl-1 and pro-apoptotic Bak are integral membrane proteins that are predominantly present in the outer mitochondrial membrane. In contrast, pro-apoptotic members such as Bax, Bid and Bad are sequestered in the cytosol prior to a death signal [37]. Upon an apoptotic stimulus, caspase 8 cleaves Bid into tBid (truncated Bid), whereas Bax oligomerizes and Bad is released from the adaptor molecule 14-13-3 by dephosphorylation. These events result in the translocation and insertion of tBid, Bax and Bad in the outer mitochondrial membrane (Figure 3) [38–40].

In the mitochondrial membrane, Bad interacts with and antagonizes anti-apoptotic Bcl-2 and/or Bcl-XL [41], whereas tBid, Bax and Bak are able to form tetrameric outer membrane channels through which cytochrome c can escape. Bax and Bak need the interaction with tBid to form pores in the mitochondrial membrane [21]. The anti-apoptotic Bcl-2 family members inhibit apoptosis by binding Bax and Bak, sequestering tBid and Apaf-1 (apoptotic protease-activating factor 1), and preventing mitochondrial release of cytochrome c and Smac (second mitochondria-derived activator of caspase)/DIABLO [direct IAP (inhibitor of apoptosis protein) binding protein with low pI] (Figure 3) [42–45].

Mitochondrial death factors

Factors released from the mitochondria are crucial for the activation of pro-apoptotic signalling. Cytochrome c, an electron shuttle molecule, is released from mitochondria during apoptosis. It complexes with Apaf-1, dATP and cytosolic pro-caspase 9 to form a caspase-activating complex called the apoptosome (Figure 3). Cytochrome c and dATP induce refolding of Apaf-1, which allows interaction with pro-caspase 9. In this way, pro-caspase 9 is activated and subsequently cleaves and activates caspase 3 [46].

Depending on cell type and stimulus, caspase 3 can be involved in the activation of pro-caspases 8, 6 and 9 and Bid, resulting in feedback amplification of the apoptotic signal (Figure 3) [47,48]. It is assumed that (pro-)caspases are localized in the cytosol; however, caspase 2 has been detected in the Golgi complex and caspase 12 is situated in the endoplasmic reticulum and activated by endoplasmic reticulum stress [49,50]. In the mitochondria, the localization of caspases is controversial. Liver mitochondria of mice, human and rat are reported to contain pre-processed caspase 9 and caspase 3 [51,52], which is in contrast with observations describing the absence of caspase activity in mitochondria of mice hepatocytes exposed to anti-Fas [53].

Other mitochondrial apoptogenic factors that are released from the intermembrane space include Smac/DIABLO and Omi/HtrA2 (Figure 3). These proteins require proteolytic processing in the mitochondria to become active [54,55]. The release of Smac/DIABLO requires active caspases, occurs downstream of cytochrome c translocation and can be controlled by Bax [56,57]. Smac/DIABLO acts as a dimer and sequesters members of the IAP family such as XIAP (X chromosome-linked IAP), cIAP1 and cIAP2 (Figure 3). This results in the release of active caspases and the propagation of caspase cascades [58,59]. Omi/HtrA2 exerts a similar function, although it also contributes to caspase-independent apoptosis due to its N-terminal serine protease catalytic domain [60].

In summary, the balance between pro-apoptotic and anti-apoptotic pathways determines the outcome of cell death in response to an apoptotic stimulus. As long as protective proteins are present in sufficient amounts, the balance will be in favour of cell survival.

CELL SURVIVAL MECHANISMS IN LIVER DISEASES

Common protective mechanisms include antioxidants such as glutathione, which is present in very high amounts in hepatocytes [61,62]. In addition, bile acid transporters undergo adaptive responses during cholestatic disorders in order to protect hepatocytes against elevated intracellular levels of apoptotic bile acids [63,64].

Another defence mechanism in the liver is preservation of its functional capacity via precise regulation of its growth and mass. When the functional capacity of the liver becomes too small, due to surgical resection of hepatic lobes or hepatocyte loss caused by toxic injury, quiescent hepatocytes become proliferative. In situations in which hepatocyte proliferation is blocked or delayed, intra-hepatic precursor cells (oval cells) are responsible for this process [65,66].

Among the cytokines, TNF-α and IL-1β are present abundantly during acute and chronic liver failure [2,3,67]. Since these inflammatory cytokines also activate survival signalling pathways, such as NF-κB, their presence can be beneficial.
NF-κB signalling

NF-κB is a major regulator of the balance between cell survival and cell death in acute and chronic liver injury. Studies on mice that are deficient in subunits of the transcription factor NF-κB demonstrated the essential role of NF-κB in preventing TNF-α-induced cell death [68,69]. Recently, it has been postulated that NF-κB inhibits TNF-α-induced accumulation of ROS that normally mediate prolonged JNK (c-Jun N-terminal kinase) activation and cell death [70]. Inhibition of NF-κB activity induces apoptosis in hepatocytes, indicating its role in the transcription of anti-apoptotic genes [71]. Advances in the identification of these genes may provide opportunities for the development of novel therapies that shift the balance towards cell survival during acute and chronic liver injury.

NF-κB is activated by inflammatory cytokines, including TNF-α and IL-1β, and endotoxin (LPS (lipopolysaccharide)), as shown in Figure 2 [72]. Other NF-κB-activating signals include PKC (protein kinase C) and PI3K (phosphoinositide 3-kinase). Evidence that ROS mediate NF-κB activation is controversial [73,74].

Although the active DNA-binding form of NF-κB exists as a heterogeneous collection of dimers, in most cells NF-κB is predominantly composed of a p65/p50 heterodimer [75]. NIK (NF-κB-inducing kinase), a member of the MAPK family, serves as a common mediator in the NF-κB signalling cascade (Figure 2). Activation of NIK often occurs after its binding to receptor-recruited TRAF-2 (TNF-α) or TRAF-6 (IL-1β). As a consequence, an IkB (inhibitor of NF-κB) kinase (IKK) complex is activated consisting of IKK-α, IKK-β and IKK-γ [76,77]. This complex is involved in the phosphorylation and thereby inactivation of IkB. Of these inhibitors, IkB-α, IkB-β and IkB-γ are the most abundant isoforms.

Upon phosphorylation at specific serine residues, IkBs are ubiquitinated and degraded via the 26 S proteasome complex, thereby allowing release of NF-κB (Figure 2). In contrast with IkB-β and IkB-γ, the degradation of IkB-α is very rapid, but IkB-α is quickly resynthesized in an NF-κB-dependent manner. In this way, cells can react adequately and regulate downstream genes differentially in response to different stimuli [78,79].

The release of NF-κB from its inhibitor exposes a nuclear localization signal sequence and permits translocation of NF-κB to the nucleus (Figure 2). In the nucleus, NF-κB binds to κB-binding sites in promoters of target genes and induces transcription of these genes. NF-κB-regulated genes include inflammation-related genes such as TNF-α, IL-1β, IL-6 (interleukin-6), ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), as well as apoptosis-related genes such as iNOS (inducible NO synthase), COX-2 (cyclo-oxygenase-2) and IAP family members [76,80].

NF-κB-inducible anti-apoptotic genes expressed in hepatocytes are prime candidates for novel therapies in liver diseases [81]. These genes include members of the Bcl-2 family, such as A1/Bfl, and the IAP family, such as cIAP2. These families contain important anti-apoptotic proteins that are strong inhibitors of cell death in different cell types (Figures 2 and 3) [33,82].

The IAP family consists of different members such as XIAP, cIAP1 and cIAP2. These members not only inhibit active caspases 3, 7 and (pro- and active) 9 (Figure 3), but are also involved in signal transduction and protein degradation [82,83]. The IAP family members contain different protein domains for their anti-apoptotic activities, such as BIRs (baculovirus IAP repeats) and a Ring finger domain. This Ring domain is involved in protein degradation, such as IκB degradation [84]. In addition, IAPs associate with TRAF proteins, implying that IAPs participate in the stimulation of NF-κB [84,85].

MAPK signalling

Besides NF-κB, other signalling pathways antagonize cell death in hepatocytes, thereby influencing the balance between pro- and anti-apoptotic signals. These survival pathways belong to the MAPK signalling cascades and PI3K.

Four distinctly regulated groups of MAPKs are present: ERK1/2 (extracellular signal-related kinases 1/2), JNK, p38 MAPK proteins and ERK5 (Figure 2). MAPK activity is regulated through a MAPKK (MAPK kinase), which is MEK1/2 (MAPK/ERK kinase 1/2) for ERK1/2 and MKK3/6 (MAPK kinase 3/6) for p38 MAPK, and a MAPKKK (MAPK kinase) (Figure 2). Each MAPKK can be activated by more than one MAPKKK [such as Ask-1 (apoptosis signal-regulating kinase-1) and MEKK1 (MEK kinase 1)], which reacts upon distinct stimuli [86]. Activation of TNF-R1 results in activation of ERK1 and p38 MAPKs through TRAF-2 sequestering (Figure 2). In addition, EGF-R (epidermal growth factor receptor) is involved in ERK1/2 activation [87,88].

The general function of MAPK cascades is the regulation of gene expression [89,90]. In this way, MAPKs regulate cell proliferation and cell survival, but also mediate cell death. However, the actual roles of each MAPK cascade are cell-type and context-dependent.

PI3K signalling

The PI3K family is a superfamily of three different classes of enzymes and is linked to different cellular functions, including cell survival (Figure 2). This family is important in controlling cell survival in hepatocytes. Class I enzymes have largely been characterized and further subdivided into two groups of PI3K, class IA and IB. The catalytic subunits (p110) of class IA interacts with adaptor proteins (p85) to mediate activation by growth factor receptors (e.g. EGF-R), whereas class IB is linked to GPCR (G-protein-coupled receptor) systems [91].

Class I PI3Ks reside mainly in the cytosol until recruited into active signalling complexes at the plasma
membrane. Once localized to the plasma membrane, they are involved in the generation of 3′-phosphorylated phosphoinositides that function as signalling intermediates in signal transduction cascades (Figure 2) [92]. Targets of PI3K, such as Akt, have been implicated in the suppression of apoptosis [93]. Akt is a serine/threonine kinase and can exert its anti-apoptotic effects in a variety of ways [94].

DEVELOPMENT OF NOVEL TREATMENTS
FOR LIVER DISEASES

The strict regulation of apoptotic cell death and survival pathways allows therapeutic intervention strategies. Hepatocytes and stellate cells contain different protective mechanisms against cytotoxic cytokines, bile acids and ROS. In fact, stellate cells proliferate in response to these factors [95]. Thus both prevention of cell death in hepatocytes and induction of apoptosis in activated stellate cells may constitute relevant therapeutic strategies.

Hepatocyte-associated therapy

In acute liver injury, inhibition of apoptosis of hepatocytes may be beneficial. Targets for anti-apoptotic interventions include caspases, through endogenous or exogenous caspase inhibitors, and preservation of mitochondrial integrity via Bcl-2 family members.

Anti-inflammatory agents are often considered to decrease liver damage during acute liver injury; however, whether this strategy is suitable for all pathological conditions remains to be seen. For example, anti-TNF-α therapy in bacterial infection-induced acute liver disease prevented liver injury, but resulted in decreased bacterial clearance and decreased overall survival [96]. Anti-inflammatory strategies will attenuate cytokine production and NF-κB activation and thus sensitize hepatocytes to apoptosis [97].

Are anti-inflammatory strategies beneficial in chronic liver diseases? The therapeutic implications of these strategies have been addressed in animal models of chronic liver injury [98,99]. Therapeutic interventions decreasing the effects of inflammatory cytokines in chronic liver inflammation will not be beneficial themselves, since suppression of NF-κB activation in hepatocytes will sensitize these cells to apoptotic stimuli. An important issue here is the mode of cell death in chronic liver injury. Although apoptotic cell death of hepatocytes has been described in chronic liver injury [100], more recent studies only report limited contribution of apoptotic cell death compared with necrotic cell death [5–7].

Apparently, hepatocytes are very well protected against apoptotic cell death during chronic liver injury, which is, at least in part, due to cytokine-mediated activation of NF-κB [81,101]. These results have important consequences for therapeutic intervention strategies in chronic (cholestatic) liver diseases. Recently, neutrophils have been identified as an important mediator of cell death in chronic liver injury. Chemokine-attracted neutrophils generate large amounts of ROS and, therefore, neutrophils are an important therapeutic target to improve chronic (cholestatic) liver injury [102].

Patients who suffer from cholestatic liver injury are often treated with UDCA (ursodeoxycholic acid), a bile acid which normally constitutes 3% of total human bile acids. Its black bear origin has been used in Chinese traditional medicine for the treatment of liver diseases [103]. Recently, it has been demonstrated that the taurine conjugate of UDCA protects against bile-acid-induced apoptosis via direct effects on the mitochondrial membrane and via the activation of survival pathways such as MAPKs [104,105].

Liver injury is almost invariably accompanied with oxidative stress in hepatocytes. However, it is not clear whether oxidative stress contributes predominantly to apoptotic cell death or necrotic cell death. In vitro, in cultured hepatocytes, antioxidants prevent both apoptotic and necrotic cell death. In animal studies, antioxidants, such as vitamin E, protect against bile-acid-induced cell damage [106]. Furthermore, infusion of the endogenous antioxidant glutathione has been reported to protect the liver from reperfusion injury after warm ischaemia in rats [107]. Other research suggests that an intact glutathione status is essential for the execution of apoptosis [108]; however, apoptotic hepatocytes were rare in livers after reperfusion injury and not different in glutathione-treated animals [107]. Because glutathione is well tolerable, it may be supplemented in human liver surgery to prevent liver damage [107].

Stellate cell-associated therapy

Acute and chronic liver injury may induce repair mechanisms that will lead to the excessive deposition of scar matrix (liver fibrosis) progressing into liver cirrhosis. In this process, activated stellate cells are the central players. Induction of apoptotic cell death may be a promising therapeutic strategy, since it has been shown that apoptosis of activated stellate cells decreases liver fibrosis [109,110]. This strategy has also been investigated with gliotoxin, a fungal metabolite [111]. Gliotoxin induces apoptosis of activated stellate cells and decreases liver fibrosis; however, it does not improve liver function, since hepatocytes are affected as well. These results confirm the need for specific therapies.

A therapeutic gene could be selectively targeted to the activated stellate cells using cell-surface markers specific for activated stellate cells, e.g. the platelet-derived growth factor (PDGF) receptor [112,113]. Target genes in stellate cells include the NF-κB, since NF-κB protects activated stellate cells against apoptotic cell death [114]. Activation of NF-κB requires the phosphorylation of IκBα. Therefore specific delivery of an NF-κB super-repressor
(a phosphorylation-resistant mutant form of \( \text{IkB}\alpha \)) to the activated stellate cells may decrease the number of activated stellate cells and hence fibrosis [109,114]. Obviously, activation of NF-\( \kappa \)B in hepatocytes should not be prevented because this will induce apoptosis in hepatocytes. Apoptosis-inducing strategies will therefore only be beneficial if cell-selective delivery is achieved.

**APOPTOSIS-RELATED TARGET GENES FOR THERAPY**

**Bcl-2 family members**

As already mentioned, the Bcl-2 family contains attractive anti-apoptotic candidates, such as Bcl-2 or A1/Bfl-1. Anti-apoptotic Bcl-2 family members contribute to the integrity of mitochondria, which is essential to avoid apoptosis in hepatocytes. Under normal conditions, Bcl-2 is absent in hepatocytes. During chronic liver injury, the expression of Bcl-2 is induced only in cholangiocytes. This implies that Bcl-2 cannot be involved in the protection of hepatocytes against bile-acid-induced liver injury. Although Bcl-2 transgenic hepatocytes are protected against Fas-induced apoptosis [115], it is questionable whether overexpression of Bcl-2 in hepatocytes will prevent necrotic cell damage in chronic liver injury [116].

**Caspases and inhibitors of caspases**

Another family member of the Bcl-2 family is A1/Bfl-1, an NF-\( \kappa \)B-regulated gene which may be important in hepatocyte survival [81]. Besides the Bcl-2 family, caspase inhibitors, such as IAP family members, may protect against apoptotic cell death by interrupting the caspase cascade. Overexpression of the human homologue of cIAP2 did protect hepatocytes against apoptosis *in vitro* [9,81]. IAP family members selectively inhibit caspases 9 and 3, which can be blocked by Smac/DIABLO. Therefore the delicate balance between the relative cytosolic concentrations of active caspases 8, 9 and 3 and Smac/DIABLO compared with IAP family members determines cell fate [117].

Depending on the type of injury, inhibition of certain caspases is a relevant strategy. Bajt et al. [118] have shown that, although inhibition of caspase 3 by a peptide-inhibitor inhibits LPS/GalN (\( \beta \)-galactosamine)-mediated apoptosis, caspase 8 inhibition is more beneficial. Furthermore, adenovirus coding for dominant-negative FADD prevented TNF-\( \alpha \)/GalN-induced hepatocyte apoptosis [119]. These studies imply that, in TNF-\( \alpha \)-induced apoptosis, the therapeutic intervention in the apoptotic cascade should be at the level of caspase 8. In this respect, therapy aimed at increasing the expression of c-FLIP [cellular FLICE (FADD-like IL-1\( \beta \)-converting enzyme) inhibitory protein], the endogenous inhibitor of caspase 8, deserves further attention [120]. In contrast, bile-acid-induced cell death in hepatocytes is mediated predominantly via action on the mitochondria and is caspase 9-dependent [81,105]. Indeed, treatment of hepatocytes with the anti-apoptotic bile acid TUDCA (taurine conjugate of UDCA) blocks cell damage at the level of mitochondria [104,105].

**Miscellaneous target genes**

There is accumulating evidence that NO, derived from iNOS, modulates apoptosis by inactivation of caspases [121]. However, the contribution of NF-\( \kappa \)B-regulated iNOS to the protection against apoptosis is not fully elucidated yet. On the other hand, inhibition of cytokine-induced iNOS by \( \beta \)-NAME (\( N^\beta \)-nitro-\( L \)-arginine methyl ester) does not sensitize hepatocytes to cytokine-induced apoptosis *in vitro*. This can be explained by the fact that the lack of iNOS-derived NO is compensated by the presence of other NF-\( \kappa \)B-regulated anti-apoptotic genes such as cIAP2 and A1/Bfl [81]. Others [122] have shown that adenovirus-mediated iNOS gene transfer inhibits hepatocyte apoptosis *in vitro*. All of these results support the idea that iNOS is protective against cytokine-induced apoptosis in hepatocytes.

Intriguingly, exogenous NO, provided by NO donors, such as V-PYRRO/NO \( \{O^2\text{-vinyl 1-{(pyrrolidin-1-yl)diazen-1-iym-1,2-diolate}}\} \) and NOC18, blocks TNF-\( \alpha \)-induced apoptosis and toxicity in the liver [123,124]. Furthermore, in acetaminophen-induced hepatotoxicity, another model of acute liver injury, V-PYRRO/NO decreased liver damage [125]. Nevertheless, one must be cautious with iNOS and its product NO as a therapeutic agent, since NO is very reactive. Harmful effects of excessive NO production are reported in several pathophysiological processes, such as endotoxaemia [126]. For example, large amounts of NO can also rapidly interact with superoxide anions yielding peroxynitrite and tissue damage. NO may react unpredictably, especially during acute liver injury when liver cells are already sensitive.

Another attractive therapeutic gene may be SOD. Adenoviral delivery of SOD genes may be useful in the treatment of oxidative-stress-related injury (metal storage disorders, cholestasis and alcohol abuse). SODs are potent superoxide anion detoxifying enzymes and have been shown to decrease liver injury [127,128].

Recently, the therapeutic use of siRNA (small interfering RNA) has been investigated to silence gene expression post-transcriptionally *in vivo*. Different groups have demonstrated in ConA (concanavalin A)-treated and Fas agonistic antibody-treated mice that RNA interference targeting Fas and caspase 8 protects mice from liver injury [129–131]. Although some hurdles must be overcome, such as effective delivery of siRNA, these strategies hold therapeutic promise to prevent liver injury by protecting hepatocytes from cytotoxicity [129–131].

The application of HGF (hepatocyte growth factor) could be considered as a novel approach. Recently, it has been demonstrated that HGF gene therapy accelerates...
regeneration in cirrhotic livers after heptectomy [132].
Moreover, HGF gene therapy induced protection and
regeneration in livers of a mouse model of acute hepatic
failure [133]. HGF also exerts anti-apoptotic functions
during Fas-mediated apoptosis [134].

Finally, the role of A2O has been studied in acute liver
failure. A2O was originally identified as a TNF-indu-
cible gene product in endothelial cells. A20 protected
endothelial cells from apoptosis and down-regulated
inflammatory responses via inhibition of NF-κB [135].
Overexpression of A2O in these cells protected against
TNF and Fas-induced apoptosis by inhibiting activation
of the caspase cascade [136]. A beneficial effect of adeno-
viral expression of A2O in LPS/GalN-induced acute liver
failure has been demonstrated [137]. However, only leth-
ality was decreased, whereas no other beneficial effects
were displayed, including inhibition of apoptosis, necro-
sis and inflammation.

**Caveats**

Important considerations to be made when designing
an anti-apoptotic therapeutic strategy need to be taken
into account. First, the existence of compensatory mech-
anisms that circumvent the therapeutic effect of caspase
inhibition, such alternate caspase activation [138], needs
consideration. Furthermore inhibition of caspases may
increase oxidative stress, thereby counteracting the anti-
apoptotic effect of caspase inhibition. Evidence for this
hypothesis has been reported recently [139,140]. These
studies have demonstrated that caspase activation in re-
ponse to TNF-α has anti-necrotic effects as well. Removal
of damaged and ROS-over-producing mitochondria is a
caspase-dependent process. Therefore caspases are also
involved in survival after TNF-α toxicity by providing a
negative feedback loop on excessive oxidative stress.
These issues are very important in developing caspase
inhibitors for therapeutic applications in liver injury.

Secondly, anti-apoptotic genes are often overexpressed
in cancer cells, resulting in inhibition of apoptosis and
subsequent chemotherapeutic drug resistance [141]. For
example, in human hepatocellular carcinoma, the IAP
family member XIAP and the Bcl-2 family member Bcl-2
are often highly expressed [141–144]. Therefore therapy
using anti-apoptotic genes may promote carcinogenesis
[145,146]. Anti-apoptotic therapy must therefore contain
options to tightly control the expression pattern of anti-
apoptotic genes.

**CONCLUDING REMARKS**

Understanding the cellular mechanisms that control
death in liver cells is of clinical and scientific importance
in the development of novel therapies. Much effort is
put into elucidating the mechanisms of cell death and the
quest for new intervention targets has not been solved
yet. To translate therapeutic approaches from a controlled
in vitro setting to a complex human liver disease with
its many interacting components will remain a major
challenge.

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into account. First, the existence of compensatory mech-
anisms that circumvent the therapeutic effect of caspase
inhibition, such alternate caspase activation [138], needs
consideration. Furthermore inhibition of caspases may
increase oxidative stress, thereby counteracting the anti-
apoptotic effect of caspase inhibition. Evidence for this
hypothesis has been reported recently [139,140]. These
studies have demonstrated that caspase activation in re-
ponse to TNF-α has anti-necrotic effects as well. Removal
of damaged and ROS-over-producing mitochondria is a
caspase-dependent process. Therefore caspases are also
involved in survival after TNF-α toxicity by providing a
negative feedback loop on excessive oxidative stress.
These issues are very important in developing caspase
inhibitors for therapeutic applications in liver injury.

Secondly, anti-apoptotic genes are often overexpressed
in cancer cells, resulting in inhibition of apoptosis and
subsequent chemotherapeutic drug resistance [141]. For
example, in human hepatocellular carcinoma, the IAP
family member XIAP and the Bcl-2 family member Bcl-2
are often highly expressed [141–144]. Therefore therapy
using anti-apoptotic genes may promote carcinogenesis
[145,146]. Anti-apoptotic therapy must therefore contain
options to tightly control the expression pattern of anti-
apoptotic genes.

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