Apoptotic cell death as a target for the treatment of acute and chronic liver injury

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Apoptotic cell death as a target for the treatment of acute and chronic liver injury

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Contents

Chapter 1
Scope of this thesis

Acute liver failure 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 acute and chronic liver injury, liver cells are exposed to increased levels of cytokines, oxidative stress and bile acids. This may result in cell damage followed by necrotic and apoptotic cell death of hepatocytes and proliferation of stellate cells. Eventually, this may lead to loss of liver function. Effective therapies for acute and chronic liver diseases are not available.

Hepatocytes, the parenchymal cells of the liver, are well equipped with protective mechanisms to prevent cell death. As long as these protective pathways 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, insight into the cellular mechanisms leading to cell death is of relevance to understand liver diseases. This can result in identification of novel intervention targets in order to prevent cell death.

The aim of this thesis is to investigate apoptotic mechanisms in acute and chronic liver injury in order to develop strategies to protect hepatocytes against apoptotic cell damage. In contrast to the loss of hepatocytes, chronic liver injury is associated with the proliferation and activation of hepatic stellate cells. These cells play a pivotal role in liver fibrosis. Thus, contrary to the prevention of cell death in hepatocytes, induction of apoptosis in activated stellate cells may constitute a relevant therapeutic strategy during chronic liver diseases.

An important anti-apoptotic mechanism is the activation of the transcription factor NF-κB which results in the activation of anti-apoptotic genes. This thesis describes the role of NF-κB in the protection against apoptosis in hepatocytes during acute liver injury (chapter 3 in vitro and chapter 4 in vivo) and chronic liver injury (chapter 5). Furthermore, NF-κB-regulated Inhibitor of Apoptosis Protein2 (cIAP2) is highlighted as a new target of therapy.

In chronic cholestatic liver diseases, the accumulation of bile acids in the liver is thought to play a role in causing hepatocyte damage. This thesis describes the extent of apoptotic cell death in hepatocytes in an experimental model of extrahepatic cholestasis and the ability of these cells to adapt to bile acid-induced apoptosis (chapter 5). Patients with cholestatic liver disease are often treated with ursodeoxycholic acid (UDCA), but its protective mechanism of action is not well defined yet. Chapter 6 of this thesis describes the possible mechanisms behind the anti-apoptotic action of taurine-conjugated UDCA.

Liver injury eventually results in excessive deposition of extracellular matrix referred to as fibrogenesis. In this process, the activation and proliferation of stellate cells (fibroblasts-like cells) is a key event. Therefore, the stellate cell is an attractive target for anti-fibrotic therapies. One promising approach is the induction of apoptotic cell death in activated stellate cells. However, it is crucial that this induction should be selectively aimed at stellate cells while leaving hepatocytes unaffected. Therefore, a gene delivery tool which selectively targets to activated stellate cells is developed and tested (chapter 7). This will allow cell selective gene modulation in this important cell type during chronic liver diseases accompanied by fibrosis.

This thesis describes some of the mechanisms leading to apoptotic cell death during liver disease and provides tools to selectively interfere with this process.

Chapter 2
General introduction

2.1 The liver

A major function of the liver is the transformation and metabolism of substances, like endobiotics (bilirubin, ammonia) and xenobiotics (drugs, alcohol), into non-toxic compounds. These compounds are subsequently eliminated from the body. In addition, the liver is essential in bile homeostasis; it regulates the synthesis, uptake and secretion of bile acids that are important components of bile. Derangement of these functions may lead to damage to the hepatocytes leading to cell death and liver failure.

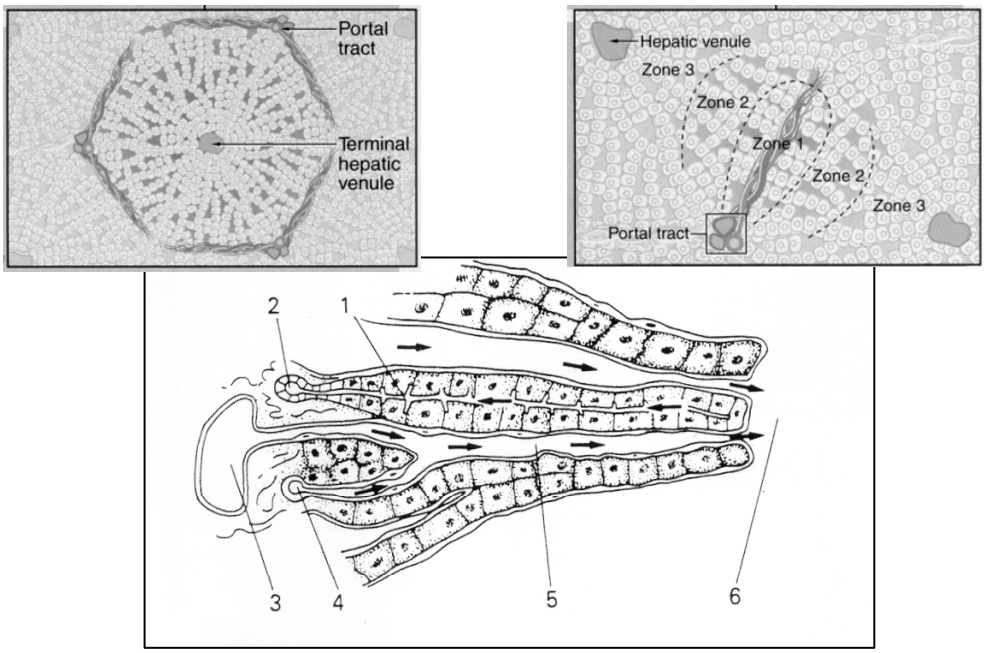

Figure 1. Schematic representation of a liver lobule (left panel) with blood and bile flow. Bile flow of canaliculus (1) towards the common bile duct (2). Blood is transported from the portal tract (3;4) to the hepatic venule (6) via sinusoids (5) establishing a zonated blood distribution (right panel). Reprinted from Bloom *et al.* (1975), a textbook of histology and from Young B and Heath JW (2000), Wheather's Functional Histology, a text and colour atlas.

2.1.1 Liver anatomy and physiology

The classical microscopic unit of the liver is a hexagonal lobule.[1] At the corners of each hexagon, blood enters via the portal tracts. Each portal tract consists of a portal vein, hepatic arteries and a common bile duct. In the centre of each hexagon, a central vein is located (Fig 1). Liver cells are situated in cell cords radially orientated around the central hepatic vein. Between these cell layers, blood is transported from the portal tracts to the central vein via sinusoids (Fig 1). In this way, a zonated distribution is established, composed of a periportal area which is provided with oxygen- and nutrient-rich blood (zone 1, surrounded by zone 2), and a pericentral area receiving oxygen-deprived blood (zone 3) (Fig 1).

The liver consists of different cell types of which the hepatocytes, the parenchymal cells, are most abundant.[2] Hepatocytes are polarised cells comprised of a basolateral membrane facing the sinusoid (portal blood) and an apical membrane facing the bile canaliculus (bile). Tight junctions separate both membranes. A bile canaliculus is present between two adjacent hepatocytes (Fig 1 and 2). Bile acids taken up or produced in the hepatoyctes are transported across the canalicular membrane and arrive via the canals of Hering in small and large bile ducts, present in the portal tracts (Fig 1). From there, bile acids reach the extrahepatic bile duct and eventually the gut. The canals of Hering are lined both by hepatocytes and biliary epithelial cells (cholangiocytes). Depending on the size of the bile duct, bile ducts are lined by small or large cholangiocytes with different metabolic functions.

The sinusoidal wall comprises endothelial cells that contain fenestrae through which transport of portal blood plasma and small particles to the hepatocytes occur. The space between the sinusoids and the hepatocytes is the space of Disse (Fig 2). This space contains Kupffer cells (liver macrophages), natural killer cells and stellate cells (fat-storing cells). Hepatocytes contain microvilli that project into the space of Disse. Via the fenestrae of the endothelium, they come directly in contact with the blood (Fig 2).

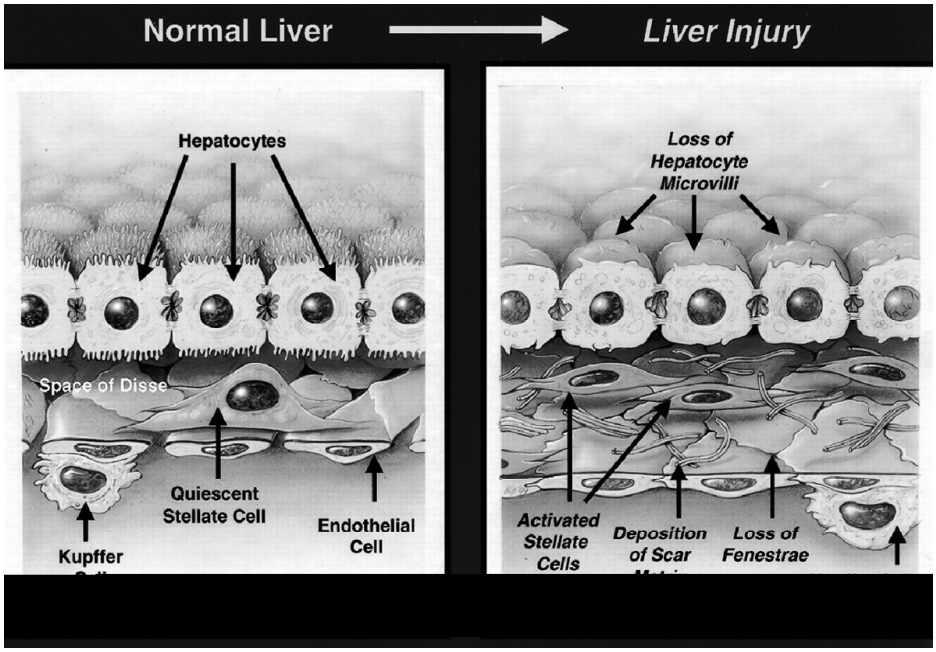

Figure 2. Liver cells during normal conditions and after liver injury. See text for details. Reprinted with permission from the copyright holder the American Society for Biochemistry and Molecular Biology; Friedman SL, J of Biol Chem 2000; 275:2247-2250.

2.2 Bile acids in health and cholestatic liver disorders

In normal conditions, between 500-800 ml bile is produced daily in a human adult. Bile is made up of 80 % water and 20 % dissolved substances of which bile acids form the main part followed by phospholipids, proteins, and products of metabolic processes like vitamins, hormones and conjugated bilirubin. In the hepatocytes, bile acids are synthesised from cholesterol via a series of enzymatic steps. Primary bile acids produced in the liver are cholic acid and chenodeoxycholic acid that are conjugated with glycine or taurine in the peroxisomes of hepatocytes. Energy-dependent transport by means of a bile salt export pump (BSEP) is required for

secretion of bile acids across the canalicular membrane into the bile canaliculus. BSEP is a member of the ATP-binding cassette (ABC) membrane transporter family. A defect in canalicular bile acid transport causes severe liver disease.[3] Cholangiocytes play a role in the final modification of bile fluid and thus contribute to the excretion of bile acids and other compounds.[4;5]

2.2.1 Enterohepatic circulation of bile acids

Bile acids follow an enterohepatic circulation in which 90 % of primary bile acids formed in the liver is absorbed from the intestine and transported back to the liver. Ten percent enters the colon. Here, bile acids are converted into secondary bile acids due to de-conjugation and bacterial transformation. In healthy humans, approximately 0.5 g bile acids is lost daily in the faeces. Primary and secondary bile acids circulate back to the liver through the portal vein. In the liver, bile acids reach the hepatocytes via the sinusoids. Uptake of bile acids is mediated in the basolateral membranes of hepatocytes predominantly by the sodium-dependent bile acid transporter NTCP and members of the sodium-independent organic anion transporting polypeptide family.[6;7] In the liver, de-conjugated bile acids will be re-conjugated with taurine or glycine. Part of the secondary bile acid deoxycholic acid is converted into ursodeoxycholic acid. In normal conditions, loss of bile acids from the enterohepatic circulation will be replaced by *de novo* bile acid synthesis in the liver. Bile acid synthesis is tightly regulated.[8]

2.2.2 Cholestasis

Cholestatic liver disorders (cholestasis) are characterised by a disturbance in the formation, secretion or drainage of bile acids. Causes of cholestasis include impairments in bile flow through intra- or extrahepatic obstruction (e.g. by gallstones), cancer (e.g. in the pancreas or gall bladder), drugs, inflammatory bile duct damage[9] or genetic disorders of bile salt transport and metabolism.[10;6]

During cholestasis, the accumulation of bile acids in the liver causes damage to cholangiocytes and hepatocytes, and may induce the activation of Kupffer cells[11]

and stellate cells.[12;13] Cholestasis is accompanied by (increased sensitivity to) endotoxemia and could result in chronic liver inflammation and liver fibrosis.[14;15].

2.3 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 in these cells. However, acute or chronic exposure to certain insults such as cytokines, reactive oxygen species and bile acids results in disturbed liver function.

2.3.1 Acute liver injury

Acute liver failure develops in a short period 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 liver transplantation.[16] Cell death can occur in different forms, that is programmed cell death (apoptosis) which is tightly controlled, and necrosis. During acute liver injury, Kupffer cells and newly recruited leukocytes and lymphocytes are activated. This results in the production of pro-inflammatory cytokines and reactive oxygen species causing apoptotic and necrotic cell death in hepatocytes.[17] Eventually, this may lead to loss of liver function, which is a life-threatening condition (Fig 3).

2.3.2 Chronic liver injury

Chronic cholestasis or long term exposure to alcohol, drugs, or chemicals can result in liver failure progressing into liver fibrosis. Liver fibrosis is characterised by deposition of scar tissue. Its end-stage is called liver cirrhosis. Other causes of chronic liver failure are viral hepatitis, metabolic diseases like Wilson's disease (copper storage disease), or auto-immune diseases (e.g. primary biliary cirrhosis, primary sclerosing cholangitis).

During chronic liver injury, the endothelial cells, hepatocytes and cholangiocytes can be damaged due to the accumulation of toxic metabolites, reactive oxygen species and bile acids. This results in the activation of Kupffer cells and the recruitment of inflammatory cells and the subsequent release of growth factors (e.g. Transforming Growth Factor-β, (TGF-β)), cytokines and reactive oxygen species that induce the activation and proliferation of hepatic stellate cells (Fig 3). These cells are major players in the development of liver fibrosis.

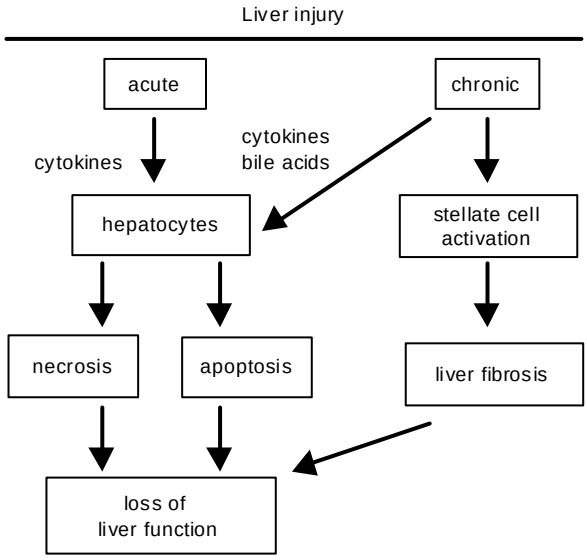

Figure 3. Highlights of this thesis

In normal conditions, stellate cells function as vitamin A stores and are crucial in regulating extracellular matrix metabolism.[18] This extracellular matrix (ECM), a complex network of collagens, glycoproteins, proteoglycans and glycosaminoglycans, is essential for maintaining the differentiated function of all resident liver cells.[19] Besides production of matrix material, stellate cells, among

other cell types, also synthesise matrix-degrading proteinases (metalloproteinases or MMPs) and tissue inhibitors of metalloproteinases (TIMPS). In this way they play a pivotal role in the turnover and deposition of the ECM.

During chronic liver injury, activated stellate cells markedly increase matrix production through the action of TGFβ1. Furthermore, the number of stellate cells increases by increased expression of the Platelet Derived Growth Factor-receptor (PDGF-R) on their cell membranes and production of its ligand PDGF. A major consequence is a remodelling of the ECM that leads to deposition of scar tissue (fibrosis) and liver dysfunction.[20] Therefore, the activation and proliferation of stellate cells are considered key events in liver fibrosis (Fig 3). Progression of the fibrotic process is associated with the loss of sinusoidal fenestrae and perturbation of hepatocyte function.

2.4 Cell death in liver diseases

During acute and chronic liver diseases, hepatocytes are exposed to increased levels of cytokines (e.g. Tumour necrosis factor-alpha (TNF-α), Interleukin-1beta (IL-1β) and Interferon-gamma (IFN-γ)), oxidative stress and bile acids.[21] Although hepatocytes have an enormous capacity to defend themselves against these agents, over-exposure will result in cell death. Therefore, accurate knowledge of the cellular mechanisms leading to cell death is of relevance to understand liver diseases.

Cell death is often 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.[22] Necrosis results from metabolic disruption with energy depletion (loss of adenosine triphosphate, ATP), mitochondrial and cellular swelling and activation of degradative enzymes. This leads to cell lysis followed by loss of cell constituents in

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 characterised by DNA condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage. Eventually, the apoptotic cell breaks into small membrane-surrounded fragments (apoptotic bodies) which are cleared by surrounding cells.[23;24] All these events are tightly controlled and well organized.

Apoptotic cell death is complementary to mitosis and crucial in embryonic development and adult tissues by controlling tissue homeostasis.[24] Hepatocyte cell death occurs in both acute and chronic liver diseases. Massive cell death occurs in acute liver failure, whereas the mode of cell death in chronic liver injury is controversial. The strict regulation of apoptotic cell death allows therapeutic intervention strategies. Therefore, this thesis focuses on the mechanisms of apoptotic cell death and cell survival in hepatocytes during acute and chronic liver injury.

2.4.1 Receptor-mediated apoptosis

Apoptotic cell death can be initiated by activation of death receptors on the cell membrane that belong to the tumour necrosis/nerve growth factor (TNF/NGF) receptor super family. Hepatocytes express Fas (CD95), Tumour necrosis factor related apoptosis inducing ligand-receptor (TRAIL-R1), TRAIL-receptor 2 (TRAIL-R2), and Tumour necrosis factor-receptor type 1 (TNF-R1).[25] 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 Tumour Necrosis Factor-alpha (TNF-α) is an important cytokine in liver injury[26], this thesis focuses on TNF-R1-mediated signalling. 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.[27-29] Upon activation by TNF-α, trimerization of TFN-R1 is followed by recruitment of the adaptor protein TRADD (TNFR-associated protein with death domain) (Fig 4). TRADD recruits signalling proteins like FADD (Fas associated death domain), TRAF-2 (TNF-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 (Fig 4). Binding of RIP to TRAF-2 initiates the activation of survival pathways like Nuclear Factor kappa B (NF-κB) and Mitogen-activated protein kinases (MAPKs).[30]

Caspase-8 contains in its prodomain a death effector domain, which binds to FADD. Local clustering of the inactive pro-form of caspase-8 to the TNF-α-activated TNF-R1 results in a death-inducing signalling complex (DISC) and auto-activation of caspase-8. In fact, internalisation of TNF receptors may be required for this process.[31]

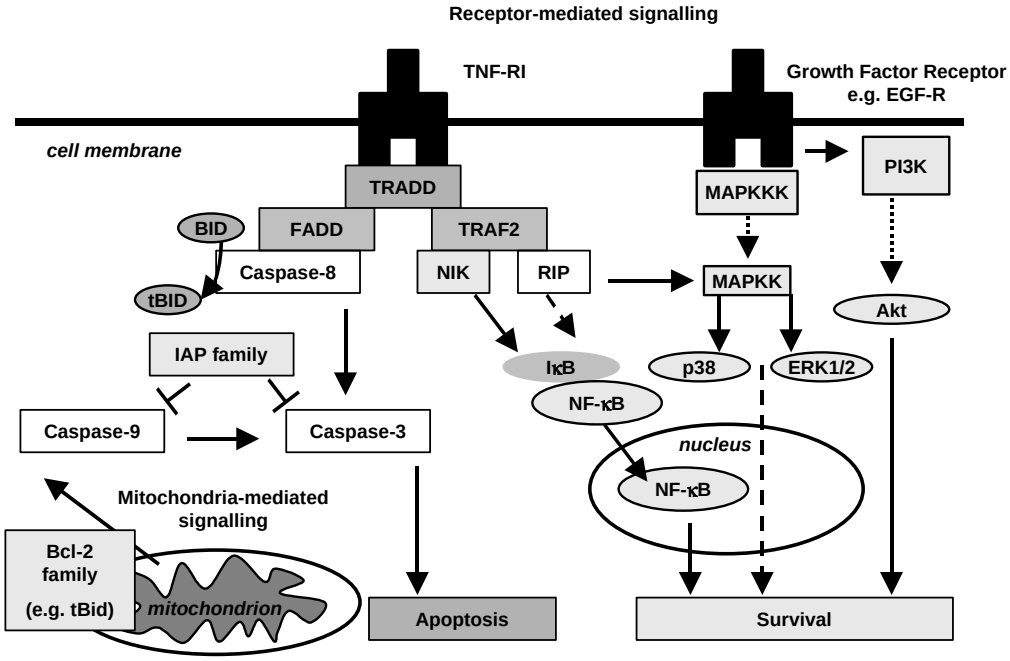

Figure 4. Schematic overview of Receptor-mediated and Mitochondria-mediated signal transduction pathways. TNF-R1-mediated intracellular signalling activates both apoptotic and survival signals. See text for details.

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 (Asp) residues in the context of tetrapeptide motifs. Active caspase-8 is involved in the cleavage and activation of effector caspase-3 (Fig 4). 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.

Dependent on the cell type and stimulus, the mitochondria play 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.[32] Thus, mitochondria play a crucial role in regulating cell death in hepatocytes (Fig 4).[22;33;34;35]

Since the mechanisms of mitochondria-mediated apoptosis may differ between different types of hepatocyte stimuli, knowledge of mitochondria-controlled apoptosis is needed to find suitable intervention targets for acute and chronic liver diseases. Although in different cell types, the mechanisms of mitochondria-mediated apoptosis have been partially unravelled, in hepatocytes further investigation is still needed. In this thesis, much attention is paid to mitochondria-controlled apoptosis in cytokine- and bile acid-exposed hepatocytes.

2.4.2 Mitochondria-mediated apoptosis

In normal conditions, the inner membrane of the mitochondria contains the protein complexes of the respiratory chain and the ATP synthase.[36] During an apoptotic stimulus, the permeability of the mitochondrial membrane is disrupted which is called the mitochondrial permeability transition (MPT). The MPT is characterised by rapid permeability of the mitochondrial membrane and release of apoptotic factors from the intermembrane space into the cytosol (Fig 5). Furthermore, there is a rapid reduction in the mitochondrial membrane potential due to disruption of the electron transport chain.[37] Consequently, the ATP production is abolished and electrons escape from the respiratory chain to form reactive oxygen species.[38] 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.

The decision between necrotic and apoptotic cell death may depend on the cellular concentration of ATP. Thus, the mitochondrial permeability transition (MPT) mediates both necrosis and apoptosis, but when the bulk of mitochondria will undergo MPT, a marked depletion of ATP can develop leading to necrotic cell death. When the MPT occurs without severe ATP depletion, apoptosis develops which may be followed by secondary necrosis if ATP eventually is depleted during apoptosis.

Mitochondria-mediated release of pro-apoptotic proteins

The mitochondrial permeability transition (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.[39,40,38] Both direct pore formation by some pro-apoptotic Bcl-2 family members and opening of the permeability transition pore (PTP) complex releases apoptotic proteins from the mitochondria. Depending on the apoptotic stimulus either pathway may predominate[41;42] (Fig 5).

The importance of Bcl-2 family members 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 heterodimerization and regulate mitochondrial-mediated apoptosis[43;44] (Fig 5). Bax and Bak double knockout cells are completely resistant to mitochondrial cytochrome c release during apoptosis.[45] Moreover, Bid-deficient hepatocytes are more resistant to TNF-α-induced and Fas-mediated apoptosis compared to wild-type mice hepatocytes.[46] In this thesis, the role of the Bcl-2 family in hepatocytes is further investigated.[42]

Anti-apoptotic members like 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 like Bax, Bid and Bad are sequestered in the cytosol prior to a death signal.[47] Upon an apoptotic stimulus, caspase-8 cleaves Bid into a truncated form (tBid) (Fig 4 and 5), whereas Bax oligomerises, and Bad is released from the adaptor molecule 14-3-3 by de-phosphorylation. These events result in the translocation and insertion of tBid, Bax and Bad in the outer mitochondrial membrane[48-50] (Fig 5).

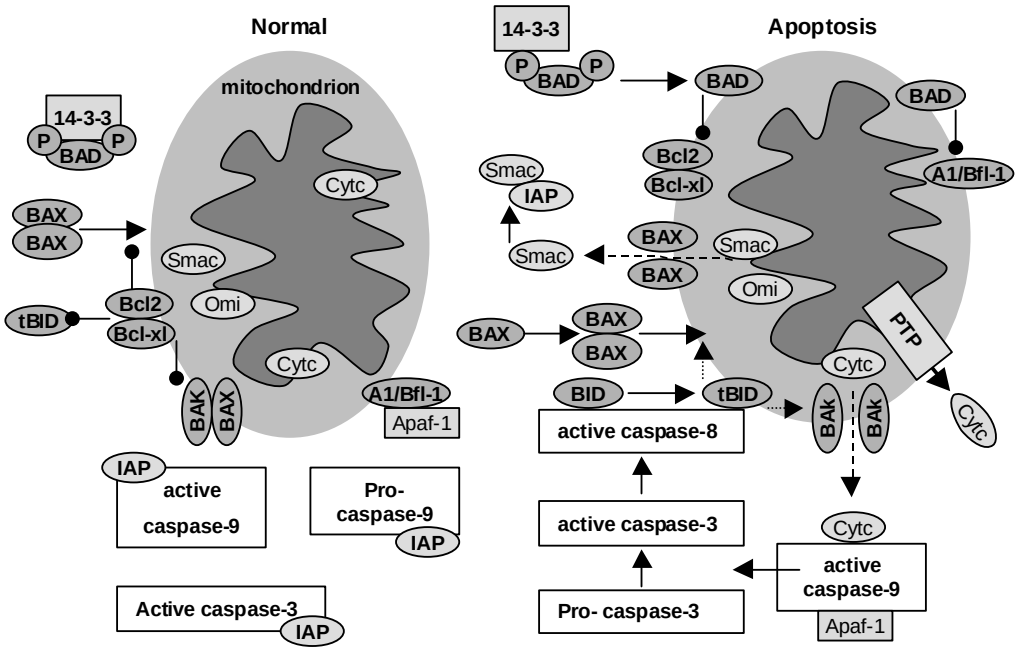

Figure 5. Schematic summary of mitochondria-controlled apoptosis. See text for details. Induction is represented as a regular arrow, whereas inhibition is demonstrated using: —● .

In the mitochondrial membrane, Bad interacts with and antagonises anti-apoptotic Bcl-2 and/or Bcl-XL[51], whereas tBid, Bax and Bak are able to form tetrameric outer membrane channels through which cytochrome c can escape (Fig 5 right panel). Bax and Bak need the interaction with tBid to form pores in the mitochondrial membrane.[36] The anti-apoptotic Bcl-2 family members inhibit apoptosis by binding Bax and Bak, sequestering tBid and Apaf-1, and preventing mitochondrial release of cytochrome c and Smac/DIABLO[52-55] (Fig 5 left panel).

Mitochondrial death factors

Factors released from the mitochondria are crucial for the activation of pro-apoptotic signalling. Therefore, some of these factors are outlined below.

Cytochrome c, an electron shuttle molecule, is released from mitochondria during apoptosis. It complexes with apoptosis protease-activator factor 1 (Apaf-1), dATP and cytosolic pro-caspase-9 to form a caspase-activating complex called the apoptosome (Fig 5). Cytochrome c and dATP induce refolding of Apaf-1, which allows interaction with pro-caspase-9. In this way, pro-caspase-9 is activated which subsequently cleaves and activates caspase-3.[56] Depending on cell type and stimulus, caspase-3 can be involved in the activation of procaspase-8, pro-caspase-6, pro-caspase-9, and Bid that results in a feedback amplification of the apoptotic signal (Fig 5).[57;58]

Other mitochondrial apoptogenic factors that are released from the intermembrane space include Smac/DIABLO and Omi/HtrA2 (Fig 5). These proteins require proteolytic processing in the mitochondria to become active.[59;60] The release of Smac/Diablo requires active caspases, occurs downstream of cytochrome c translocation and can be controlled by Bax.[61;62] Smac/DIABLO acts as a dimer and sequesters members of the Inhibitor of Apoptosis Family (IAP family) such as XIAP, cIAP1 and cIAP2 (Fig 5). This results in the release of active caspases and the propagation of caspase cascades.[63;64] Omi/HtrA2 exerts a similar function although it also contributes to caspase-independent apoptosis due to its N-terminal serine protease catalytic domain.[65]

The balance between pro-apoptotic and anti-apoptotic pathways determines the outcome of cell death upon a stimulus. As long as protective proteins are present in high amounts, the balance will be in favour of cell survival. Thus, understanding of cell survival mechanisms is essential to find therapeutic interventions for acute and chronic liver diseases.

2.5 Cell survival mechanisms in liver diseases

Hepatocytes and stellate cells contain different protective mechanisms against cytotoxic cytokines, bile acids and reactive oxygen species (ROS). In fact, stellate cells proliferate in response to these factors.[13] Common protective mechanisms include antioxidants like reduced glutathione, which is present in very high amounts in hepatocytes.[66;67] In addition, bile acid transporters undergo adaptive responses during cholestatic disorders in order to protect hepatocytes against elevated intracellular levels of bile acids.[68;69]

Another defence mechanism of the liver is the 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. The replication of hepatocytes allows restoration of the liver capacitiy. In situations in which hepatocyte proliferation is blocked or delayed, intra-hepatic precursor cells (oval cells) are responsible for this process.[70;71]

Among the cytokines, TNF-α, Interleukin-1β (IL-1β), and Interferon-γ (IFN-γ) are abundantly present during acute and chronic liver failure.[17;21;72] Since these inflammatory cytokines also activate survival signalling pathways, such as NF-κB, their presence can be beneficial. In the studies described in this thesis, a mixture of these cytokines is used to investigate the apoptosis-related effects on hepatocytes during acute and chronic liver injury. In this way, the *in vivo* situation is best mimicked, as some NF-κB-regulated genes are solely expressed in response to this cytokine mixture.

2.5.1 Survival pathways

NF-κB signalling

Since NF-κB is a major regulator of the balance between cell survival and cell death, the studies in this thesis investigate its role in acute and chronic liver injury. Other 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.[73;74] Recently, it has been postulated that NF-κB inhibits TNF-α-induced accumulation of ROS that normally mediate prolonged c-Jun N-terminal kinase (JNK) activation and cell death.[75] Indeed, inhibition of NF-κB activity induces apoptosis in hepatocytes, indicating its role in the transcription of anti-apoptotic genes.[76] Advances in our understanding of these genes may provide opportunities for the development of novel therapies that favour 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)[77] (Fig 4). Other NF-κB activating signals include protein kinase C (PKC) and phosphatidylinositol-3 kinase (PI3-kinase). Evidence that reactive oxygen species mediate NF-κB activation is controversial.[78;79]

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.[80] The NF-κB-inducing kinase (NIK), a member of the MAP-kinase family, serves as a common mediator in the NF-κB signalling cascade (Fig 4). Activation of NIK often occurs after its binding to receptor-recruited TRAF-2 (TNF-α) or TRAF-6 (IL-1β). As a consequence, an IκB kinase-comples (IKK) is activated consisting of IKK-α, IKK-β and IKK-γ.[81;82] This complex is involved in the phosphorylation and thereby inactivation of inhibitors of NF-κB. Of these inhibitors, IκB-α, IκB-β and IκB-γ (IκBs) are most abundantly present. Upon phosphorylation at specific serine residues, IκBs are ubiquitinated and degraded via the 26S

proteasome complex allowing release of NF-κB (Fig 4). In contrast to IκB-β and IκB-γ, the degradation of IκB-α is very rapid but IκB-α is quickly re-synthesised in an NF-κB-dependent manner. In this way, cells can react adequately and regulate downstream genes differentially upon different stimuli.[83;84]

The release of NF-κB exposes a nuclear localization signal sequence and permits translocation of NF-κB to the nucleus (Fig 4). In the nucleus, NF-κB binds to κB binding sites in promoters of target genes and induces transcription of these genes. Among NF-κB-regulated genes, inflammation-related genes are present like TNF-α, IL-1β, IL-6, intercellular and vascular cellular adhesion molecules (ICAM-1, VCAM-1), as well as apoptosis-related genes like inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and Inhibitor of Apoptosis (IAP) family members.[81; 85]

This thesis investigates which apoptosis-related genes are regulated by NF-κB in hepatocytes and which apoptosis-related genes can be used as a novel therapy. Attractive candidates belong to the Bcl-2 family and IAP family. These families contain important anti-apoptotic proteins that are strong inhibitors of cell death in different cell types[43;86] (Fig 4 and 5).

The Inhibitor of Apoptosis Protein (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) caspase–9 (Fig 5), but are also involved in signal transduction and protein degradation.[86;87] The IAP family members contain different protein domains for their anti-apoptotic activities, such as baculovirus IAP repeats (BIRs) and a Ring finger domain. This Ring domain is involved in protein degradation, like IκB degradation.[88] In addition, IAPs associate with TRAF proteins implying that IAPs participate in the stimulation of NF-κB.[88;89]

Besides NF-κB, other signalling pathways may antagonise cell death in hepatocytes, thereby influence the balance between pro- and anti-apoptotic signals. Of these survival pathways, the mitogen-activated protein kinase signalling cascades (MAPKs) and phosphoinositide (PI) 3-kinase are good candidates.

MAP-Kinase signalling

Four distinctly regulated groups of MAPKs are present: extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK), p38 proteins and ERK5 (Fig 4). Until recently, selective inhibitors of JNK were not available. Therefore, only ERK1/2 and p38 MAPKs are highlighted in this thesis.

MAPK activity is regulated through a MAPK kinase (MAPKK), which is MEK1/2 for ERK1/2 and MKK3/6 for p38, and a MAPKK kinase (MAPKKK) (Fig 4). Each MAPKK can be activated by more than one MAPKKK (like apoptosis signal-regulating kinase-1 and MEKK1), which reacts upon distinct stimuli.[90] Activation of TNF-R1 results in activation of ERK1/2 and p38 MAPKs through TRAF2 sequestering (Fig 4). In addition, the epidermal growth factor receptor (EGF-R) is involved in ERK1/2 activation.[91;92]

The general function of MAPK cascades is the regulation of gene expression[93;94] 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 and are not fully elucidated in hepatocytes yet.

PI-3 Kinase signalling

The PI 3-kinase family is a super family of three different classes of enzymes and linked to different cellular functions, including cell survival (Fig 4). This family may be important in controlling cell survival in hepatocytes. Class I enzymes have largely been characterised and further subdivided into two groups of PI3-kinases, 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 G-protein-coupled receptor systems.[95]

PI3-kinases of class I reside mainly in the cytosol until recruited into active signalling complexes at the plasma membrane. Once localised to the plasma membrane, they are involved in the generation of 3'-phosphorylated phosphoinositides that function as signalling intermediates in signal transduction

cascades (Fig 4).[96] Targets of PI3-kinases, such as Akt, have been implicated in the suppression of apoptosis.[97] Akt is a serine kinase and can exert its anti-apoptotic effects in a variety of ways.[98]

2.6 Development of novel treatments

As indicated above, much is known about cell signalling pathways and the regulation of proteins involved in apoptosis and cell survival. Yet, much of this knowledge has been derived from cancer cells and may not be directly applicable to liver cells.

Cell death of hepatocytes and the proliferation of activated stellate cells are principal events that lead to liver failure in acute and chronic liver injury. Therefore, the target cell for anti-apoptotic therapy in liver failure is the hepatocyte, whereas induction of apoptosis in the activated stellate cell may be beneficial. For this purpose, the characterisation of death and survival signalling pathways and the identification of therapeutic genes are needed in hepatocytes. On the other hand, a selective and efficient gene carrier is essential to induce apoptosis solely in activated stellate cells

The strict regulation of apoptotic cell death allows therapeutic intervention strategies in hepatocytes and activated stellate cells during acute and chronic liver injury. However, these intervention strategies must be chosen very carefully because of the complexity of cell signalling.

2.6.1 Gene Therapy in acute and chronic liver injury

In the delicate system of cell death and survival, selective interference is crucial. For this purpose, gene therapy is a promising approach. In this way, a therapeutic transgene is transduced into a cell through a vector system. This vector system is either of viral or non-viral origin, such as adenovirus, retrovirus, herpes simplex virus, adeno-associated virus, and lipofection. Retroviral and adenoviral

vectors have so far been the most commonly used vectors in gene-transfer clinical trials as these vectors are very efficient.[99] Most of these gene therapy trials are targeted to cancer.[100]

In the studies described in this thesis, adenoviral-mediated gene delivery is used. Adenoviruses are non-enveloped, linear double-stranded DNA viruses. Fifty serotypes of adenovirus have been identified and the prototype adenoviral vector for gene therapy is based on adenovirus type 5 (Ad5). Adenoviral-based vectors are promising vehicles for liver diseases because they most efficiently transfer genes into the liver. This type of vector is rapidly taken up by the hepatocytes in the liver, thereby avoiding transgene expression in other organs. In addition, activated stellate cells are more susceptible for adenoviral gene delivery compared to non-viral vectors.[29]

In general, adenovirus type 5 attaches to cells via binding to the coxsackievirus and adenovirus receptor (CAR) on cells. Binding of the virus is mediated through a tripartite knob of one of the 12 fibers that extend from the viral capsid. Viral uptake is mediated via binding of a RGD peptide (at the capsid base of a fiber) to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins on the cell membrane. Receptor-mediated endocytosis via clathrin coated pits results in gene delivery into the cell.

To achieve specific gene expression in the activated stellate cell, targeting of the adenovirus is needed to avoid uptake by the hepatocytes. This can be accomplished by transductional and transcriptional targeting.[101] Transductional targeting means selective gene transfer by the vector to a specific cell type, e.g. the activated stellate cell, whereas transcriptional targeting is achieved by placing the transgene under control of a cell-specific promotor. In this thesis, transductional targeting to the activated stellate cells is investigated.

Although adenoviral-based vectors are extremely efficient in the transduction of liver cells, the main limitation of this vector is that the adenoviral capsid can mediate an inflammatory response, which can result in shorter duration of the transgene expression. In addition, virus-neutralizing antibodies can be elicited. As

most humans have been exposed to wild-type adenovirus and harbour these antibodies, adenoviral vectors may be cleared when administered in clinical trials.[99]

Much effort is put into the development of clinical safe gene delivery vectors with improved efficiency and specificity. First-generation adenovirus vectors were deleted of certain genes (E1 and E3) resulting in replication-deficient viruses[99] Second- and third-generation vectors contain additional deletions in other genes (E2 and / or E4), thereby reducing toxicity. Furthermore, there is a promising development of helper-dependent adenoviruses that are deleted for all viral genes.[102] In combination with selective targeting to liver cells, the inflammatory response against the vector will be further reduced due to increased infection efficiency and thus a lower vector dose.

2.7 Concluding remarks

In contrast to the prevention of cell death in hepatocytes, induction of apoptotic cell death in activated stellate cells may constitute a relevant therapeutic strategy during liver diseases. For this purpose, this thesis describes some of the mechanisms leading to apoptosis and provides tools to selectively interfere with this process.

2.8 Reference list

1. Millward-Sadler GH, Jezequel AM. Normal Histology and Ultrastructure. In: Wright R, Alberti KGMM KS, and Millward-Sadler GH, eds. Liver and Biliary Disease, Pathophysiology, Diagnosis, Management. London: W.B.Saunders Company Ltd, 1979:13-43.
2. Desmet VJ. Organizational Principles. In: Arias AM et al., ed. The Liver, Biology and Pathobiology. fourth ed. Lippincott Williams and Wilkins, 2001:3-15.

3. Thompson RJ, Strautnieks SS. Genetic defects of canalicular transport. In: Arias AM et al., ed. The Liver, Biology and Pathobiology. fourth ed. Lippincott Williams and Wilkins, 2001:383-388.
4. Kanno N, LeSage G, Glaser S, Alvaro D, Alpini G. Functional heterogeneity of the intrahepatic biliary epithelium. Hepatology 2000;31:555-561.
5. Boyer JL. Bile duct epithelium: frontiers in transport physiology. Am J Physiol 1996;270:G1-G5.
6. Kullak-Ublick GA, Stieger B, Hagenbuch B, Meier PJ. Hepatic transport of bile salts. Semin Liver Dis 2000;20:273-292.
7. Rost D, Herrmann T, Sauer P, Schmidts HL, Stieger B, Meier PJ, Stremmel W, Stiehl A. Regulation of rat organic anion transporters in bile salt-induced cholestatic hepatitis: effect of ursodeoxycholate. Hepatology 2003;38:187-195.
8. Trauner M, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev 2003;83:633-671.
9. Kullak-Ublick GA, Meier PJ. Mechanisms of cholestasis. Clin Liver Dis 2000;4:357-385.
10. Jansen PL, Muller M, Sturm E. Genes and cholestasis. Hepatology 2001;34:1067-1074.
11. Sugata K, Kamimura S, Shijo H. Differential effects between tauroursodeoxycholic and taurochenodeoxycholic acids in hepatic fibrosis: an assessment by primary cultured Ito and Kupffer cells from the rat liver. J Gastroenterol Hepatol 1996;11:451-458.
12. Brady LM, Beno DW, Davis BH. Bile acid stimulation of early growth response gene and mitogen-activated protein kinase is protein kinase C-dependent. Biochem J 1996;316:765-769.
13. Svegliati-Baroni L, Ridolfi F, Schoemaker MH, Saccomanno S, Homan M, Jansen PLM, Benedetti A, Moshage H. Bile acids induce survival signals in hepatic stellate cells through a PKC- and ERK-dependent mechanism. Hepatology 36[No. 4, Pt. 2 of 2], 316A. 2002. Ref Type: Abstract
14. Harry D, Anand R, Holt S, Davies S, Marley R, Fernando B, Goodier D, Moore K. Increased sensitivity to endotoxemia in the bile duct-ligated cirrhotic Rat. Hepatology 1999;30:1198-1205.
15. Grinko I, Geerts A, Wisse E. Experimental biliary fibrosis correlates with increased numbers of fat-storing and Kupffer cells, and portal endotoxemia. J Hepatol 1995;23:449-458.
16. Saunders SJ, Seggie J, Kirsch RE, Terblanche J. Acute Liver Failure. In: Wright R, Alberti KGMM, Karran S, and Millward-Sadler GH, eds. Liver and Biliary Disease, Pathophysiology, Diagnosis and Management. London: W.B. Saunders Company Ltd., 1979:569-584.
17. Ramadori G, Armbrust T. Cytokines in the liver. Eur J Gastroenterol Hepatol 2001;13:777-784.
18. Kawada N. The hepatic perisinusoidal stellate cell. Histol Histopathol 1997;12:1069-1080.

19. Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis 2001;21:351-372.
20. Friedman SL. Liver fibrosis - from bench to bedside. J Hepatol 2003;38 Suppl 1:S38-53.:S38-S53.
21. Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. Toxicol Sci 2002;65:166-176.
22. Lemasters JJ. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. Am J Physiol 1999;276:G1-G6.
23. Reed JC. Mechanisms of apoptosis. Am J Pathol 2000;157:1415-1430.
24. Granville DJ, Carthy CM, Hunt DW, McManus BM. Apoptosis: molecular aspects of cell death and disease. Lab Invest 1998;78:893-913.
25. Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH, Lee TH. Involvement of two NF-kappa B binding elements in tumor necrosis factor alpha -, CD40-, and epstein-barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. J Biol Chem 2000;275:18022-18028.
26. Bradham CA, Plumpe J, Manns MP, Brenner DA, Trautwein Mechanisms of hepatic toxicity. I. TNF-induced liver injury. Am J Physiol 1998;275:G387-G392.
27. Trauner M, Fickert P, Stauber RE. Inflammation-induced cholestasis. J Gastroenterol Hepatol 1999;14:946-959.
28. Bortolami M, Venturi C, Giacomelli L, Scalerta R, Bacchetti S, Marino F, Floreani A, Lise M, Naccarato R, Farinati F. Cytokine, infiltrating macrophage and T cell-mediated response to development of primary and secondary human liver cancer. Dig Liver Dis 2002;34:794-801.
29. Weiskirchen R, Kneifel J, Weiskirchen S, van de Leur E, Kunz D, Gressner AM. Comparative evaluation of gene delivery devices in primary cultures of rat hepatic stellate cells and rat myofibroblasts. BMC Cell Biol 2000;1:4
30. Beyaert R, van Loo G, Heyninck K, Vandenabeele P. Signaling to gene activation and cell death by tumor necrosis factor receptors and Fas. Int Rev Cytol 2002;214:225-272.
31. Harper N, Hughes M, MacFarlane M, Cohen GM. Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis. J Biol Chem 2003;278:25534-25541.
32. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;17:1675-1687.
33. Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA, Lemasters JJ. The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. Mol Cell Biol 1998;18:6353-6364.
34. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1998;1366:177-196.

35. Sokol RJ, Straka MS, Dahl R, Devereaux MW, Yerushalmi B, Gumpricht E, Elkins N, Everson G. Role of oxidant stress in the permeability transition induced in rat hepatic mitochondria by hydrophobic bile acids. Pediatr Res 2001;49:519-531.
36. Newmeyer DD, Ferguson-Miller S. Mitochondria: releasing power for life and unleashing the machineries of death. Cell 2003;112:481-490.
37. Ricci JE, Gottlieb RA, Green DR. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. J Cell Biol 2003;160:65-75.
38. van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P. Mitochondrial intermembrane proteins in cell death. Biochem Biophys Res Commun 2003;304:487-497.
39. Kim JS, He L, Lemasters JJ. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem Biophys Res Commun 2003;304:463-470.
40. Fesik SW. Insights into programmed cell death through structural biology. Cell 2000;103:273-282.
41. Reed JC, Jurgensmeier JM, Matsuyama S. Bcl-2 family proteins and mitochondria. Biochim Biophys Acta 1998;1366:127-137.
42. Zhao Y, Ding WX, Qian T, Watkins S, Lemasters JJ, Yin XM. Bid activates multiple mitochondrial apoptotic mechanisms in primary hepatocytes after death receptor engagement. Gastroenterology 2003;125:854-867.
43. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609-619.
44. Diaz JL, Oltersdorf T, Horne W, McConnell M, Wilson G, Weeks S, Garcia T, Fritz LC. A common binding site mediates heterodimerization and homodimerization of Bcl-2 family members. J Biol Chem 1997;272:11350-11355.
45. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727-730.
46. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 1999;400:886-891.
47. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 1999;274:1156-1163.
48. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491-501.
49. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 1997;139:1281-1292.
50. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 1996;87:619-628.

51. Kelekar A, Thompson CB. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol 1998;8:324-330.
52. Werner AB, de Vries E, Tait SW, Bontjer I, Borst J. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. J Biol Chem 2002;277:22781-22788.
53. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J Biol Chem 1999;274:2225-2233.
54. Sun XM, Bratton SB, Butterworth M, MacFarlane M, Cohen GM. Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. J Biol Chem 2002;277:11345-11351.
55. Vander Heiden MG, Thompson CB. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1999;1:E209-E216.
56. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997;91:479-489.
57. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 1999;6:1067-1074.
58. Degli EM, Ferry G, Masdehors P, Boutin JA, Hickman JA, Dive C. Post-translational modification of Bid has differential effects on its susceptibility to cleavage by caspase 8 or caspase 3. J Biol Chem 2003;278:15749-15757.
59. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999;397:441-446.
60. van Loo G, Schotte P, van Gurp M, Demol H, Hoorelbeke B, Gevaert K, Rodriguez I, Ruiz-Carrillo A, Vandekerckhove J, Declercq W, Beyaert R, Vandenabeele P. Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ 2001;8:1136-1142.
61. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J 2001;20:6627-6636.
62. Yamaguchi H, Bhalla K, Wang HG. Bax plays a pivotal role in thapsigargin-induced apoptosis of human colon cancer HCT116 cells by controlling Smac/Diablo and Omi/HtrA2 release from mitochondria. Cancer Res 2003;63:1483-1489.
63. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102:43-53.
64. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33-42.

65. Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, Day CL, Tikoo A, Burke R, Wrobel C, Moritz RL, Simpson RJ, Vaux DL. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem 2002;277:445-454.
66. Okabe H, Irita K, Taniguchi S, Kurosawa K, Tagawa K, Yoshitake J, Takahashi S. Endotoxin causes early changes in glutathione concentrations in rabbit plasma and liver. J Surg Res 1994;57:416-419.
67. Xu Y, Jones BE, Neufeld DS, Czaja MJ. Glutathione modulates rat and mouse hepatocyte sensitivity to tumor necrosis factor toxicity. Gastroenterology 1998;115:1229-1237.
68. Trauner M, Meier PJ, Boyer JL. Molecular regulation of hepatocellular transport systems in cholestasis. J Hepatol 1999;31:165-178.
69. Bohan A, Chen WS, Denson LA, Held MA, Boyer JL. Tumor necrosis factor alpha-dependent up-regulation of Lrh-1 and Mrp3(Abcc3) reduces liver injury in obstructive cholestasis. J Biol Chem 2003;278:36688-36698.
70. Fausto N. Liver regeneration. J Hepatol 2000;32:19-31.
71. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 2003;120:117-130.
72. Bradham CA, Plumpe J, Manns MP, Brenner DA, Trautwein C. Mechanisms of hepatic toxicity. I. TNF-induced liver injury. Am J Physiol 1998;275:G387-G392.
73. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 1996;274:782-784.
74. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science 1996;274:787-789.
75. Sakon S, Xue X, Takekawa M, Sasazuki T, Okazaki T, Kojima Y, Piao JH, Yagita H, Okumura K, Doi T, Nakano H. NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. EMBO J 2003;22:3898-3909.
76. Bellas RE, FitzGerald MJ, Fausto N, Sonenshein GE. Inhibition of NF-kappa B activity induces apoptosis in murine hepatocytes. Am J Pathol 1997;151:891-896.
77. Jobin C, Sartor RB. The I kappa B/NF-kappa B system: a key determinant of mucosalinflammation and protection. Am J Physiol Cell Physiol 2000;278:C451-C462.
78. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K. Evidence that reactive oxygen species do not mediate NF-kappaB activation. EMBO J 2003;22:3356-3366.
79. Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. Free Radic Biol Med 1997;22:1115-1126.
80. Rothwarf DM, Karin M. The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci STKE 1999;1999:RE1.
81. Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, Goeddel DV. Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. Immunity 1999;10:421-429.

82. Zandi E, Karin M. Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. Mol Cell Biol 1999;19:4547-4551.
83. Ting AY, Endy D. Signal transduction. Decoding NF-kappaB signaling. Science 2002;298:1189-1190.
84. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science 2002;298:1241-1245.
85. Vos TA, Van Goor H, Tuyt L, Jager-Krikken A, Leuvenink R, Kuipers F, Jansen PL, Moshage H. Expression of inducible nitric oxide synthase in endotoxemic rat hepatocytes is dependent on the cellular glutathione status. Hepatology 1999;29:421-426.
86. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes Dev 1999;13:239-252.
87. Huang H, Joazeiro CA, Bonfoco E, Kamada S, Leverson JD, Hunter T. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. J Biol Chem 2000;275:26661-26664.
88. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappaB control. Proc Natl Acad Sci U S A 1997;94:10057-10062.
89. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998;281:1680-1683.
90. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410:37-40.
91. Roberts RA, James NH, Cosulich SC. The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis. Hepatology 2000;31:420-427.
92. Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, Hylemon PB. Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. Hepatology 2002;35:307-314.
93. Treisman R. Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 1996;8:205-215.
94. Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, Haegeman G. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. J Biol Chem 1998;273:3285-3290.
95. Foster FM, Traer CJ, Abraham SM, Fry MJ. The phosphoinositide (PI) 3-kinase family. J Cell Sci 2003;116:3037-3040.
96. Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem 1999;274:8347-8350.
97. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905-2927.
98. Khwaja A. Akt is more than just a Bad kinase. Nature 1999;401:33-34.

99. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 2003;4:346-358.

100. Noguchi P. Risks and benefits of gene therapy. N Engl J Med 2003;348:193-194.

101. Rots MG, Curiel DT, Gerritsen WR, Haisma HJ. Targeted cancer gene therapy: the flexibility of adenoviral gene therapy vectors. J Control Release 2003;87:159-165.

102. Morsy MA, Caskey CT. Expanded-capacity adenoviral vectors: the helper- dependent vectors. Mol Med Today 1999;5:18-24.

Chapter 3

Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-κB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis

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3.1 Abstract

In acute liver failure, hepatocytes are exposed to various cytokines that activate both cell survival and apoptotic pathways. NF-κB is a central transcription factor in these responses. Recent studies indicate that blocking NF-κB causes apoptosis, suggesting the existence of NF-κB-regulated anti-apoptotic genes. In the present study the relationship between NF-κB activation and apoptosis has been investigated in hepatocytes. *Methods*: Primary rat hepatocytes were exposed to a cytokine mixture of TNF-α, Il-1β, IFN-γ, and LPS. Modulation of signalling pathways was performed by using dominant negative adenoviral constructs. Apoptosis and NF-κB activation were determined by caspase-3 activity and Hoechst staining, and Electrophoretic Mobility Shift Assay, respectively. Furthermore, expression and regulation of apoptosis-related genes was investigated. *Results*: (1) Inhibition of NF-κB activation results in apoptosis. (2) Inhibitor of Apoptosis (IAP) family members, cIAP1, and XIAP, are expressed in rat hepatocytes. cIAP2 is induced by cytokines in an NF-κB-dependent manner and overexpression of cIAP2 inhibits apoptosis. (3) The anti-apoptotic Bcl-2 family member A1/Bfl-1 and the pro-apoptotic members Bak and Bid are induced by cytokines and NF-κB-dependent. (4) Nitric oxide (NO) inhibits caspase-3 activity in hepatocytes. *Conclusions:* In inflammatory conditions, hepatocyte survival is dependent on NF-κB activation and cIAP2 contributes significantly to this protection.

3.2 Introduction

In acute liver failure or acute viral hepatitis, hepatocytes are exposed to high levels of a variety of cytokines. These cytokines, in particular tumour necrosis factor α (TNF-α), activate both cell survival and apoptotic pathways in hepatocytes.[1;2] Trimerisation of the TNF-α type I receptor (TNFR-1) by TNF-α results in docking of adaptor proteins such as Fas-associated protein with death domain (FADD), TNFR-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2) and the serine/threonine kinase receptor interacting protein (RIP).[3] Recruitment of FADD to TNFR-1-bound TRADD results in the activation of caspase-8 followed by the activation of effector caspases, in particular caspase-3, resulting in apoptosis.[4]

TRADD is also a docking protein for TRAF2 and RIP. These proteins are involved in the induction of the NF-κB and Jun Kinase (JNK) survival pathways. The NF-κB survival pathway is initiated by the activation of NF-κB-inducing kinase (NIK). This protein activates an IκB kinase (IKK) complex, which results in the specific phosphorylation and proteasomal degradation of the inhibitor of NF-κB, IκBα. The release of NF-κB is followed by its migration to the nucleus, where it activates the transcription of NF-κB-responsive genes.[5]

Blocking of the NF-κB pathway in TNF-α-stimulated hepatocytes results in a shift towards apoptosis. This implies the existence of NF-κB-regulated anti-apoptotic genes.[6-8] Recent data suggest that members of the Inhibitor of Apoptosis (IAP) family may represent these genes. In different cell types, but not yet in hepatocytes, it has been demonstrated that the expression of members of the IAP family is regulated by NF-κB.[9-11] The IAP family was originally discovered in baculovirus and subsequently identified in human cells. This family includes cIAP2 (also known as human IAP1: HIAP1), cIAP1 (also known as human IAP2: HIAP2), X chromosome-linked IAP (XIAP), Survivin[11] and Livin[12]. These proteins have been reported to directly bind and inhibit the activation of caspase-3, -7 and –9.

Signalling initiated from caspase-8 is therefore blocked at the effector caspase level.[13] Another family, which is also critical in regulating cell death, is the Bcl-2 protein family.[14-16] This family consists of pro- (e.g. Bak, Bid and Bax) and anti-apoptotic (e.g. Bcl-2, Bcl-XL and A1/Bfl-1) members, which together regulate the integrity of the mitochondrial membrane.[17;18] The regulation of the Bcl-2 family by cytokines has been investigated in different cell types, but little is known about its expression and regulation in hepatocytes.

Finally, inducible nitric oxide synthase (iNOS) may act as an anti-apoptotic gene. Protection against TNF-α-induced apoptosis has been achieved by nitric oxide (NO).[19-21] NO is a product of iNOS, which has been found to be an NF-κB-regulated gene.[22] Although the inhibitory effect of exogenous nitric oxide on caspase activity has been demonstrated before[20], it is not clear whether inhibition of iNOS activity would result in apoptosis in cytokine-exposed hepatocytes.

In this study we want to investigate the relationship between NF-κB activation and apoptosis in hepatocytes. To mimick acute liver inflammation, in this study a mixture of cytokines is used containing tumour necrosis factor-α, interleukin-1β, interferon-γ, and LPS. The results of our study demonstrate that in inflammatory conditions hepatocyte survival is dependent on activation of NF-κB. The NF-κB-regulated gene cIAP2 inhibits caspase-3 activity and prevents apoptosis.

3.3 Materials and methods

Animals

Specified pathogen-free Male Wistar rats (220-250 g) were purchased from Harlan, Zeist, the Netherlands. They were housed under standard laboratory conditions with free access to standard laboratory chow and water. The study as presented was approved by the local Committee for Care and Use of Laboratory Animals.

Hepatocyte isolation and experimental design

Hepatocytes were isolated as described previously.[23] Cell viability was consistently more than 90 % as determined by trypan blue exclusion. Isolated hepatocytes were plated at a density of 125000 cells per cm^2 in William's medium E (Life Technologies Ltd., Breda, The Netherlands) supplemented with 50 μg/ml gentamycin, 2 μg/ml fungizone (Biowhittaker, Verviers, Belgium) and 20 mU/ml insulin (Novo Nordisk, Bagsvaerd, Denmark). During the attachment period (4 hours) 50 nmol/l dexamethasone (Sigma, St Louis, MO) and 5 % FCS (Life Technologies Ltd.) were added to the medium. Cells were cultured in a humidified incubator at 37 °C / 5 % CO_2.

Experiments were started twenty-four hours after isolation. Hepatocytes were exposed to 10 μg/ml LPS (*Escherichia coli*, serotype 0127:B8, Sigma, St. Louis, MO) and a cytokine mixture (CM) composed of 20 ng/ml recombinant mouse tumour necrosis factor α (TNFα, R&D Systems, Abingdon, United Kingdom), 10 ng/ml recombinant human interleukin-1β (IL-1β, R&D Systems) and 100 U/ml recombinant rat interferon-γ (IFNγ, Life Technologies Ltd.). Fifteen hours prior to CM exposure, cells receiving adenoviral constructs were infected with a Multiplicity of Infection (MOI) of 10 (as determined by plaque assay). In the case of double virus infections, cells received each virus at an MOI of 10 simultaneously. Thirty minutes prior to addition of cytokines, some cultures were exposed to 200 ng/ml of the transcriptional inhibitor actinomycin-D (ActD) or 250 μmol/l NO-donor V-

PYRRO/NO[24], or 2.5 mmol/l of the aspecific NOS-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, Sigma).

Each experimental condition was performed in triplicate wells. Two hours after addition of CM, cells were harvested for preparation of nuclear extracts. Ten hours after the addition of CM or at the indicated time-points in the time course study, cells were harvested and rinsed 3 times with ice-cold PBS prior to the addition of Trizol reagent (RNA isolation) (Life Technologies Ltd.) or hypotonic cell lysis buffer (protein analysis). This buffer consisted of 25 mmol/l HEPES (Sigma), 5 mmol/l $MgCl_2$ (MERCK**,** Darmstadt, Germany), 5 mmol/L EDTA (Sigma), 2 mmol/l PMSF (Sigma), 10 μg/ml Pepstatin A (Roche Biochemicals, Almere, The Netherlands) and 10 μg/ml Leupeptin (Roche Biochemicals), pH 7.5. Cells were stored at –80 °C (RNA) or –20 °C (protein). Each experiment was performed three times, using hepatocytes from different isolations.

Adenoviral constructs

Recombinant, replication-deficient adenovirus Ad5IκBAA was used to inhibit NF-κB activation as described previously.[25] This adenovirus contains a construct driven by the cytomegalovirus promoter-enhancer in which IκBα has been mutated at serines 32 and 36. Therefore, mutant IκBα cannot be phosphorylated and binds NF-κB irreversibly, preventing its activation. As a control virus Ad5LacZ was used, which contains the *E. coli* β-galactosidase gene. The Ad5dnFADD expresses a FADD mutant lacking the death effector domain. It is therefore unable to bind caspase-8.[26] The AdHIAP1 virus contains human IAP1 which is the human homologue of rat cIAP2.[27]

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using a final concentration of 0.25% Nonidet P-40 as described previously.[28] EMSA for NF-κB was performed as described previously.[28]

Caspase-3 enzyme activity assay

Caspase-3 enzyme activity was assayed in cells using a caspase-3 activity kit with fluorimetric detection (Promega) according to the manufacturer's instructions. 20 μg of protein was used.

Microscopic determination of apoptosis

Hoechst 33342 (Sigma-Aldrich Chemie, Schnelldorf, Germany) was used to detect apoptotic nuclei in hepatocytes. Hepatocytes were seeded on glass coverslides and exposed to CM with or without ActD or Ad5IκBAA. Fifteen hours after addition of CM, cells were incubated with 4.7 μg/ml Hoechst 33342 for 5 minutes at 37 °C / 5 % CO_2. Glass coverslides were rinsed twice in HBSS (Life Technologies Ltd.) and placed upside down on microscope slides. Fluorographs were visualised and monitored using a Leitz fluorescence microscope.

RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated using the Trizol method (Life Technology Ltd.) according to the manufacturer's instructions. Reverse trancription was performed on 5 μg of total RNA using random primers in a final volume of 75 μl (Reverse Transcription System, Promega, Madison, WI). Each PCR was performed as described previously.[29] PCR primes are listed in table 1. For every PCR, GAPDH was used as internal control. Each PCR product was loaded on a 2 % agarose gel and stained with ethidium bromide.

Table 1. Oligonucleotide primers used for the analysis of pro- and anti apoptotic genes by RT-PCR

Primers (species)	Sense and Antisense	PCR product (bp)	# cycles	Annealing temp(°C)
cIAP2 (rat)	5'-ACATTTCCCCAGCTGCCCATTC -3'	622	30	60
	5'-CTCCTGCTCCGTCTGCTCCTCT-3'			
cIAP1 (rat)	5'-CCAGCCTGCCCTCAAACCCTCT-3'	502	30	61
	5'-GGGTCATCTCCGGGTTCCCAAC-3'			
XIAP (rat)	5'-CGCGAGCGGGGTTTCTCTACAC-3'	510	28	61
	5'-ACCAGGCACGGTCACAGGGTTC-3'			
iNOS (rat)	5'-CGAGGAGGCTGCCTGCAGACTGG-3'	1383	26	60
	5'-CTGGGAGGAGCTGATGGAGTAGTA-3'			
Bcl-2 (rat)	5'-GCTACGAGTGGGATACTGGAGA-3'	446	30	58
	5'-AGTCATCCACAGAGCGATGTT-3'			
Bcl-XL (rat)	5'-GCATATCAGAGCTTTGAACAGGT-3'	534	30	56
	5'-CTTTCACAGAAGCGTGGTAGATT-3'			
Bak (mouse)	5'-TCTCCACCACGACCTGAAAAAT-3'	494	30	56
	5'-GATATCAGCCAAAAAGCAGGTC-3'			
Bid (mouse)	5'-AGTCAGGAAGAAATCATCCACAA-3'	361	30	58
	5'-CTCCTCAGTCCATCTCGTTTCTA-3'			
Bax (rat)	5'-AGGATGATTGCTGATGTGGATAC-3'	300	30	56
	5'-CACAAAGATGGTCACTGTCTGC-3'			
A1/Bfl-1 (rat)	5'-ATCCACTCCCTGGCTGAGAACT-3'	311	30	56
	5'-ACATCCAGGCCAATCTGCTCTT-3'			
GAPDH (rat)	5'-CCATCACCATCTTCCAGGAG-3'	576	22	58
	5'-CCTGCTCACCACCTTCTTG-3'			

Western blot analysis

Hepatocytes were scraped and cell lysates were obtained by three cycles of freezing (-80 °C) and thawing (37 °C) followed by centrifugation for 5 minutes at 13.000 rpm. Western blot analysis for iNOS was performed as described before.[30] Blots were incubated with a dilution of 1:1000 of rabbit anti-rat iNOS antibody.[30]

Statistical analysis

Results are presented as the mean ± standard deviation. One-way ANOVA and Student-Newman-Keuls test were used to determine the significance of differences between experimental groups. A P value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3.4 Results

Specific inhibition of the NF-κB pathway results in apoptosis

To investigate the relationship between NF-κB activation and caspase-3 activity, hepatocytes were incubated with a cytokine mixture (CM) for 10 hours with or without adenoviral constructs that selectively inhibit various signalling pathways. Functionality of Ad5IκBAA and Ad5dnFADD was demonstrated by EMSA and caspase-3 assay, respectively. In the presence of CM alone, NF-κB was clearly activated as determined by EMSA (Fig 1).

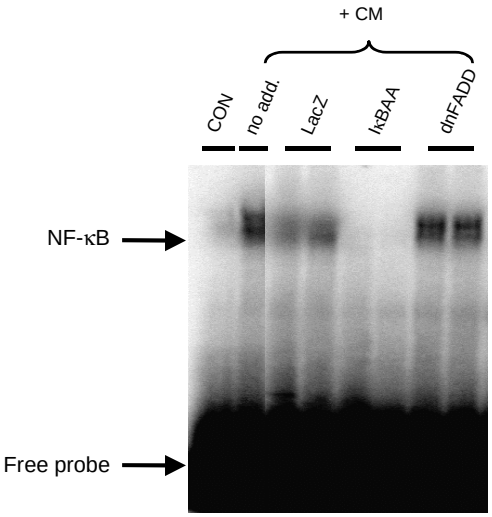


Figure 1. NF-κB activation is FADD-independent and inhibited by Ad5IκBAA. Cultured rat hepatocytes were treated as indicated in the figure. NF-κB activation was measured by EMSA. One or two representative samples per condition are shown. (Con = control hepatocytes; no add. = no additives; CM = cytokine mixture; Ad5dnFADD and Ad5IκBAA express mutated FADD, and IκB-α, respectively; Ad5LacZ = control virus).

The activation of NF-κB was inhibited by the dominant negative IκB-construct, whereas control (LacZ) virus had no effect (Fig 1).

Next, the corresponding effect on caspase-3 activity was determined. A positive control experiment was performed with CM in the presence of ActD, which induces apoptosis in many cell types, including hepatocytes. The induction of caspase-3 activity was F ADD-dependent, as shown in Figure 2A. ActD alone or

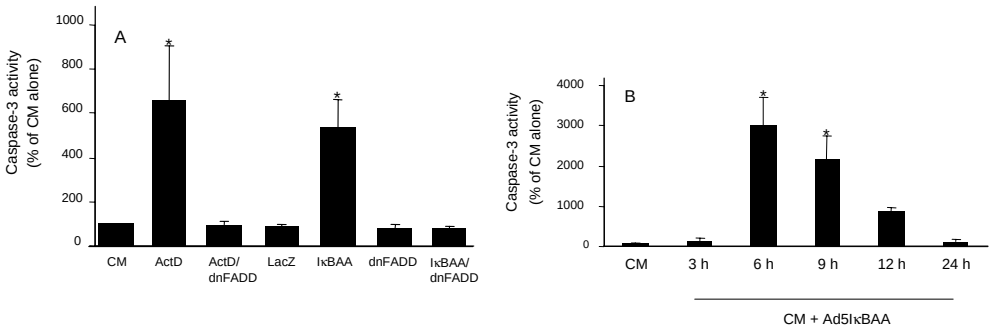


Figure 2. Specific inhibition of NF-κB results in a FADD-dependent increase of caspase-3 activity, peaking at 6 hours after CM exposure. (A) Cultured rat hepatocytes were treated as indicated in the figure. All conditions were performed in the presence of CM. (B) Time course study on hepatocytes exposed to CM for 3, 6, 9, and 12 hours (h) and the adenoviral construct Ad5IκBAA. Caspase-3 enzyme activity is shown as percentage of CM alone. The data represent mean of 3 independent experiments ± SD. *P <0.05 (compared with other groups). (CM = cytokine mixture; ActD = Actinomycin-D; adenoviral constructs (Ad5) expressing dnFADD, and IκBAA; LacZ = control virus).

CM alone had no effect on caspase-3 activity compared to control hepatocytes (data not shown). Specific inhibition of NF-κB activation by Ad5IκBAA resulted in FADD-dependent induction of caspase-3 activity. Control (LacZ) virus had no effect on caspase-3 activity. Caspase-3 activity started to rise at least 3 hours after cytokine addition in hepatocytes with a blocked NF-κB pathway (Fig 2B). Caspase-3 activity peaked at 6 hours and returned to normal 24 hours after cytokine addition.

To confirm that activation of caspase-3 activity results in apoptosis, staining with Hoechst 33342 was performed to detect apoptotic nuclei (Fig 3). Nuclear fragmentation and condensation of chromatin were observed in many hepatocytes exposed to CM in the presence of actinomycin-D or Ad5IκBAA, but not in control hepatocytes or hepatocytes exposed to CM alone.

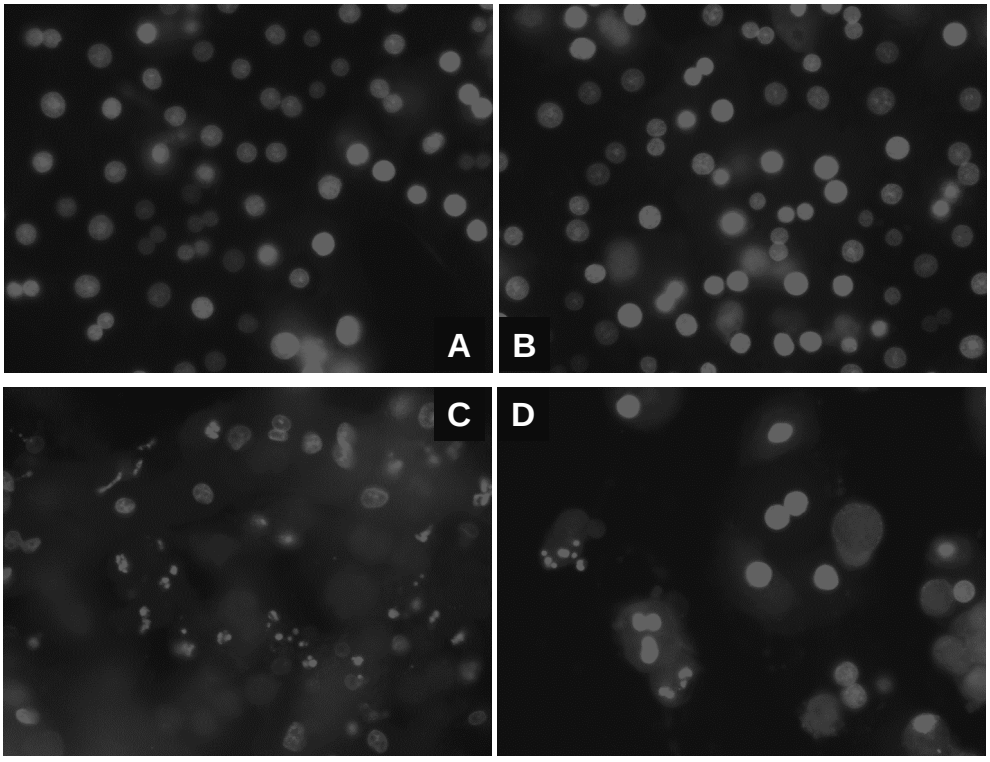


Figure 3. Inhibition of NF-κB results in apoptotic nuclei. Control hepatocytes (A); hepatocytes exposed to CM (B), CM + ActD (C); and CM + Ad5IκB (D) were stained by Hoechst 33342 for determination of nuclear morphological alterations. The original magnification of all panels is 400x.

These data indicate the importance of NF-κB-regulated transcription of anti-apoptotic genes that inhibit caspase-3 activity. Therefore, we investigated their expression and their effect on caspase-3 activity. In order to approximate the *in*

vivo situation, experiments were performed in the presence of a mixture of cytokines (CM).

cIAP2 is an NF-κB-regulated gene and prevents apoptosis in hepatocytes

IAP protein family members are potent inhibitors of caspases and may play an important role in maintaining the pro-/anti-apoptotic balance after NF-κB activation. Both cIAP1 and XIAP, but not cIAP2 are clearly expressed in freshly isolated rat hepatocytes corresponding to hepatocytes *in vivo* (Fig 4A). However, cIAP2 expression is strongly increased 3 hours after cytokine addition (Fig 4B) preceding the 6-hour-peak of caspase-3 activity.

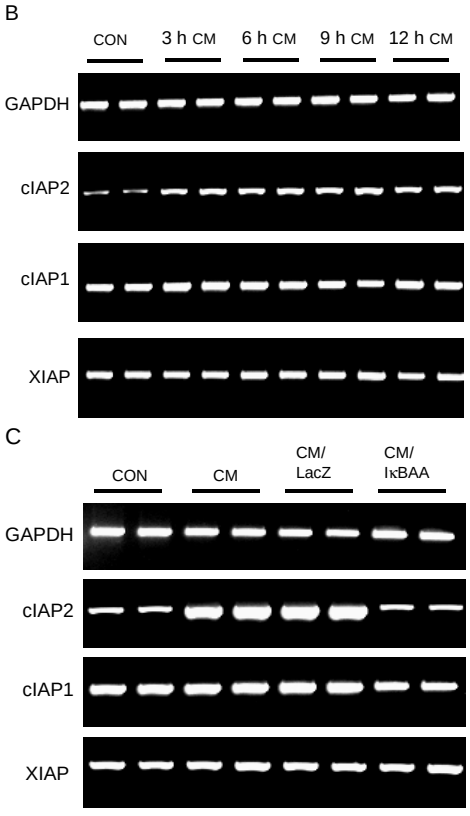


Figure 4. cIAP2 is induced 3 hours after cytokine exposure and is NF-κB dependent. (A) cIAP2, cIAP1 and XIAP mRNA expression in freshly isolated rat hepatocytes. (B) mRNA expression of GAPDH, cIAP2, cIAP1 and XIAP in a time course study on cultured hepatocytes exposed to CM for 3, 6, 9, and 12 hours (h). (C) mRNA expression of GAPDH, cIAP2, cIAP1 and XIAP in cultured rat hepatocytes treated with or without CM in the presence of adenoviral (Ad5) LacZ or IκBAA. Expression of mRNA was determined by RT-PCR. One of three representative experiments of n = 2 per condition is shown. (Con = control hepatocytes; CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; Ad5LacZ = control virus).

Elevated cIAP2 expression persists at least 12 hours after cytokine exposure. cIAP1 and XIAP expression are not increased by cytokines. Experiments on hepatocytes exposed to CM alone or in the presence of Ad5IκBAA reveal that cIAP1 and XIAP are not regulated by NF-κB (Fig 4C), whereas the cytokine-induced expression of cIAP2 is NF-κB dependent.

To demonstrate that cIAP2 is able to inhibit caspase-3, hepatocytes were incubated with CM alone or in the presence of Ad5IκBAA and/or AdHIAP1 (Fig 5). The increase in caspase-3 activity obtained with CM + Ad5IκBAA is completely prevented by AdHIAP1, demonstrating inhibition of caspase-3 by cIAP2 in hepatocytes.

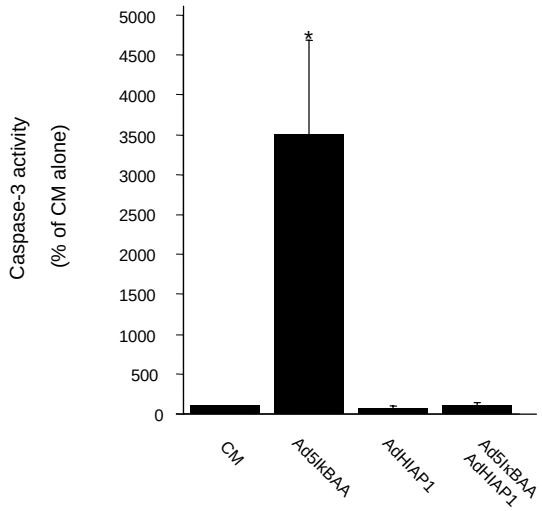
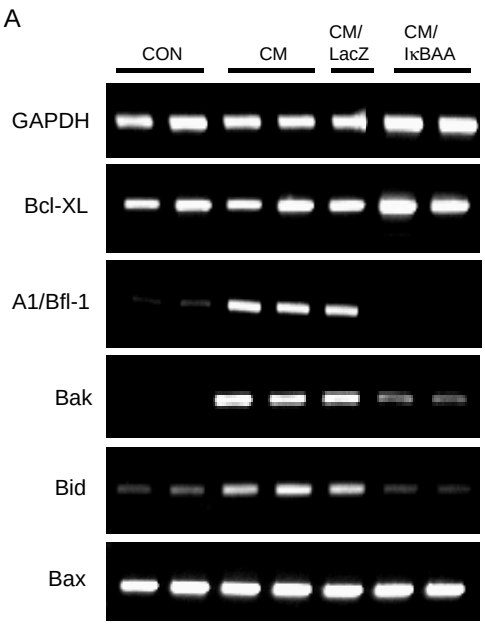


Figure 5. cIAP2 prevents apoptosis in hepatocytes. Cultured rat hepatocytes were treated as indicated in the figure. All conditions were performed in the presence of CM. Caspase-3 activity is shown as percentage of CM alone. The data represent mean of 3 independent experiments ± SD. *P<0.05 (compared with other groups). (CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; AdHIAP1 express HIAP1; Ad5LacZ = control virus).

Anti-apoptotic A1, and Pro-apoptotic Bak and Bid are NF-κB regulated genes

Since the Bcl-2 family plays an important role in regulating apoptosis, the regulation of Bcl-2 family members by cytokines in hepatocytes was investigated.



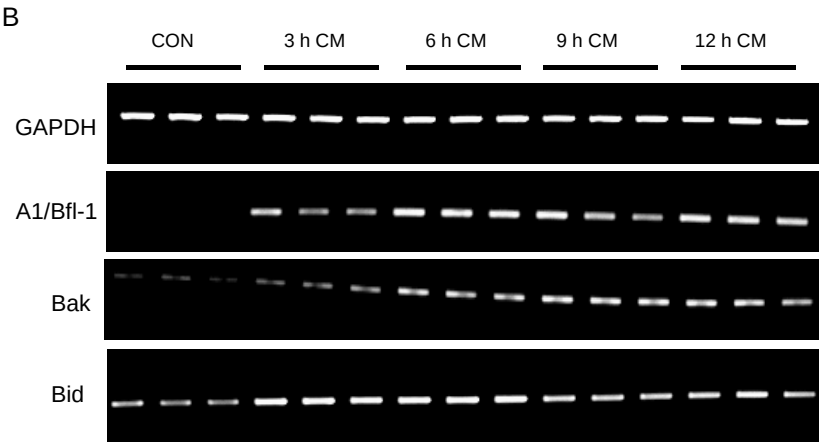


Figure 6. A1/Bfl, Bak and Bid are induced by cytokines in an NF-κB-dependent manner. (A) Cultured rat hepatocytes were treated with or without CM in the presence of adenoviral (Ad5) LacZ or IκBAA. mRNA expression of anti-apoptotic Bcl-XL and A1/Bfl-1, and pro-apoptotic Bak, Bid and Bax was determined by RT-PCR. (B) A time course study on hepatocytes exposed to CM for 3, 6, 9, and 12 hours (h). mRNA expression of A1/Bfl, Bak and Bid. One of three representative experiments of n = 2 or 3 per experimental condition is shown. GAPDH served as internal control. (Con = control hepatocytes; CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; Ad5LacZ = control virus).

With the exception of Bcl-2, mRNA levels of all investigated Bcl-2 family members were identical in freshly isolated hepatocytes, and primary cultured hepatocytes (data not shown). Bcl-2 expression was only shown in late-cultured hepatocytes, but not in freshly isolated and early cultured hepatocytes. This allowed further investigation of these Bcl-2 family members, whereas Bcl-2 was not examined in detail.

Exposure of hepatocytes to CM induced the expression of anti-apoptotic A1/Bfl-1 and pro-apoptotic Bak and Bid. This induction is abrogated in the presence of Ad5IκBAA, indicating that these genes are NF-κB-dependent. CM had no effect on anti-apoptotic Bcl-XL and pro-apoptotic Bax (Fig 6A). mRNA expression of A1/Bfl is already increased 3 hours after CM exposure (Fig 6B) preceding the peak of caspase-3 activity and it is still elevated 12 hours after CM exposure. Although Bid and Bak expression followed approximately the time-course of A1/Bfl, the induction of Bak expression was somewhat later.

Study on iNOS and its anti-apoptotic role in hepatocytes

Since exogenous NO is able to inhibit apoptosis in various cell types, in the present report the regulation of iNOS and its importance as an anti-apoptotic protein were investigated. Using Ad5IκBAA, we confirm that the cytokine-induced expression of iNOS in hepatocytes is NF-κB-dependent both at the mRNA and protein level (Fig 7A). Control (LacZ) virus has no effect on iNOS expression. mRNA expression of iNOS is clearly induced after 3 hours of CM exposure and remains unchanged after 12 hours of CM addition (Fig 7B).

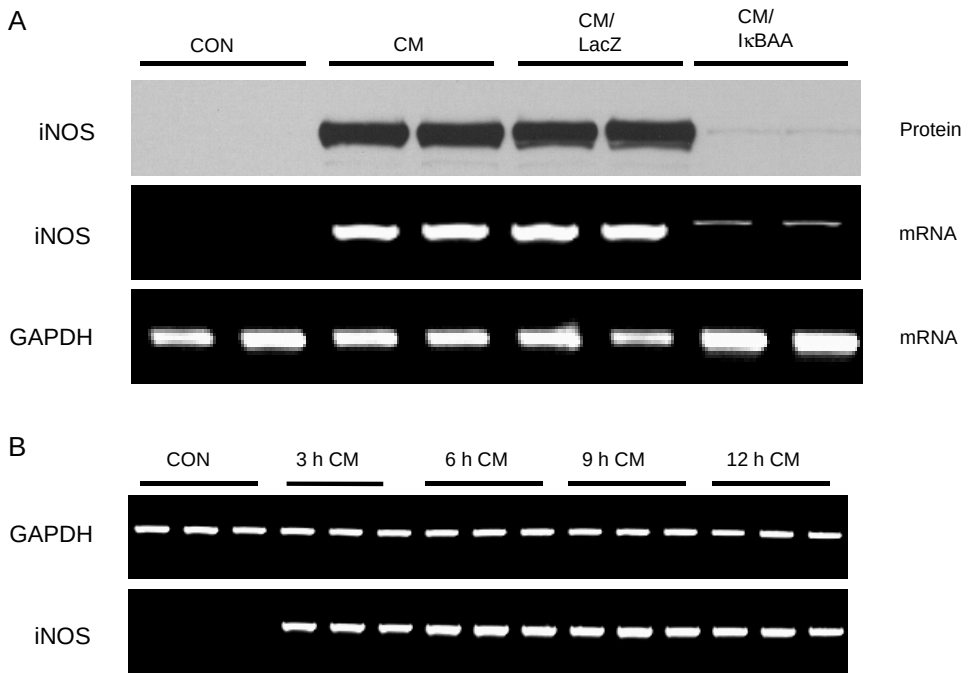


Figure 7. iNOS is induced 3 hours after cytokine addition and NF-κB-dependent. (A) iNOS protein and mRNA (iNOS and GAPDH) expression in cultured rat hepatocytes treated with or without CM in the presence of adenoviral (Ad5) LacZ or IκBAA. One of three representative experiments of n = 2 per condition is shown. (B) mRNA expression of iNOS and GAPDH in a time course study on hepatocytes exposed to CM for 3, 6, 9, and 12 hours (h). One of three representative experiments of n = 3 per condition is shown. (Con = control hepatocytes; CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; Ad5LacZ = control virus).

Addition of V-PYRRO/NO to hepatocytes exposed to CM and ActD resulted in a 50 % decrease of caspase-3 activity (Fig 8). This confirms previous reports[20,21] and demonstrates that NO inhibits caspase-3 activity. Inhibition of iNOS activity in CM-exposed hepatocytes by L-NAME did not increase caspase-3 activity, as shown in Figure 8.

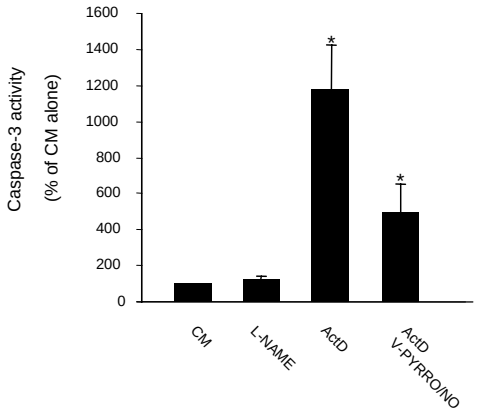


Figure 8. Inhibition of iNOS does not affect caspase-3 activity, whereas exogenous NO is anti-apoptotic. Hepatocytes were treated as indicated in the figure. All conditions were performed in the presence of CM. Caspase-3 activity is shown as percentage of CM. The data represent mean of 3 independent experiments ± SD. *P<0.05 (compared with other groups). (Con = control hepatocytes; CM = cytokine mixture; ActD = Actinomycin-D; L-NAME = inhibitor of iNOS; V-PYRRO/NO = NO-donor).

Comparison of cytokine mixture- to TNF-α- induced gene expression

Since TNF-α alone is often used to study apoptotis in hepatocytes, we compared TNF-α and CM-induced expression of NF-κB-regulated genes (Fig 9). TNF-α and CM induced the expression of anti-apoptotic cIAP2 to the same extent. The expression of anti-apoptotic A1/Bfl is more strongly induced by CM compared to TNF-α alone, whereas iNOS and Bak are hardly induced by TNF-α.

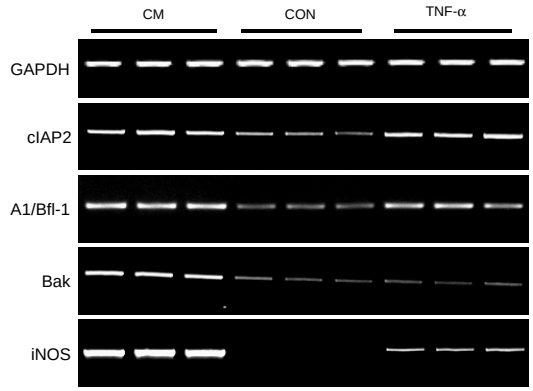


Figure 9. Comparison of CM- to TNF-α-induced NF-κB-regulated gene expression. Hepatocytes were treated as indicated in the figure. mRNA expression of GAPDH, anti-apoptotic cIAP2 and A1/Bfl-1, and iNOS, and pro-apoptotic Bak was determined by RT-PCR. One of three representative experiments of n = 3 per experimental condition is shown. (Con = control hepatocytes; CM = cytokine mixture; TNF-α = tumor necrosis factor α).

3.5 Discussion

In acute liver failure or acute viral hepatitis, hepatocytes are exposed to high levels of a variety of cytokines, such as TNF-α, IL-1β, IFN-γ and LPS. These cytokines simultaneously activate both survival and apoptotic pathways and it depends on the level of pro- and anti-apoptotic activities whether the balance will tip to one side or the other. Unlike Fas, cytokines like TNF-α are by themselves not sufficient to induce apoptosis.[6] Disturbance of NF-κB activation, either by ActD or an adenovirus expressing a mutated IκB-α, results in a shift towards apoptosis, as shown here by an increased FADD-dependent caspase-3 activity and nuclear fragmentation. Our study demonstrates that the anti-apoptotic effect of NF-κB is, to a large extent, due to the NF-κB-dependent transcription of the anti-apoptotic cIAP2 which inhibits caspase-3 activity.

The NF-κB-dependence of rat cIAP2 in hepatocytes correlates with findings in other cell types.[9;31;32] Overexpression of the human homologue of rat cIAP2 (HIAP1) prevents caspase-3 activation in hepatocytes in which NF-κB activation is prevented and cIAP2 expression is absent. These results clearly demonstrate the importance of cIAP2 in inhibiting apoptosis: in cytokine-exposed hepatocytes in which NF-κB activation is blocked, cIAP1 and XIAP are normally expressed, whereas expression of cIAP2 is abolished. Furthermore, the time course of cIAP2 mRNA induction precedes the activation of caspase-3. Our findings correlate with observations in neurons in which overexpression of cIAP2 delays cell death.[27;33] Although cIAP1 (HIAP2) and XIAP are not NF-κB-dependent in hepatocytes, XIAP and cIAP1 are NF-kB-regulated in human endothelial cells[11;34] and human skin epithelial cells[35] and in the human fibrosarcoma cell line HT1080.[10]

Little is known about the regulation of Bcl-2 family members in hepatocytes. The present report demonstrates that NF-κB is strongly involved in the cytokine-induced expression of pro-apoptotic Bak and Bid, and the anti-apoptotic A1/Bfl-1 in hepatocytes. Since inhibition of NF-κB tips the balance towards apoptosis, the

upregulation of some pro-apoptotic genes appears surprising. On the other hand, the anti-apoptotic Bcl-2 family member A1/Bfl-1 is a potent inhibitor of apoptosis.[14] Moreover one may conclude from the observed effects of NF-κB inhibition, that the NF-κB-mediated activation of anti-apoptotic cIAP2 and A1/Bfl is dominant over the NF-κB activation of pro-apoptotic Bak and Bid. Since anti-apoptotic Bcl-2 is only expressed in late-cultured hepatocytes, this expression is a culture artefact. Therefore, the role of Bcl-2 has not been investigated further.

Since TNF-α is frequently used to study apoptosis[6;26;36], we compared the effects of CM to TNF-α alone. This study demonstrates that some NF-κB-dependent genes are hardly induced by TNF-α alone. TNF-α induces similar induction of Bid and A1 compared to cytokine mixture indicating that IL-1β and IFN-γ play a minor role in the induction of these genes. In contrast, TNF-α alone only partially induced Bak and iNOS expression, suggesting the presence of IL-1β and IFN-γ responsive elements in the Bak and iNOS promoter.

Although some studies reported NF-κB-regulated expression of anti-apoptotic Bcl-XL in various cell types[15;16], we do not observe NF-κB-regulated induction of Bcl-XL in hepatocytes. Also the mRNA expression of pro-apoptotic Bax is not regulated by NF-κB in hepatocytes.

The protective role of NO in hepatocytes has been established in different studies.[20;21;37] Our report confirms that NO is an inhibitor of caspase-3 activity. Furthermore, we confirmed that iNOS is regulated by NF-κB. However, the exact importance of iNOS-derived NO in the inhibition of caspase-3 remains to be clarified. Inhibition of NO synthesis using L-NAME in cytokine-exposed hepatocytes did not increase caspase-3 activity. An explanation of this observation is that cIAP2 is a very effective inhibitor of caspase-3. In cytokine-exposed hepatocytes in the presence of L-NAME, the NF-κB pathway is still intact (data not shown) and cIAP2 is induced and active. In this situation, the lack of NO is compensated by the presence of active cIAP2. In cytokine-exposed hepatocytes in which the NF-κB pathway is inhibited, we demonstrate that cIAP2 and iNOS are

not expressed. In these hepatocytes, exogenous NO inhibits caspase-3 which is also shown by others.[20] From these observations we conclude that during inflammation various anti-apoptotic signals exist to protect hepatocytes against apoptosis. Exogenous NO could be useful as an anti-apoptotic agent in conditions in which NF-κB activation is compromised.

The results of the present report demonstrate that in inflammatory conditions hepatocyte survival is dependent on NF-κB activation. NF-κB activation results in the transcription of anti-apoptotic genes, in particular cIAP2, which in turn inhibits caspase-3 activity and apoptosis.

3.6 Acknowledgements

We thank Dr. Larry K. Keefer (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland) for providing V-PYRRO/NO, Ronald Boonstra (Department of Pathology, Universital Hospital Groningen, Groningen, The Netherlands) for his assistance with the fluorescence microscope and Rick Havinga (Department of Pediatrics, University Hospital Groningen, Groningen, The Netherlands) for isolating rat hepatocytes.

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3.7 References

1. Kaplowitz, N. Mechanisms of liver cell injury. J Hep 2000;32:39-47.
2. Bradham CA, Plümpe J, Manns MP, Brenner DA and Trautwein C. Mechanisms of hepatic toxicity I. TNF-induced liver injury. Am J Physiol 1998;275 (Gastrointest Liver Physiol 38):G387-G392.
3. Strasser A, O'Connor L and Dixit VM. Apoptosis Signaling. Annu Rev Biochem 2000;69:217-45
4. Reed JC. Warner-Lambert/Parke-Davis award lecture, mechanisms of apoptosis. Am J Pathol 2000;157:1415-1430.
5. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV and Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 1999;17:331-67.
6. Xu Y, Bialik S, Jones BE, Iimuro Y, Kitsis RN, Srinivasan A, Brenner DA et al. NF-κB inactivation converts a hepatocyte cell line TNF-α response from proliferation to apoptosis. Am J Pathol 1998;275 (Cell Physiol 44):C1058-C1066.
7. Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA and Lemasters JJ. The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. Mol Cell Biol 1998;18:6353-6364.
8. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH and Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc Natl Acad Sci USA 1997;94:10057-10062.
9. Wang CY, Mayo MW, Korneluk RG, Goeddel DV and Baldwin AS Jr. NF-κB anti-apoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998;281:1680-1683.
10. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR and Lipp J. Nuclear factor (NF)-KappaB-regulated X-chromosome-linked gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J Exp Med 1998; 188:211-216.
11. Deveraux QL and Reed JC. IAP family proteins, suppressors of apoptosis. Genes & Dev 1999;13:239-252.
12. Kasof GM and Gomes BC. Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem. 2001;276:3238-3246.
13. Hay BA. Understanding IAP function and regulation: a view from Drosophila. Cell Death and Diff. 2000;7:104501056.
14. Zong WX, Edelstein LC, Chen C, Bash J and Gélinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNF-α-induced apoptosis. Genes & Dev 1999;13:382-387.
15. Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T et al. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NF-κB activation in primary hippocampal neurons. J Biol Chem 1999;274:8531-8538.
16. Chen C, Edelstein LC and Gélinas C. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-XL. Mol Cell Biol 2000;20:2687-2695.

17. Chao DT and Korsmeyer SJ. Bcl-2 family: regulators of cell death. Annu Rev Immunol 1998;16:395-419.
18. Gross A, McDonnell JM and Korsmeyer SJ. Bcl-2 family members and the mitochondria in apoptosis. Genes & Dev 1999;13:1899-1911.
19. Kim YM, Talanian RV and Billiar TR. Nitric Oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 1997;272:31138-31148.
20. Li J, Bombeck CA Yang S, Kim YM and Billiar TR. Nitric Oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. J Biol Chem 1999;274:17325-17333.
21. Kim, YM, Kim TH, Chung HT, Talanian RV, Yin XM and Billiar TR. Nitric Oxide prevents tumor necrosis factor-α-induced rat hepatocyte apoptosis by the interruption of mitochondrial apoptotic signaling through s-nitrosylation of caspase-8. Hepatology 2000;32:770-778.
22. Vos TA, van Goor H, Tuyt L, de Jager-Krikken A, Leuvenink R, Kuipers F, Jansen PLM et al. Expression of inducible nitric oxide synthase in endotoxemic rat hepatocytes is dependent on the cellular glutathione status. Hepatology 1999;29:421-426.
23. Moshage H, Casini A and Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990;12:511-518.
24. Saavedra JE, Billiar TR, Williams DL, Kim YM, Watkins, SC and Keefer LK. Targeting Nitric Oxide (NO) delivery in vivo. Design of a liver-selective NO donor prodrug that blocks tumor necrosis factor-κ-induced apoptosis and toxicity in the liver. J Med Chem1997;40:1947-1954.
25. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW and Brenner DA. NF-κB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998;101:802-811.
26. Hatano E, Bradham CA, Stark A, Limuro Y, Lemasters JJ and Brenner DA. The mitochondrial permeability transition augments Fas-induced apoptosis in mouse hepatocytes. J Biol Chem 2000;275:11814-11823.
27. Perrelet D, Ferri A, Mackenzie AE, Smith GM, Korneluk RG, Liston P, Sagot Y et al. IAP family proteins delay motoneuron cell death in vivo. Eur J Neuroscience 2000;12:2059-2067.
28. Tuyt LML, Bregman K, Lummen C, Dokter WHA and Vellenga E. Differential binding activity of the transcription factor LIL-Stat in immature and differentiated normal and leukemic myeloid cells. Blood 1998;92:1364-1373.
29. Ros JE, Schuetz JD, Geuken M, Streetz K, Moshage H, Kuipers F, Manns MP et al. Induction of Mdr1b expression by Tumor Necrosis Factor-α in rat liver cells is independent of p53 but requires NF-κB signaling. Hepatology 2001 ;33 :1425-1431.
30. Vos TA, Gouw ASH, Klok PA, Havinga R, van Goor H, Huitema S, Roelofsen H et al. Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. Gastroenterology 1997;113:1323-1333.

31. Erl W, Hansson GK, de Martin R, Draude G, Weber KSC and Weber C. Nuclear factor-κB regulates induction of apoptosis and Inhibitor of Apoptosis protein-1 expression in vascular smooth muscle cells. Circ Res 1999;84:668-677.
32. Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH and Lee TH. Involvement of two NF-κB binding elements in tumor necrosis factor-α-, CD40-, and Epstein-Barr virus latent membrane protein 1-mediated induction of the cellular Inhibitor of Apoptosis protein 2 gene. J Biol Chem 2000;275:18022-18028.
33. Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, Mackenzie AE, Bähr M et al. Adenovirus-mediated gene transfer of Inhibitors of Apoptosis proteins delays apoptosis in cerebellar granule neurons. J Neurochem 1999;72:292-301.
34. Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J and de Martin R. Activation of NF-κB by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. J Biol Chem 2000;275:22064-22068.
35. Seitz CS, Freiberg RA, Hinata K and Khavari PA. NF-κB determines localization and features of cell death in epidermis. J Clin Invest 2000;105:253-260.
36. Jones BE, Lo CR, Liu H, Srinivasan A, Streetz K, Valentino KL and Czaja MJ. Hepatocytes sensitized to tumour necrosis factor-α cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. J Biol Chem 2000;275:705-712.
37. Li J and Billiar TR. The anti-apoptotic actions of nitric oxide in hepatocytes. Cell Death and Diff 1999;6:952-955.

Chapter 4

Adenoviral overexpression of caspase inhibitor HIAP1 is not protective in a mouse model of severe acute liver failure

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In preparation

4.1 Abstract

Upon acute liver injury, Kupffer cells and newly recruited leukocytes are activated followed by the production of pro-inflammatory cytokines and reactive oxygen species. These events induce massive apoptotic and necrotic cell death of hepatoctyes. Previously, we have shown that under these conditions, apoptosis can be blocked in hepatocytes *in vitro* through adenoviral expression of the human Inhibitor of Apoptosis protein1 (HIAP1). The hypothesis of the present study is that HIAP1 gene transfer rescues hepatocytes from apoptosis during acute liver injury. To investigate this, BALB/c mice were treated i.v. with control recombinant adenovirus (Ad5LacZ) or adenovirus encoding HIAP1 (AdHIAP1) using 5 x 10^9 pfu per animal. After three days, these mice and control mice were injected i.p. with 20 mg GalN and 250 ηg LPS. Seven hours after this treatment, livers were screened for transgene expression, morphology, inflammation and cell death. In addition, XIAP transgenic mice were exposed to LPS/GalN. Our results demonstrated that HIAP1 was only expressed in AdHIAP1-pretreated livers. The adenoviral vector itself was non-toxic to normal livers as shown by normal vitality and absence of necrosis, apoptosis and inflammation. However, when acute liver injury was induced with LPS/GalN in HIAP1-positive livers, apoptosis, necrosis and liver damage were increased dramatically compared to animals receiving only LPS/GalN. In contrast, inflammation in LPS/GalN-exposed livers was clearly decreased by viral-pretreatment. LPS/GalN treatment of XIAP-transgenic animals exaggerated apoptosis as well. In conclusion, these data imply that IAP overexpression is detrimental in LPS/GalN-induced liver injury. This study is important for gene therapy as a treatment of acute liver diseases.

4.2 Introduction

Acute liver failure is associated with high mortality. It develops as a consequence of viral hepatitis, toxin- or drug-induced toxicity or due to rejection after liver transplantation.[1] In acute liver injury, Kupffer cells and newly recruited leukocytes are activated which results in the production of cytokines, like Tumour Necrosis Factor-α (TNF-α), and reactive oxygen species. This triggers apoptotic and necrotic cell death of hepatocytes. In hepatocytes, apoptosis is only induced when NF-κB activation is prevented,[2; 3] implicating the importance of NF-κB-regulated anti-apoptotic genes. Recently, we demonstrated that cIAP2 is an NF-κB-regulated gene which prevents apoptosis in cytokine-exposed hepatocytes *in vitro*. The protein cIAP2 belongs to the Inhibitor of Apoptosis Protein (IAP) family and is also known as human IAP1 (HIAP1). Overexpression of HIAP1 protects against apoptosis in hepatocytes *in vitro*.[2] The IAP family also includes XIAP1, HIAP2, NAIP, Survivin and Livin, of which Survivin and Livin are predominantly expressed in cancer cells. IAP family members are potent inhibiters of pro- and active caspase-9, and active caspase-3, thereby arresting the apoptotic cascade initiated by caspase-8.[4] The IAP family does not inhibit active caspase-8 itself.

Since hepatocyte apoptosis is a strictly regulated process and a key event in the development of acute liver failure, inhibition of apoptosis by means of gene therapy may prevent lethal liver failure. In this study, we therefore investigate whether HIAP1 gene expression rescues hepatocytes from apoptosis during development of acute liver failure.

Animal models have been developed to reflect human acute liver injuries. Well characterized models are T-cell-mediated injury in ConA-treated and Fas agonistic antibody-treated mice. Another widely used model of acute liver injury is D-galactosamine (GalN)-sensitized mice treated with lipopolysaccharide (LPS).[5; 6] GalN selectively depletes UTP in the liver and specifically inhibits RNA synthesis in hepatocytes. LPS is needed to initiate an acute inflammatory response (e.g. IL-1β,

IL-6, TNF-α) inducing apoptosis and necrosis in hepatocytes.[7;8]

In the present study, the GalN/LPS model is utilized to study the anti-apoptotic effects of HIAP1 expression in acute liver injury. For this purpose, a gene encoding the HIAP1 protein will be introduced in the liver using a recombinant adenoviral vector. Furthermore, XIAP-transgenic mice are used. The adenoviral vector very efficiently delivers genes into hepatocytes.[9-11] Except for several gene therapy studies in T-cell-mediated liver failure[12-15], (adenoviral) gene therapy in the GalN/LPS model has not been extensively investigated yet.[11;16] Since the GalN/LPS model mimics acute toxic hepatitis, it is relevant to explore novel therapeutic strategies. The present report contributes to this search by investigating the therapeutic effects of HIAP1 gene delivery.

4.3 Materials and methods

Animals

Specified pathogen-free BALB/c male mice (25g) were purchased from Harlan, Zeist, the Netherlands. Ub-6myc-XIAP transgenic mice were generated in the Apoptosis Research Center of Children's Hospital of Eastern Ontario, Ottawa, Canada. These transgenic mice express a 6myc-tagged version of XIAP under the control of the Ubiquitin C promotor. Mice were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local Committee for Care and Use of Laboratory Animals. Adenovirus studies were in compliance with the Dutch regulations regarding the use of genetically modified organisms.

Experimental design

In mice, adenoviral mediated gene transfer in hepatocytes was accomplished by intravenous injection of 5 x 10^9 pfu/mouse of recombinant adenoviral vectors coding for HIAP1 or β-galactosidase. Control mice received PBS. Three days following virus infection, mice were treated intraperitoneally with PBS or 800 mg/kg D-galactosamine (GalN; Sigma, St. Louis, MO, USA) plus 10 µg/kg LPS (*E.coli* serotype 0127:B8; Sigma). Some mice were only treated with AdHIAP1 or 10 mg/kg LPS, whereas other mice only received LPS/GalN. Ub-6myc-XIAP transgenic mice were also exposed to 800 mg/kg GalN plus 10 µg/kg LPS. Each group contained five to ten mice.

Seven hours after administration of LPS/GalN, mice were bled for biochemical analysis and sacrificed. Specimens of livers were either snap-frozen in liquid nitrogen for isolation of RNA and protein, stored in formalin or frozen in iso-pentane for immunohistochemical studies.

Recombinant Adenoviral constructs

Recombinant, replication-deficient adenovirus AdHIAP1 was up-scaled using standard techniques.[17] AdHIAP1 is an adenoviral type 5 based vector in which E1 and E3 genes have been deleted. It contains human IAP1, which is the human homologue of mouse IAP1.[18; 19] As a control virus Ad5LacZ was used, which contains the *E. coli* β-galactosidase gene.

Expression of transgenes in mice liver

Transgene expression and localization were determined by X-Gal staining (Sigma) on cryostat sections. Shortly, sections were fixed in 0.5 % glutaraldehyde for 5 min. and incubated in iron-buffer (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 3 mM $MgCl_2$ in PBS) containing X-Gal (final concentration of 1 µg/µl) at 37°C. Sections were counter stained with eosin and evaluated on a microscope. The expression of HIAP1 was measured using RT-PCR as described elsewhere in this report.

Liver enzymes

In mice sera, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) served as markers for liver damage. These enzymes were measured by routine clinical chemistry.

RT-PCR

Total RNA was extracted from livers using the Trizol method according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands). Reverse transcription (RT) was performed on 5 µg of total RNA using random primers, 50 U AMV Reverse-Transcriptase in a final volume of 75 µl. (Reverse Transcription System, Promega, Madison, WI). RT program included 3 steps: 10 min at 25°C followed by 50°C for 1 hr and 5 min at 95°C. PCR reactions were performed with 3 µl of RT reaction mixture supplemented with 5 U Taq Polymerase (Eurogentec, Seraing, Belgium), 50 pmol sense and 50 pmol antisense primers (Invitrogen) and 50mM $MgCl_2$. The final reaction volume was 50 µl. PCR primers are listed in table 1. For every PCR, GAPDH was used as standard control.

Caspase-3 enzyme activity assay

Livers were mechanically homogenized in a hypotonic cell lysis buffer[2] followed by two centrifugation-steps of 13.000 rpm for 10 min at 4 °C. Protein amount in the supernatant was determined with a BioradDC protein assay according to the manufacturer's instructions. (Biorad Laboratories, Veenendaal, The Netherlands). Caspase-3 enzyme activity was assayed using a caspase-3 activity kit with fluorimetric detection (Promega) according to the manufacturer's instructions. 20 µg of protein was used.

Table 1. Oligonucleotide primers used for RT-PCR analysis

Primers	Sense and antisense
HIAP1	5'-CAGTGGATATTTCCGTGGCT-3'
	5'-ATTTTCCACCACAGGCAAAG-3'
TNF-α	5'-CACCATGAGCACGGAAAGCA-3'
	5'-GCAATGACTCCAAAGTAGACC-3'
IL-1β	5'-CAGGCAGGCAGTATCACTCA-3'
	5'-AGGCCACAGGTATTTTGTCG-3'
IFNγ	5'-ACTGGCAAAAGGATGGTGAC-3'
	5'-TGAGCTCATTGAATGCTTGG-3'
mMIP2	5'-GCCAAGGGTTGACTTCAAGA-3'
	5'-TTAGCCTTGCCTTTGTTCAG-3'
Gro-1	5'-GCTGGGATTCACCTCAAGAA-3'
	5'-TGGGGACACCTTTTAGCATC-3'
GAPDH	5'-CCATCACCATCTTCCAGGAG-3'
	5'-CCTGCTTCACCACCTTCTTG-3'

Western Blot analysis

SDS-Page was performed with liver homogenates using 40 μg protein on a 15 % gel followed by Western blot analysis using 1:1000 diluted antibody against cleaved caspase-3 by courtesy of G. Robertson and D. Nicholson (Merck, Montreal, Canada).

Histology and immunohistochemistry

Liver sections fixed in formalin were embedded in paraffin. For morphologic examinations, hematoxylin-eosin staining and a periodic acid schiff (PAS) staining were performed. Paraffin sections were also used for the detection of active caspase-3.[20] Briefly, after antigen retrieval and quenching of endogenous peroxidase, slides were incubated for 1 hr with 1:100 diluted polyclonal antibody against cleaved caspase-3 (Cell Signaling Technology, Beverly MA, USA). This step was followed by 1:50 diluted Goat-Anti-Rabbit horseradish peroxidase (GARpo (DAKO, Glostrup, Denmark) for 30 min and another amplification step with

1:50 Rabbit-Anti-Goat horseradish perixodase (RAGpo, DAKO) Color was developed using diaminobenzidine as substrate. All slides were evaluated on a Zeiss microscope.

Statistical analysis

Results are presented as the meant of 5 to 10 animals per experimental group ± standard deviation. A Mann-Whitney test was used to determine the significance of differences between two experimental groups. A *P* value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

4.4 Results

HIAP1 expression in acute liver injury

To investigate our hypothesis that HIAP1 rescues hepatocytes from apoptosis in acute liver injury, mice were pre-treated with adenoviral vector coding HIAP1 (AdHIAP1). The experimental design is shown in Figure 1.

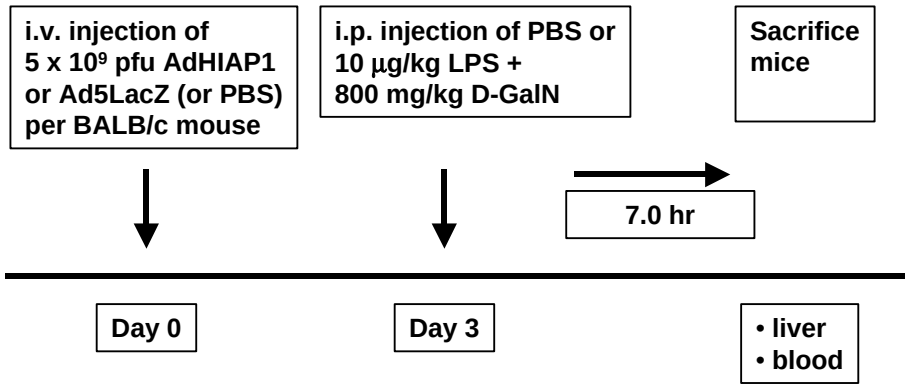

Figure 1. Experimental design: adenoviral mediated gene transfer of HIAP1 in a model of acute liver failure. See Material and Methods for details.

Mice received intravenously 5 x 10^9 pfu of adenovirus/animal. Adenovirus containing a β-galactosidase reporter gene served as control virus. After three days, control and adenovirus-infected mice were treated with or without galactosamine (GalN) and LPS (10 µg/kg). Livers were harvested seven hours after LPS/GalN treatment. At this time-point, apoptosis was determined by caspase-3 activity assay and consistently peaked in non-virus LPS/GalN-treated livers (data not shown).

Figure 2a demonstrates that HIAP1 is expressed in all AdHIAP1 pre-treated livers. As expected, control and AdLacZ-pretreated mice did not express HIAP1.

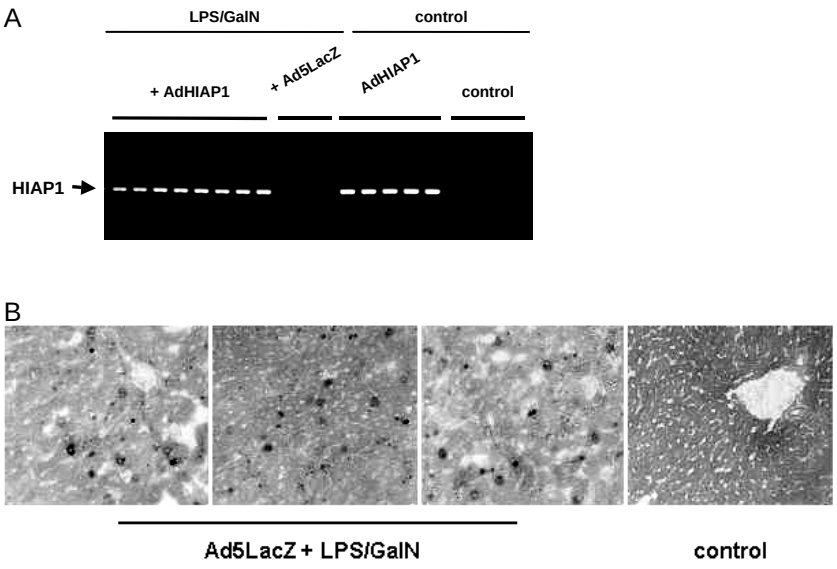

Figure 2. Adenoviral transgene expression after LPS/GalN-induced acute liver injury in mice. (A) mRNA levels of human Inhibitor of Apoptosis Protein1 (HIAP1) in livers of control mice or LPS/GalN-treated animals pre-exposed to 5 x 10^9 pfu of AdHIAP1. Ad5LacZ served as control virus. (B) Detection of β-galactosidase in mice livers pretreated with 5 x 10^9 pfu of Ad5LacZ followed by induction of acute liver injury. Enzymatic processing of X-Gal by β-galactosidase results in the release of a blue dye (original magnification 400x). Representative data of 5 to 10 animals per group are shown.

The strong expression of the transgene in LPS/GalN-treated livers was present in 30 % of hepatocytes. This was shown by the enzymatic processing of X-Gal, the substrate for β-galactosidase (Fig 2b).

Liver histology and morphology

The liver architecture in mice was assessed using hematoxylin-eosin staining. Control mice had normal liver morphology as demonstrated in Figure 3a (appendix). Mice that were exposed to a high dose of LPS only also showed a normal liver architecture (Fig 3f, appendix). However, administration of LPS/GalN resulted in severe liver damage with hemorrhagic and disarraying areas and apoptotic and necrotic hepatocytes (Fig 3b, appendix). Pretreatment of LPS/GalN mice with control virus (Ad5LacZ) even worsened the liver architecture (Fig 3c). Although a severe hemorrhagic pattern was shown in these livers, increased inflammation seemed to be absent. Remarkably, pre-treatment of mice with adenovirus coding for HIAP1 did not decrease LPS/GalN-induced liver damage (Fig 3d, appendix). Therefore, the effects of the adenovirus itself on control livers were examined more closely. As shown in Figure 3e (appendix), livers treated only with adenovirus demonstrated some variety of the nucleus size in hepatocytes, but without a significant number of apoptotic and necrotic cells. In addition, these livers displayed normal morphology of hepatocytes (Fig 4).

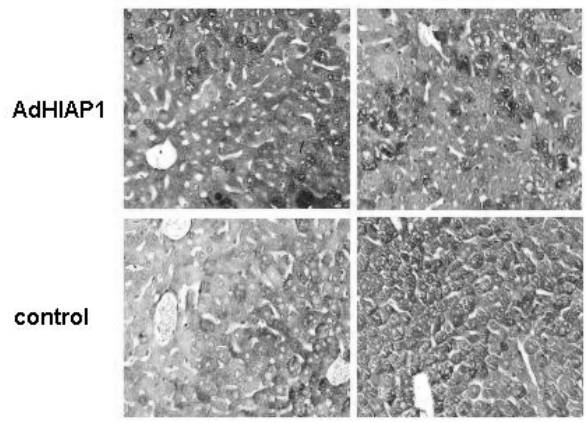

Figure 4. Liver glycogen content displayed by periodic acid schiff (PAS) staining. Control mouse livers and livers pre-treated with 5 x 10^9 pfu of AdHIAP1 were examined for glycogen content at 3 days after injection of the recombinant adenovirus AdHIAP1 (original magnification 400x). Representative data of 5 to 10 animals per group are shown.

Moreover, serum levels of the liver enzymes ASAT and ALAT in AdHIAP1-treated control mice where similar to control levels (Fig 5). These data demonstrate that the adenoviral vector only minimally affects liver morphology and that adenoviral toxicity in these livers is not a prominent feature.

Mice treated with LPS/GalN demonstrated highly increased serum levels of AST and ALT compared to control and LPS-exposed animals. These levels increased 7-fold in LPS/GalN mice that were pre-treated with Ad5LacZ (Fig 5). This was similar in the AdHIAP1-pretreated mice demonstrating that HIAP1 did not prevent adenoviral-mediated AST /ALT release in LPS/GalN-exposed mice.

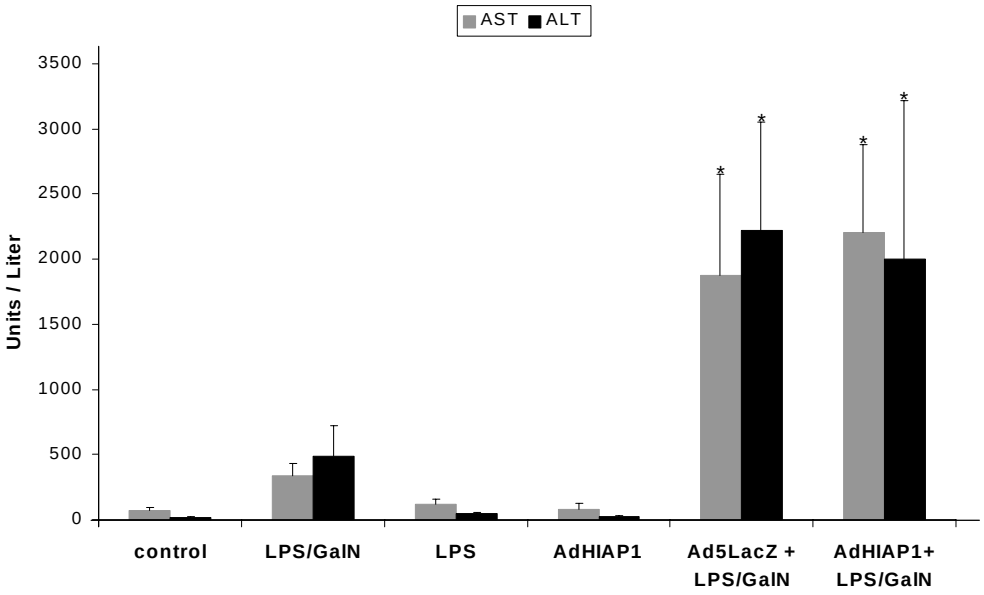

Figure 5. Serum levels of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in control mice or mice pretreated with 5 x 10^9 pfu of AdHIAP1 or Ad5LacZ followed by the onset of acute liver injury using LPS/GalN. Some animals were treated with LPS/GalN, AdHIAP1, or 10 mg/kg of LPS. Data represent mean of 5 to 10 animals per group ± SD. *P<0.05 for AdHIAP1 + LPS/GalN vs LPS/GalN and for Ad5LacZ + LPS/GalN vs LPS/GalN.

Effects of AdHIAP1 on apoptosis in acute liver injury

Next, the anti-apoptotic effects of HIAP1 were investigated. Since activation of caspase-3 is very indicative for apoptotic cell death, we measured this enzyme activation in liver homogenates. As shown in Figure 6a, LPS/GalN treatment resulted in a marked increase of caspase-3 activity compared to control mice livers. Apoptotic cell death increased even more when animals had been pretreated with Ad5LacZ. Also livers of AdHIAP1-treated animals that subsequently received LPS/GalN displayed high levels of active caspase-3 (Fig 6a). No significant reduction was seen in caspase-3 levels as compared to Ad5LacZ-treated animals. In contrast, adenovirus itself did not induce caspase-3 activation (Fig 6a).

Besides these quantitative data, analysis of the localization of active caspase-3 was performed by immunohistochemistry. Staining for active caspase-3 confirmed our results obtained with the caspase-3 assay (Fig 6b). Thus, the number of caspase-3 positive hepatocytes in LPS/GalN-exposed animals was not reduced upon pretreatment of mice with AdHIAP1.

AdHIAP1 does not induce inflammation in the liver during acute injury

Adenoviral vectors can induce pro-inflammatory events in the liver that can amplify the inflammatory response to LPS. To rule out the possibility that this mechanism obscured any beneficial effects of HIAP1, we investigated the hepatic expression of different cytokines seven hours after LPS/GalN exposure. Livers of mice that were treated only with a high dose of LPS (10 mg/kg) served as positive control.

In livers of LPS-treated mice, mRNA levels for IL-1β, IFN-γ and TNF-α were induced compared to control livers (Fig 7). This was similar for the chemokines Gro-1 (human homologue of IL-8) and murine MIP2 (mMIP2). Furthermore, in GalN/LPS-treated mice the expression of mMIP2 and TNF-α increased slightly compared to LPS-treated mice (Fig 7).

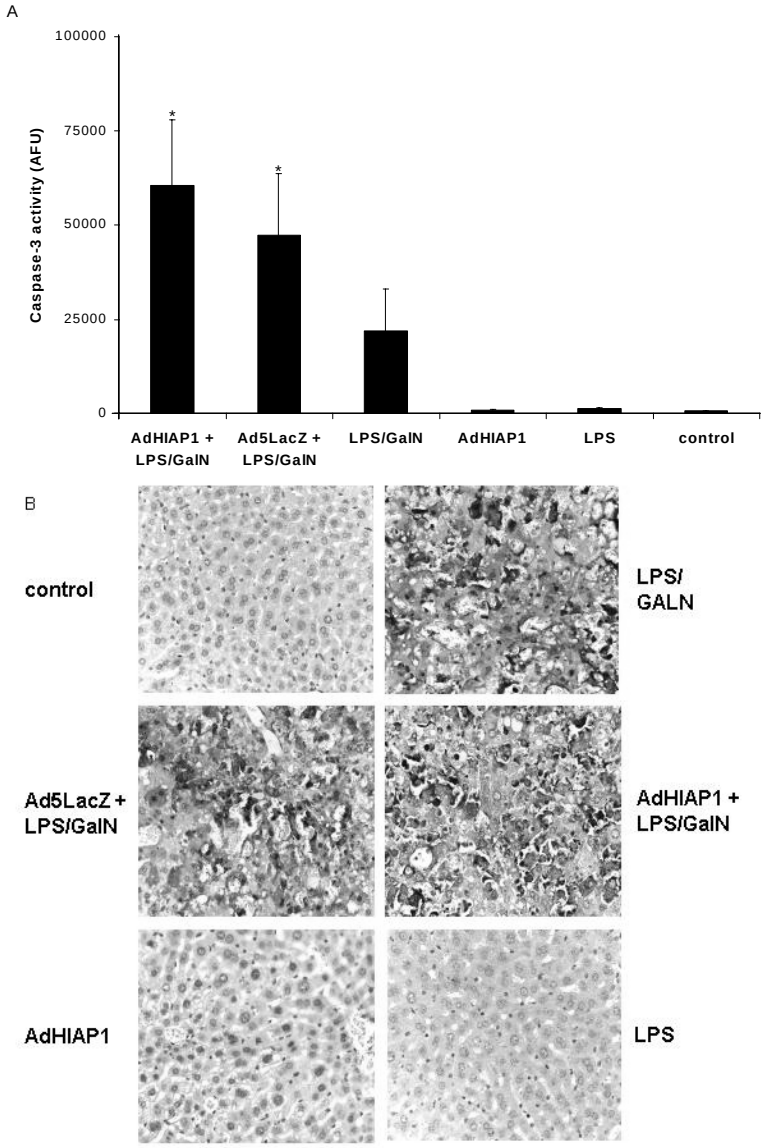

Figure 6. Apoptotic cell death after LPS/GalN-induced acute liver injury in mice. (A) Caspase-3 activity in liver homogenates of control mice or mice pretreated with 5 x 10^9 pfu of AdHIAP1 or Ad5LacZ followed by exposure to LPS/GalN. Some animals were treated with LPS/GalN, AdHIAP1 or 10 mg/kg of LPS. Data represent mean of 5 to 10 animals per group ± SD. *P<0.05 for AdHIAP1 + LPS/GalN vs LPS/GalN and for Ad5LacZ + LPS/GalN vs LPS/GalN. (B) Active caspase-3 staining (original magnification 400x).

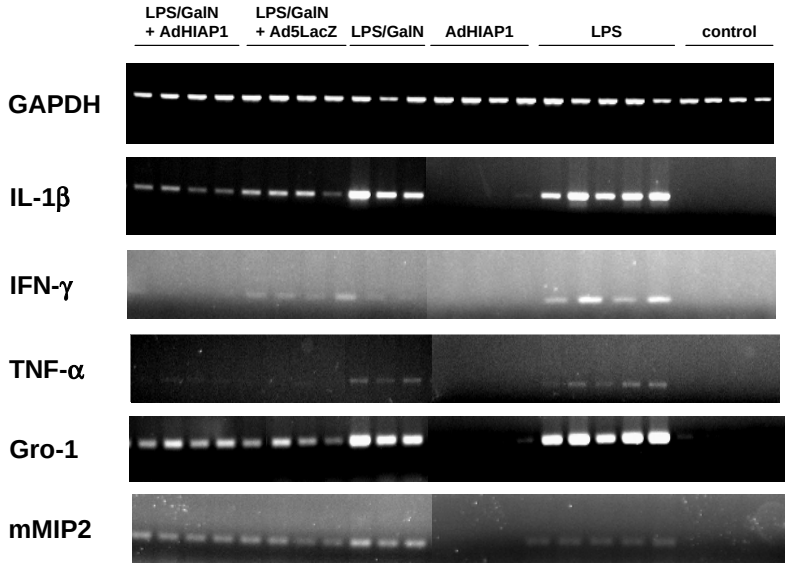

Figure 7. Cytokine and chemokine expression in liver homogenates. Mice were pretreated with or without 5 x 10^9 pfu of AdHIAP1 or Ad5LacZ followed by exposure to LPS/GalN. Some animals were treated with AdHIAP1 or 10 mg/kg of LPS. mRNA levels for the cytokines IL-1β, IFN-γ, TNF-α, and the chemokines Gro-1 (Human Interleukin 8) and mouse MIP2 (mMIP2) are displayed.

In contrast, LPS/GalN-exposed animals pretreated with adenovirus demonstrated a clear decrease in mRNA levels of IL-1β, TNF-α, Gro-1 and mMIP2 compared to LPS/GalN alone, indicating a decreased inflammatory and chemotactic response. Interestingly, in LPS/GalN-exposed animals pretreated with AdHIAP1, mRNA for IFN-γ was not expressed at all. Moreover, in AdHIAP1-treated control mice livers, mRNA for IL-1β, IFN-γ, TNF-α, Gro-1 and mMIP2 was not induced (Fig 7), again indicating the lack of an inflammatory response to adenovirus.

Acute liver injury in XIAP-transgenic mice

To discriminate between the effects of the IAP transgene and non-specific adenoviral effects, we induced acute liver injury in XIAP-transgenic mice. These mice were exposed to the same doses of LPS/GalN and sacrificed 7 hours after LPS/GalN treatment. Strikingly, caspase-3 activity was increased in XIAP-transgenic mice compared to their wild-type littermates (Fig 8a). This was also confirmed by Western blot analysis (Fig 8b), demonstrating caspase-3 processing. These results confirm data obtained from adenoviral overexpression of HIAP1 in LPS/GalN-treated animals (Fig 6), indicating that IAP overexpression is detrimental in this model of liver injury.

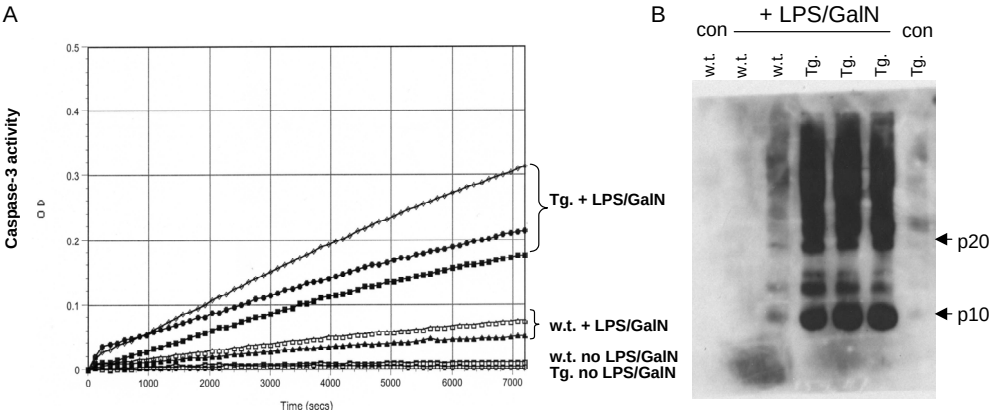

Figure 8. Apoptosis in XIAP-transgenic mice after LPS/GalN treatment. Ub-6myc-XIAP transgenic mice were exposed to LPS/GalN and sacrificed 7 hours after treatment. (A) Caspase-3 activity in livers from (LPS/GalN-exposed) XIAP-transgenic mice (Tg.) and their wild-type littermates (w.t.). (B) Western blot detection of active caspase-3 in livers from (LPS/GalN-exposed) XIAP-transgenic mice and their wild-type littermates. Representative data of 4 to 8 animals per group are shown.

4.5 Discussion

The role of adenoviral gene transfer in the liver and the suitability of HIAP1 as a therapeutic candidate for acute liver injury are highlighted in this paper. The protection of human IAP1 (HIAP1) against hepatocyte apoptosis in acute liver injury was investigated using the LPS/GalN model. Although LPS in the absence of GalN caused inflammation in the liver, rodent liver cells were insensitive to cell damage, which confirms other reports.[6; 8] To obtain an acute model that reflects the clinical situation, GalN-sensitization, which blocks transcription specifically in hepatocytes, was needed. In the present study, mice were pretreated with adenovirus encoding HIAP1 before the onset of acute liver injury. However, our experimental design could still be relevant since in humans, acute liver injury takes several days to develop (e.g. overdose of paracetamol or the mushroom-derived amantin toxin).

Contrary to our hypothesis, AdHIAP1 did not inhibit apoptotic cell death in acute liver injury, but in fact aggravated LPS/GalN-induced damage. This is in contrast to our *in vitro* study in which AdHIAP1 blocked cytokine-induced apoptosis completely in hepatocytes that were unable to activate NF-κB.[2]

There are several possible explanations for the failure of HIAP1 to prevent acute liver injury. In some reports, the adenoviral gene transfer efficiency in normal livers was reported to be 70 percent.[10;21] In our study, the transgene expression in acute liver injury was comparable to other groups demonstrating significant expression in 30 % of the hepatocytes.[10;11] Although this percentage is relatively low, in a recently published study, 30 % transgene expression was beneficial in the same model.[11] Moreover, in the present report, the number of β-galactosidase positive hepatocytes may have been underestimated since in necrotic areas with cell debris, β-galactosidase positive hepatocytes are hard to discriminate. This result is consistent with a previous study, which reported that decreased expression of β-galactosidase was due to loss of liver cells containing the Ad5LacZ

DNA and not due to downregulation via cytokines.[22] Nevertheless, the percentage of transduction may have been too low to be protective in this model.

Priming of the immune system by AdHIAP1 may be another explanation for the lack of anti-apoptotic action of HIAP1 in acute liver injury. Pretreatment with adenovirus could induce cytokines (e.g. TNF-α) in the liver that might increase the inflammatory response upon LPS/GalN-induced inflammation such, that Hiap1 is not able to block apoptosis anymore. As previously reported, overproduction of TNF-α leads to a lethal inflammatory response.[23] Indeed, we demonstrated increased liver damage, which was associated with elevated apoptotic and necrotic cell death in adenovirus-treated LPS/GalN mice compared to the LPS/GalN group alone. Although the spleens of adenovirus-infected mice were enlarged after LPS/GalN treatment (data not shown), increased cytokine and chemokine levels were absent in these livers. In addition, adenovirus-infected LPS/GalN animals exhibited lower hepatic expression of cytokines and chemokines compared to the LPS/GalN group alone, indicating a reduced inflammatory response. Indeed, histology of the liver did not demonstrate an enlarged number of infiltrating inflammatory cells. These data are in line with a recent study, which implies that recombinant E1/E3-deleted adenoviral vectors decrease the expression of pro-inflammatory mediators in acute liver injury by preconditioning.[24] The underlying mechanism remains to be elucidated. Overall, it is not very likely that priming of the immune system exacerbates the inflammatory response in our model explaining the lack of anti-apoptotic action of HIAP1.

Although several studies reported adenoviral toxicity in the liver,[10;11;22] we did not find evidence for adenoviral toxicity of AdHIAP1. Apoptotic and necrotic cell death, as well as inflammation were clearly absent in AdHIAP1-exposed livers. Our results imply that in normal livers, HIAP1 inhibits adenoviral toxicity, whereas in acute liver injury, AdHIAP1 sensitizes the liver to damage.

We found increased necrosis in adenovirus-treated mice after LPS/GalN-exposure compared to LPS/GalN-treated animals. It is known that necrosis follows apoptosis at a certain time-point after treatment with LPS/GalN.[25;26] Lawson et al.

postulated that in response to apoptosis, neutrophil migration is responsible for the induction of necrosis. It is possible that neutrophil-induced necrotic cell death caused the hemorrhagic liver damage in adenovirus-treated LPS/GalN livers. However, we could not find evidence for increased chemokine expression in adenovirus-treated LPS/GalN-livers compared to the LPS/GalN group alone.
Non-specific adenoviral effects are further excluded by our study in XIAP-transgenic animals. LPS/GalN dramatically exaggerate active caspase-3 activation in XIAP-transgenic mice. These results support our data obtained with adenoviral overexpression of HIAP1, implying that the increase in liver injury is not caused by adenovirus. Thus, IAP overexpression is detrimental in this model of liver injury. Further evidence comes from preliminary data of LPS-exposed HIAP1 knockout mice that display decreased lethality (data not shown).

An alternative explanation for the lack of HIAP1 therapy in acute liver failure could be related to the relative cytoplasmatic concentrations of active caspase-8, -9, -3 and Smac/DIABLO versus HIAP1. This delicate balance determines cell fate.[4] Besides the activation of caspase-8, the activation of caspase-9 is most important during cytokine-induced apoptosis. It is generally believed that in the LPS/GalN model, the activation of caspase-9 via the mitochondrial loop is required for the activation of caspase-3, whereas caspase-8 plays only a minor role, at least during the first hours after LPS/GalN treatment. [7;27] This is in support of a beneficial role for HIAP1 since HIAP1 only inhibits caspase-9 and caspase-3. When the amount of active caspase-8 increases in time via feedback amplification by caspase-3, the caspase cascade is strongly amplified. Consequently HIAP1 can not rescue hepatocytes anymore from cytokine-induced apoptosis.[7] Different studies support this hypothesis. Bajt et al. have shown that although inhibition of caspase-3 by a peptide-inhibitor inhibits LPS/GalN-mediated apoptosis, caspase-8 inhibition is more beneficial.[28] Furthermore, adenovirus coding for dominant negative Fas-associated death domain (FADD) inhibited hepatocyte apoptosis in the liver after TNF-α/GalN treatment.[21] Our study supports these data and implicates that

apoptosis can only be inhibited at the level of caspase-3 and/or caspase-9 within a narrow window of time.

Finally, HIAP1 can also be blocked by the pro-apoptotic mitochondrial factor Smac/DIABLO. Therefore, the effectiveness of HIAP to block cell death is also dependent on the magnitude of mitochondrial damage and the amount of active Smac released from mitochondria.[4]

Although a recent report demonstrated a beneficial effect of adenoviral expression of A20 in the LPS/GalN model, only lethality was decreased, whereas no other beneficial effects were displayed, like inhibition of apoptosis, necrosis and inflammation.[11] Since in our study, HIAP1 was also not able to block apoptosis in LPS/GalN- exposed livers, the quest for protective genes has not been solved yet.

4.6 Acknowledgements

We are grateful to H. Breukelman and M. de Ruiter (Laboratory Center, Universital Hospital Groningen, the Netherlands) for their assistance with liver enzyme analysis. This work is supported by grants from the Groningen University Institute for Drug exploration, the Dutch Digestive Diseases foundation (WS 99-28) and the J.K. de Cock Foundation, The Netherlands.

4.7 References

1. Shakil AO, Kramer D, Mazariegos GV, Fung JJ, Rakela J. Acute liver failure: clinical features, outcome analysis, and applicability of prognostic criteria. Liver Transpl 2000;6:163-169.
2. Schoemaker MH, Ros JE, Homan M, Trautwein C, Liston P, Poelstra K, Van Goor H, Jansen PL, Moshage H. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. J Hepatol 2002;36:742-750.

3. Xu Y, Bialik S, Jones BE, Iimuro Y, Kitsis RN, Srinivasan A, Brenner DA, Czaja MJ. NF-kappaB inactivation converts a hepatocyte cell line TNF-alpha response from proliferation to apoptosis. Am J Physiol 1998;275:C1058-C1066.
4. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998;17:2215-2223.
5. Bradham CA, Plumpe J, Manns MP, Brenner DA, Trautwein C. Mechanisms of hepatic toxicity. I. TNF-induced liver injury. Am J Physiol 1998;275:G387-G392.
6. Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc Natl Acad Sci U S A 1979;76:5939-5943.
7. Mignon A, Rouquet N, Fabre M, Martin S, Pages JC, Dhainaut JF, Kahn A, Briand P, Joulin V. LPS challenge in D-galactosamine-sensitized mice accounts for caspase-dependent fulminant hepatitis, not for septic shock. Am J Respir Crit Care Med 1999;159:1308-1315.
8. Zhou BR, Gumenscheimer M, Freudenberg M, Galanos C. A striking correlation between lethal activity and apoptotic DNA fragmentation of liver in response of D-galactosamine-sensitized mice to a non-lethal amount of lipopolysaccharide. Acta Pharmacol Sin 2003;24:193-198.
9. Morsy MA, Alford EL, Bett A, Graham FL, Caskey CT. Efficient adenoviral-mediated ornithine transcarbamylase expression in deficient mouse and human hepatocytes. J Clin Invest 1993;92:1580-1586.
10. Nakatani T, Kuriyama S, Tominaga K, Tsujimoto T, Mitoro A, Yamazaki M, Tsujinoue H, Yoshiji H, Nagao S, Fukui H. Assessment of efficiency and safety of adenovirus mediated gene transfer into normal and damaged murine livers. Gut 2000;47:563-570.
11. Arvelo MB, Cooper JT, Longo C, Daniel S, Grey ST, Mahiou J, Czismadia E, Abu-Jawdeh G, Ferran C. A20 protects mice from D-galactosamine/lipopolysaccharide acute toxic lethal hepatitis. Hepatology 2002;35:535-543.
12. Rodriguez I, Matsuura K, Khatib K, Reed JC, Nagata S, Vassalli P. A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. J Exp Med 1996;183:1031-1036.
13. Lasarte JJ, Sarobe P, Boya P, Casares N, Arribillaga L, de Cerio AL, Gorraiz M, Borras-Cuesta F, Prieto J. A recombinant adenovirus encoding hepatitis C virus core and E1 proteins protects mice against cytokine-induced liver damage. Hepatology 2003;37:461-470.
14. Fujino M, Kawasaki M, Funeshima N, Kitazawa Y, Kosuga M, Okabe K, Hashimoto M, Yaginuma H, Mikoshiba K, Okuyama T, Suzuki S, Li XK. CrmA gene expression protects mice against concanavalin-A-induced hepatitis by inhibiting IL-18 secretion and hepatocyte apoptosis. Gene Ther 2003;10:1781-1790.
15. Poussin D, Malassagne B, Tran VN, Trebeden H, Guery L, Chereau C, Soubrane O, Calmus Y, Weill B, Batteux F. Biliary administration of naked DNA encoding Fas-Fc protein prevents acute liver failure in mice. Hum Gene Ther 2002;13:901-908.
16. Nomi T, Shiota G, Isono M, Sato K, Kawasaki H. Adenovirus-mediated hepatocyte growth factor gene transfer prevents lethal liver failure in rats. Biochem Biophys Res Commun 2000;278:338-343.

17. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 1998;95:2509-2514.
18. Liston P, Lefebvre C, Fong WG, Xuan JY, Korneluk RG. Genomic characterization of the mouse inhibitor of apoptosis protein 1 and 2 genes. Genomics 1997;46:495-503.
19. Perrelet D, Ferri A, MacKenzie AE, Smith GM, Korneluk RG, Liston P, Sagot Y, Terrado J, Monnier D, Kato AC. IAP family proteins delay motoneuron cell death in vivo. Eur J Neurosci 2000;12:2059-2067.
20. van der Woude CJ, Kleibeuker JH, Tiebosch AT, Homan M, Beuving A, Jansen PL, Moshage H. Diffuse and intestinal type gastric carcinomas differ in their expression of apoptosis related proteins. J Clin Pathol 2003;56:699-702.
21. Streetz K, Leifeld L, Grundmann D, Ramakers J, Eckert K, Spengler U, Brenner D, Manns M, Trautwein C. Tumor necrosis factor alpha in the pathogenesis of human and murine fulminant hepatic failure. Gastroenterology 2000;119:446-460.
22. Zhang HG, Xie J, Xu L, Yang P, Xu X, Sun S, Wang Y, Curiel DT, Hsu HC, Mountz JD. Hepatic DR5 induces apoptosis and limits adenovirus gene therapy product expression in the liver. J Virol 2002;76:5692-5700.
23. Xuan D, Nicolau DP, Nightingale CH, Quintiliani R. Circulating tumor necrosis factor-alpha production during the progression of rat endotoxic sepsis. Chemotherapy 2001;47:194-202.
24. Yarovinsky TO, Powers LS, Butler NS, Bradford MA, Monick MM, Hunninghake GW. Adenoviral infection decreases mortality from lipopolysaccharide-induced liver failure via induction of TNF-alpha tolerance. J Immunol 2003;171:2453-2460.
25. Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J Immunol 1998;160:3480-3486.
26. Lawson JA, Fisher MA, Simmons CA, Farhood A, Jaeschke H. Parenchymal cell apoptosis as a signal for sinusoidal sequestration and transendothelial migration of neutrophils in murine models of endotoxin and Fas-antibody-induced liver injury. Hepatology 1998;28:761-767.
27. Osawa Y, Banno Y, Nagaki M, Nozawa Y, Moriwaki H, Nakashima S. Caspase activation during hepatocyte apoptosis induced by tumor necrosis factor-alpha in galactosamine-sensitized mice. Liver 2001;21:309-319.
28. Bajt ML, Vonderfecht SL, Jaeschke H. Differential protection with inhibitors of caspase-8 and caspase-3 in murine models of tumor necrosis factor and Fas receptor-mediated hepatocellular apoptosis. Toxicol Appl Pharmacol 2001;175:243-252.

Chapter 5

Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation

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5.1 Abstract

Aim: To examine the extent and mechanisms of apoptosis in cholestatic liver injury and to explore the role of the transcription factor NF-κB in protection against bile acid-induced apoptosis. *Methods*: Cholestatic liver injury was induced by bile duct ligation in Wistar rats. Furthermore, primary cultures of rat hepatocytes were exposed to glycochenodeoxycholic acid (GCDCA), tauroursodeoxycholic acid (TUDCA), taurochenodeoxycholic acid (TCDCA) and to cytokines. Apoptosis was determined by TUNEL-staining, active caspase-3 staining, activation of caspase-8, caspase-9 and caspase-3. *Results*: Limited hepatocyte apoptosis and an increased expression of NF-κB-regulated anti-apoptotic genes A1 and cIAP2 were detected in cholestatic rat livers. Bcl-2 expression was restricted to bile duct epithelium. In contrast to TCDCA and TUDCA, GCDCA induced apoptosis in a FADD-independent pathway in hepatocytes. Although bile acids do not activate NF-κB, NF-κB activation by cytokines (induced during cholestasis) protected against GCDCA-induced apoptosis *in vitro* by upregulating A1 and cIAP2. *Conclusions*: GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a FADD-independent manner. However, bile acid-induced apoptosis in cholestasis is limited. This could be explained by cytokine-induced activation of NF-κB-regulated anti-apoptotic genes like A1 and cIAP2.

5.2 Introduction

During cholestatic liver injury, accumulation of bile acids in the liver is thought to play a role in causing hepatocyte damage.[1;2] Toxic hydrophobic bile acids disrupt cell membranes resulting in the release of intracellular constituents.[3] In addition, exposure of hepatocytes to deoxycholic acid[4] and glycochenodeoxycholic acid (GCDCA)[5,6] at concentrations far below their critical micelle concentration, results in apoptotic cell death. However, the extent of hepatocyte apoptosis in cholestatic liver injury remains to be clarified. The present report focuses on apoptotic cell death during cholestasis.

It has been postulated that toxic bile acids directly activate the Fas death receptor.[7;8] Furthermore, (glycocheno)deoxycholic acid induces a decrease in the mitochondrial membrane potential and cytochrome c release from mitochondria.[9;10] Other studies suggest that oxidative stress is involved in GCDCA-induced decrease of the mitochondrial membrane potential in hepatocytes.[11]

Besides GCDCA, taurine conjugates of chenodeoxycholate (TCDCA) and ursodeoxycholate (TUDCA) accumulate in rat liver in cholestasis. TCDCA has been postulated to activate a phosphatidylinositol 3-kinase (PI3-K)-mediated survival pathway that involves the induction of NF-κB activation.[12] Ursodeoxycholic acid is used to treat patients with cholestatic liver injury[13], but the protective mechanisms are not fully understood yet.[10;14]

An important survival pathway in hepatocytes is the activation of the transcription factor NF-κB.[15;16] This results in the induction of NF-κB-regulated survival genes and inhibition of apoptotic cell death.[15] It is known that endotoxin levels in blood are increased in cholestatic liver diseases. Endotoxin induces cytokine production in Kupffer cells, resulting in the activation of the NF-κB survival pathway in hepatocytes. Therefore, we also investigated the expression of cytokines in cholestatic livers and the role of NF-κB in cholestatic liver injury.

5.3 Materials and methods

Animals

Specified pathogen-free male Wistar rats (220-250 g) were purchased from Harlan, Zeist, the Netherlands. They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local Committee for Care and Use of Laboratory Animals.

Animal model

Male Wistar rats were anaesthetised with halothane/O_2/N_2O and subjected to bile duct ligation (BDL).[17] At the indicated times after BDL**,** the rats (n=4 per group) were sacrificed, livers were perfused with saline and removed. Control rats (n=4) for each of these time points received a sham operation (SHAM). Specimens of these livers were either snap-frozen in liquid nitrogen for isolation of RNA and protein, or stored in formalin for immunohistochemical studies. For determination of markers of liver damage and cholestasis, heparinized blood samples were obtained by cardiac puncture.

Hepatocyte isolation and experimental design

Hepatocytes were isolated and cultured as described previously.[15;18] Twenty hours after isolation, hepatocytes were exposed to 50 μM glycochenodeoxycholic acid (GCDCA, Calbiochem, La Jolla, CA), 50 μM tauroursodeoxycholic acid (TUDCA, Calbiochem) or 50 μM taurochenodeoxycholic acid (TCDCA, Calbiochem) and/or 50 μM of the caspase-8 inhibitor Ac-IETD-CHO (BIOMOL, Plymouth Meeting, USA). In some experiments, hepatocytes were pre-incubated with 10 μg/ml LPS (*Escherichia coli*, serotype 0127:B8, Sigma, St. Louis, MO) and a cytokine mixture (CM) composed of 20 ng/ml recombinant tumour necrosis factor-α (TNF α, R&D Systems, Abingdon, United Kingdom), 10 ng/ml recombinant interleukin-1β (IL-1 β,

R&D Systems), 100 U/ml recombinant interferon-γ (IFN γ, Life Technologies Ltd.) as described before.[15] Hepatocytes received adenovirus (MOI of 10) fifteen hours prior to exposure of bile acids or cytokine mixture. Each experimental condition was performed in triplicate wells. Each experiment was performed three times, using hepatocytes from different isolations.

Cells were harvested at the indicated time-points and rinsed 3 times with ice-cold phosphate buffered saline (PBS) prior to the addition of Trizol reagent (RNA isolation) (Life Technologies Ltd.) or hypotonic cell lysis buffer (protein analysis, caspase-3 assay) as described previously.[15] For measurement of caspase-8 activity, cells were harvested in cell lysis buffer using a caspase-8 fluorometric Protease Assay Kit (BioVision, Mountain View, USA).

Adenoviral constructs

Adenoviral constructs have been described previously.[15;19-21]

Electrophoretic Mobility Shift Assay (EMSA)

To demonstrate the presence of NF-κB in the nucleus upon activation, an EMSA was performed. Nuclear extracts and EMSA for NF-κB were prepared using a final concentration of 0.25% Nonidet P-40 as described previously.[22] The probe containing the NF-κB consensus sequence is 5'-AGCTGCGGGGATTTTCCCTG-3'.

Caspase-3 and caspase-8 enzyme activity assay

Caspase-3 enzyme activity was assayed as described before.[15] Caspase-8 activity was measured using a Caspase-8 Fluorometric Protease Assay Kit (BioVision) according to the manufacturer's instructions. Assays were performed with 20 μg of protein.

Immunohistochemical evaluation

TUNEL staining was performed according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Livers of D-Galactosamine (D-Gal)/LPS treated rats were used as positive control.[23] Staining of Bcl-2 and active caspase-3 was performed on formalin fixed, paraffin-embedded portions of the liver as described previously[24] and according to the manufacturer's instructions. Bcl-2 antibody (DAKO, Glostrup, Denmark) and active caspase-3 antibody (New England Biolabs, Beverly, USA) were used for 1 hr at 1:50 and 1:100, respectively. Goat-anti-rabbit horseradish peroxidase and rabbit-anti-goat horseradish peroxidase were used to detect active caspase-3 antibody.

RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA isolation, reverse trancription and PCR were performed as described previously.[15] For every PCR, expression of 18S was used as internal control. Primers are listed in table 1.

Table 1. Oligonucleotide primers used for RT-PCR analysis

Primers (rat)	Sense and antisense
18S	5'-GTATTGCGCCGCTAGAGGTG-3'
	5'-CTGAACGCCACTTGTCCCTC-3'
TNF-α	5'-CACCATGAGCACGGAAAGCA-3'
	5'-GCAATGACTCCAAAGTAGACC-3'
IL-1β	5'-CAGGAAGGCAGTGTCACTCA-3'
	5'-GGGATTTTGTCGTTGCTTGT-3'
IFNγ	5'-GCCCTCTCTGGCTGTTTACTG-3'
	5'-CTTTTCCGCTTCCTTAGGCT-3'
Collagen type I	5'-GCCCTGCTGGTCCCAAAGGTTC-3'
	5'-CATCTTTGCCAGCGGGACCAAC-3'

Western blot analysis

Western blot analysis of cell lysates was performed using antibodies against Bcl-2 (Dako) and cleaved caspase-9 (Cell Signaling Technology, Beverly, MA) both at a dilution of 1:1000. Equal loading was confirmed by Ponceau S staining.

Immunocytochemistry

Analysis of active caspase-9 was performed on hepatocytes cultured on coverslips and exposed to 50 µM GCDCA for 4 hours. Coverslips were washed in PBS, fixed in 4 % paraformaldehyde for 10 min followed by incubation in 1 % Triton-X100 for 5 min. Before adding primary antibodies, cells were washed twice with PBS. Antibody against active caspase-9 was used at a dilution of 1:50 for 30-60 min. Goat-anti-Rabbit antibody coupled to FITC (GAR-FITC, Molecular Probes, Eugene, Oregon, USA) was added at a dilution of 1:600 for 45 min. Slides were evaluated on a Leica confocal laser scanning microscope.

Statistical analysis

Results are presented as the mean of at least three independent experiments ± standard deviation. A Mann-Whitney test was used to determine the significance of differences between two experimental groups. A P value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

5.4 Results

Apoptosis is very limited in cholestatic liver injury

To investigate the extent of hepatocyte apoptosis in cholestatic liver injury, rats were sacrificed 4 days and 1, 2, 3 and 4 weeks after bile duct ligation. Sham operated animals of all time points were averaged and served as control. Total bilirubin, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyltransferase (GGT) levels were elevated within the first week after BDL and still persisted 4 weeks after BDL (Fig 1a). Furthermore, mRNA levels of collagen type I, TNF-α, IL-1β and IFN-γ were clearly increased in cholestatic livers (Fig 1b).

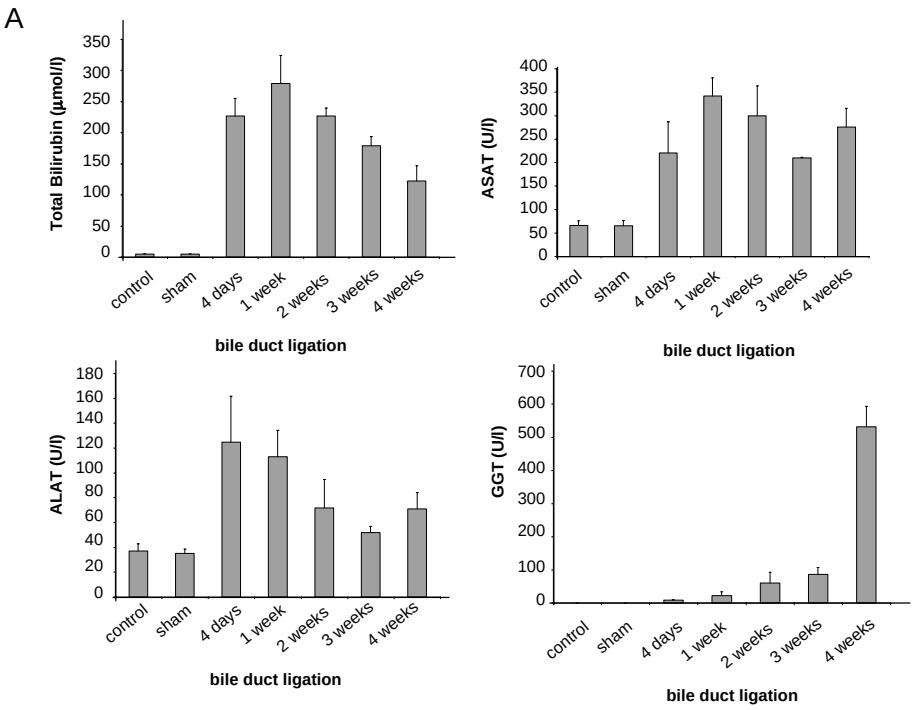
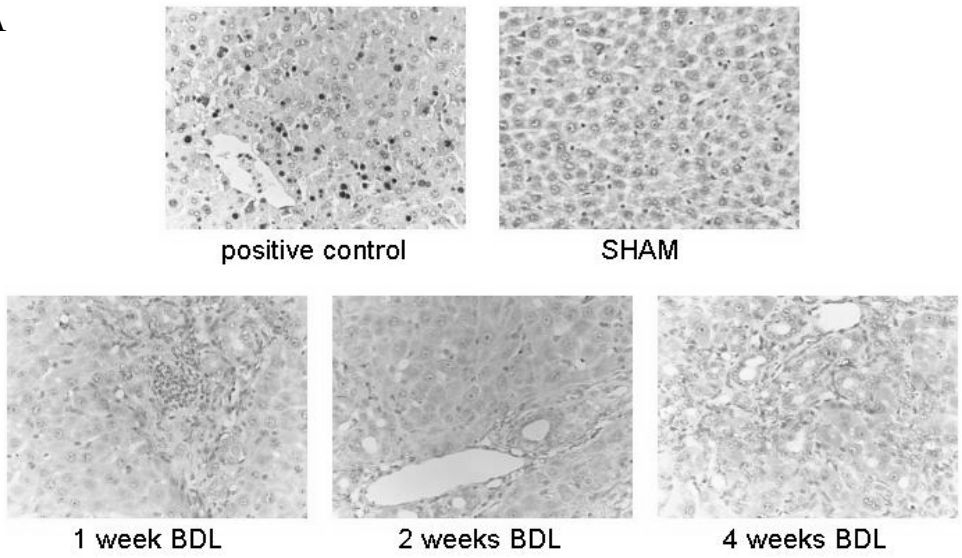


Figure 1. Liver injury and inflammation after bile duct ligation. (A) Serum levels of total bilirubin, ASAT, GGT and ALAT. (B) mRNA levels of Collagen type I (Coll I), TNF-α, IL-1β and IFN-γ. 18S mRNA served as internal control. Data represent mean of 4 animals a time point ± SD.

TUNEL staining was performed to detect apoptotic nuclei. In cholestatic livers, TUNEL staining demonstrated hardly any apoptosis while apoptotic hepatocytes were clearly present in D-Gal/LPS-treated livers (Fig 2a). To confirm these data, a

more specific staining was performed demonstrating active caspase-3. Again, only positive control livers were stained (Fig 2b, appendix). Finally, we determined caspase-3 activity in liver homogenates. As shown in Figure 2c, there is a 3-fold increase of caspase-3 activity within the first week after bile duct ligation, which declines after 1 week and remains at control level up to 4 weeks after BDL. All together, these data demonstrate that apoptotic cell death in cholestatic liver injury is limited and suggests adaptation against the apoptotic actions of bile acids.

A



C

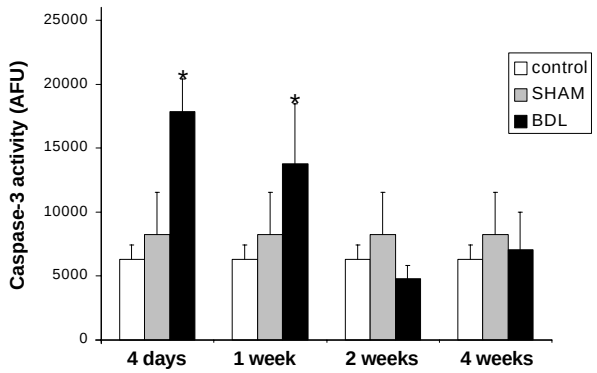


Figure 2 (A+C). Limited apoptosis in rat livers beyond 1 week of bile duct ligation (BDL). (A) TUNEL staining. (C) Caspase-3 activity in liver homogenates. Data represent mean of four animals a time point ± SD. $^{*}P<0.05$ for BDL 4 days and 1 week versus control and SHAM animals.

In previous work, we demonstrated that cIAP2 (inhibitor of apoptosis protein2) and A1/Bfl are important NF-κB-regulated anti-apoptotic genes in hepatocytes.[15] Since TNF-α, IL-1β and IFN-γ mRNA levels are increased in cholestatic livers (Fig 1b), we investigated whether cIAP2 and A1/Bfl-1 are induced during cholestatic liver injury. The mRNA expression of both anti-apoptotic cIAP2 and A1/Bfl-1 genes was strongly increased (Fig 3).

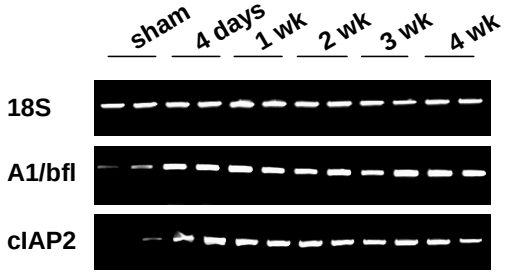
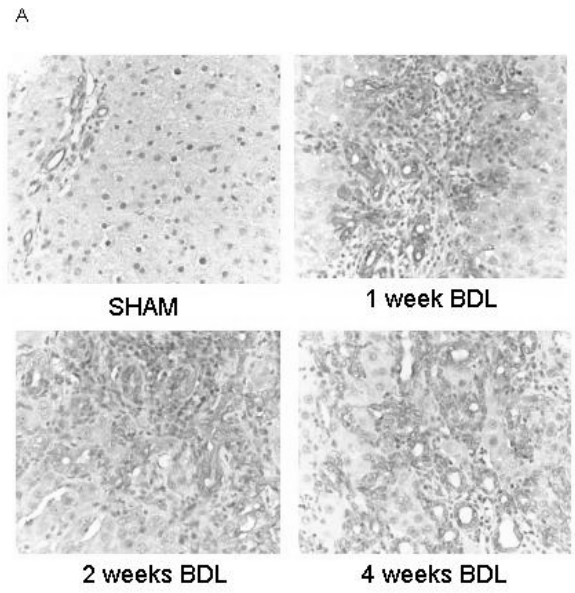


Figure 3. Induced mRNA expression of NF-κB-regulated genes A1/Bfl-1 and cIAP2 in rat livers after bile duct ligation (BDL). 18S mRNA was used as internal control. SHAM animals of 1 week served as control. Two of four representative animals are shown.

Bcl-2, an anti-apoptotic protein was clearly induced in cholestatic livers (Fig 4b), but immunohistochemical evaluation demonstrated that expression of this protein was restricted to bile duct epithelium (Fig 4a).



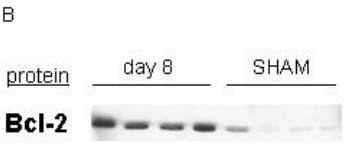


Figure 4. Bcl-2 expression in cholestatic rat livers is restricted to bile duct epithelium. SHAM animals of 1 week served as control. (A) Immunohistochemistry on formalin fixed, paraffin-embedded liver sections after 1-4 weeks of BDL. (B) Protein expression after 8 days of bile duct ligation (BDL).

GCDCA, but not TUDCA, induces apoptosis in a FADD-independent manner

A time course study on isolated rat hepatocytes exposed to the toxic bile acid GCDCA confirmed that apoptosis was induced one hour after exposure, peaking at 4 hours and returning to control levels within 24 hours (Fig 5). This confirmed previous reports.[6] TUDCA and TCDCA did not induce apoptosis.

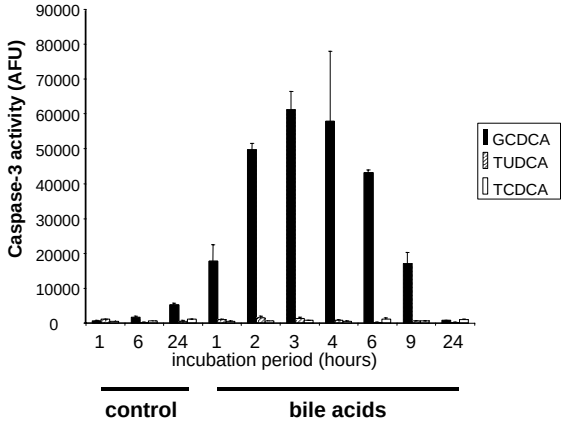


Figure 5. Glycochenodeoxycholic acid (GCDCA) induces caspase-3 activity. Time course study of caspase-3 activity in primary hepatocytes exposed to 50 μM of GCDCA, TUDCA or TCDCA. Representative data of 3 independent experiments are shown and presented as mean of n = 3 per condition.

In hepatocytes, a 3-fold increase of caspase-8 activity was demonstrated 2 hours after GCDCA exposure, which was still elevated after 4 hours (Fig 6a**)**. TUDCA did not induce caspase-8 activity (data not shown). Interestingly, GCDCA-induced activation of caspase-8 (Fig 6a) and caspase-3 (Fig 6b) was shown to be FADD-independent. Functionality of Ad5dnFADD was demonstrated by its complete inhibition of cytokine-induced caspase-3 activation in hepatocytes in which NF-κB activation is inhibited (Fig 6c). Caspase-8 inhibition by Ac-IETD-CHO (Fig 6a) had no effect on GCDCA-induced activation of caspase-3 (Fig 6b).

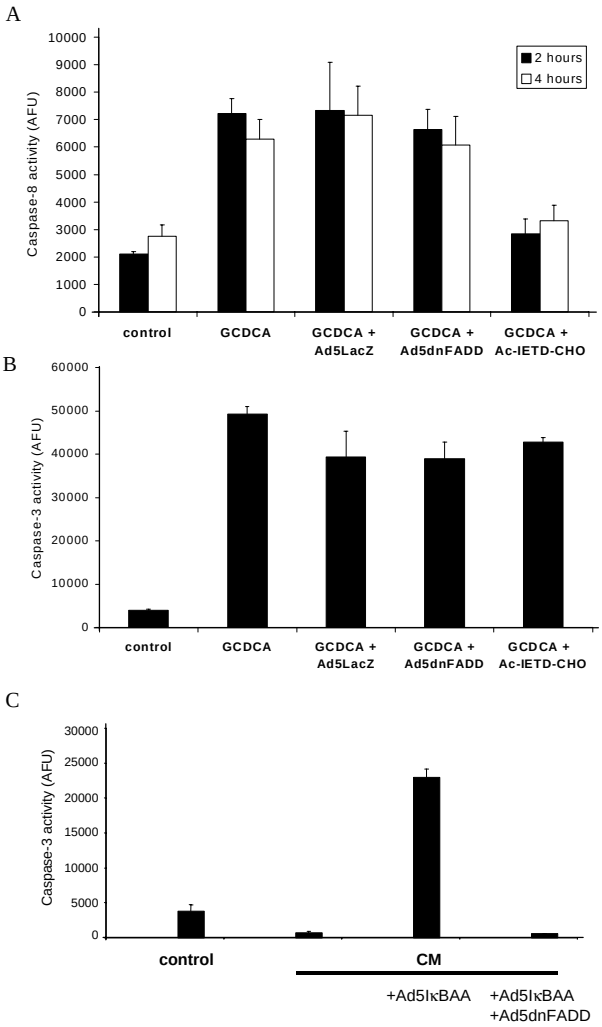


Figure 6. GCDCA induces FADD-independent caspase-3 and caspase-8 activation in primary hepatocytes. (A) Caspase-8 activity after 2, and 4 hours of exposure to GCDCA with or without caspase-8 inhibitor (Ac-IETD-CHO) or adenoviral expression of dominant negative FADD (AD5dnFADD). LacZ virus (Ad5LacZ) served as control. (B) Caspase-3 activity after 4 hours of exposure to GCDCA with or without Ad5LacZ, Ad5dnFADD or Ac-IETD-CHO. (C) Caspase-3 activity after 6 hours of exposure to cytokines (CM) and specific inhibition of NF-κB (Ad5IkBAA) plus or minus Ad5dnFADD. One representative of 3 independent experiments is shown. Mean of n = 3 per condition is presented.

Immunocytochemistry on GCDCA-exposed hepatocytes demonstrated a clear caspase-9 staining after 4 hours which was not observed in control hepatocytes or TUDCA-exposed hepatocytes (Fig 7a). Western blot confirmed these results, demonstrating active caspase-9 only in GCDCA-exposed hepatocytes, but not in control or TUDCA-exposed hepatocytes (Fig 7b).

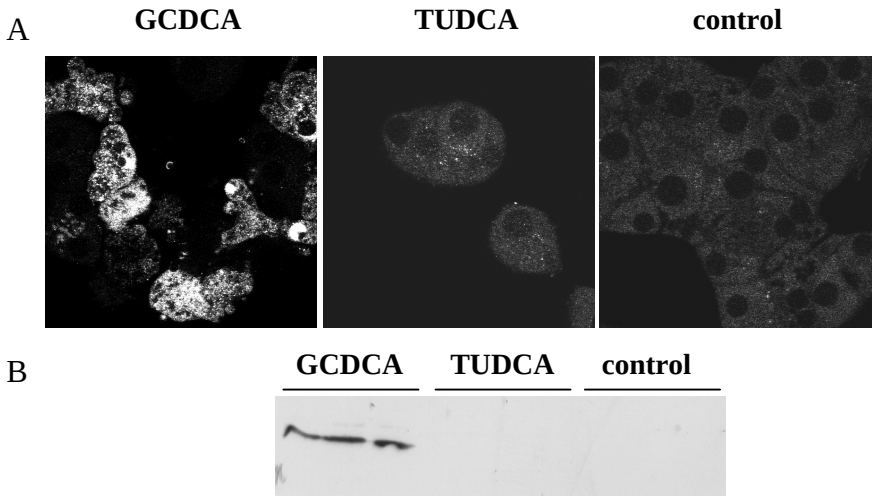


Figure 7. Caspase-9 is activated in GCDCA-induced primary hepatocytes. (A) Immunocytochemistry and (B) Western blot on primary hepatocytes after 4 hours of exposure to GCDCA and TUDCA. One representative of 3 independent experiments is presented.

Bile acids do not activate the transcription factor NF-κB in hepatocytes

We demonstrated that NF-κB-regulated genes are induced in cholestatic livers (Fig 3). Therefore, we investigated whether NF-κB is involved in the protection against bile acid-induced apoptosis. EMSA, performed on nuclear extracts of primary hepatocytes, demonstrated that GCDCA, TCDCA and TUDCA did not activate NF-κB, whereas cytokines clearly activated this transcription factor (Fig 8a). mRNA expression of NF-κB-regulated anti-apoptotic A1/Bfl-1 and cIAP2 is not induced by GCDCA, TCDCA and TUDCA as shown in Figure 8b. NF-κB-regulated inducible Nitric Oxide synthase (iNOS) was included as positive control. Finally, inhibition of NF-κB by recombinant adenovirus expressing dominant negative IκB did not increase GCDCA-induced caspase-3 activity and did not influence the effects of TUDCA and TCDCA on caspase-3 activity (Fig 8c).

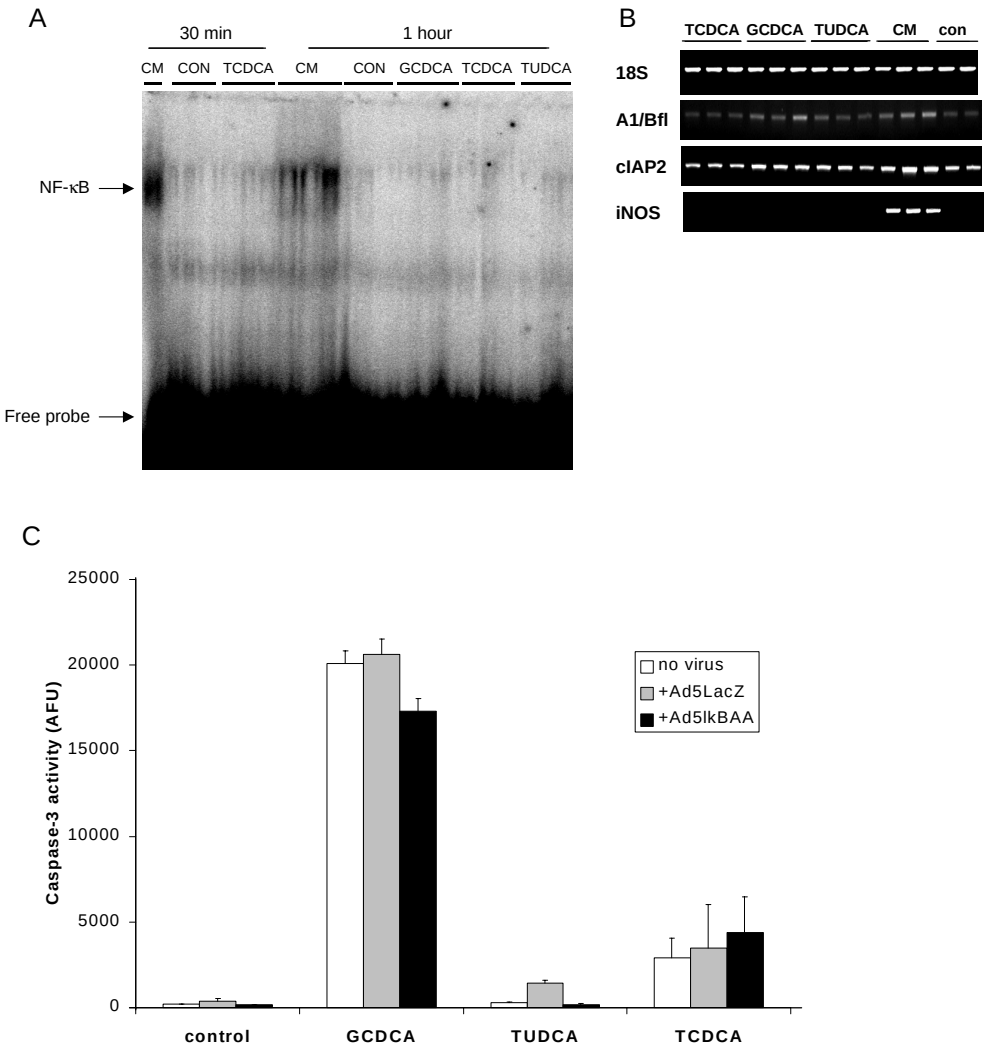


Figure 8. Bile acids do not activate NF-κB in primary hepatocytes. One representative (mean of n = 3 per condition) of 3 independent experiments is shown. (A) EMSA on nuclear extracts of hepatocytes treated for 30 min to 1 hour with 50 μM GCDCA, TUDCA, TCDCA, or cytokines (CM). (B) mRNA expression of NF-κB-regulated genes (A1/Bfl and cIAP2) 10 hours after incubation with bile acids or cytokines. 18S and iNOS mRNA served as internal and positive control, respectively. (C) Caspase-3 activity 4 hours after bile acid incubation. Adenoviral expression of dominant negative IκB-α (Ad5IκBAA) was used to inhibit NF-κB. LacZ virus (Ad5LacZ) served as control.

Cytokines inhibit GCDCA-induced apoptosis of hepatocytes

The protective role of cytokine-induced NF-κB activation against bile acid-induced apoptosis was investigated. Since in chronic cholestatic livers TNF-α, IL-1β, and IFN-γ are elevated (shown in Figure 1c), we used these cytokines *in vitro* together with LPS. Primary hepatocytes were pre-incubated for different time intervals with cytokines prior to the addition of GCDCA. Pre-stimulation with cytokines for 3 hours significantly inhibited GCDCA-induced apoptosis (Fig 9a). As shown in Figure 9b, adenoviral overexpression of the human homologue of rat cIAP2 in primary hepatocytes completely inhibited GCDCA-induced caspase-3 activation. These results indicate that cytokine-induced activation of NF-κB inhibits GCDCA-induced apoptosis in primary hepatocytes and that this protection is at least partly due to cIAP2.

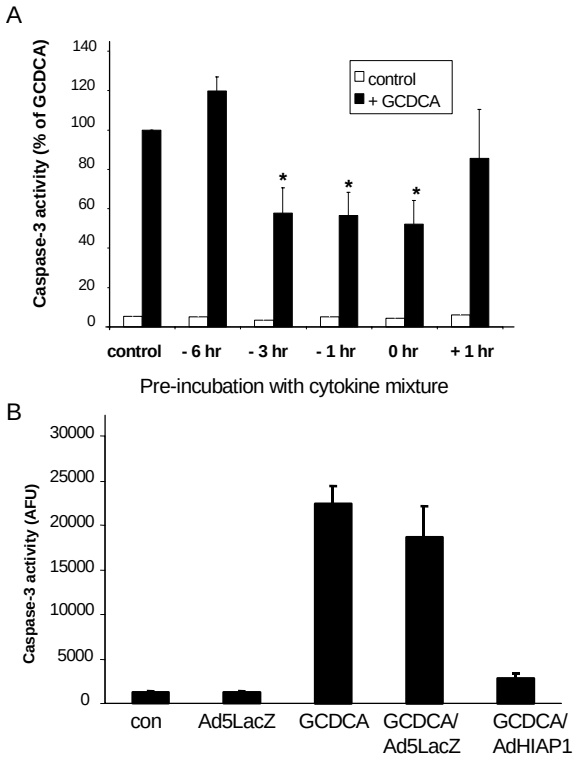


Figure 9. Cytokines inhibit GCDCA-induced apoptosis. (A) Primary hepatocytes were pre-incubated for differend time intervals (hours(hr)) with cytokines (TNF-α, IL-1β, IFN-γ) + LPS, before adding glycochenodeoxycholic acid (GCDCA). Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of 3 independent experiments ± SD. *P < 0.05 for -3 hr, -1 hr and 0 hr vs. GCDCA alone (control). (B) Caspase-3 activity in primary hepatocytes exposed to GCDCA with or without adenoviral overexpression of the human homologue cIAP2 (AdHIAP1). LacZ virus served as control (Ad5LacZ). Representative data of 3 independent experiments are shown and presented as mean of n= 3 per condition.

5.5 Discussion

Bile acids are in part responsible for liver injury during cholestasis. Apoptosis has been postulated as an important mechanism for this liver injury.[25] However, in the present study using three different methods to detect apoptosis, we could not demonstrate apoptotic hepatocytes in cholestatic livers. In previous studies[25], TUNEL staining was used to demonstrate apoptosis. Since the TUNEL assay frequently yields false-positive results[26], we have also used a more apoptosis-selective staining directed against active caspase-3 and an assay to measure caspase-3 activity. In contrast to previous studies investigating apoptosis only within one week after BDL[27;28], we investigated apoptotic cell death up to 4 weeks after BDL. We demonstrate that caspase-3 activity is only elevated within one week after BDL confirming previous results. However, after 1 week, caspase-3 activity rapidly declines to control levels. Taken these data together, we conclude that hepatocytes are well protected against apoptosis during cholestasis. Increased levels of ASAT and ALAT (Fig 1) after bile duct ligation must be due to necrotic cell damage.

During cholestasis, the NF-κB pathway is activated[27] and we demonstrated in this report that the NF-κB-dependent anti-apoptotic genes A1/Blf-1 and cIAP2[15] are highly induced in BDL livers. However, we found that none of the bile acids tested activates NF-κB in hepatocytes and that inhibition of NF-κB activation did not result in increased bile acid-induced apoptosis. This contrasts to reports describing NF-κB activation by TCDCA.[12] However, these latter results were obtained in hepatoma cells and therefore, conclusions from these studies cannot be extrapolated to primary hepatocytes.

Activation of the NF-κB pathway in cholestasis can be explained by the elevated expression of cytokines as part of the inflammatory response. We observed increased expression of TNF-α, IL-1β and IFNγ mRNA in BDL livers. Moreover in TNF-receptor type I knock-out mice, the activation of NF-κB is not

inhibited after BDL.[27] Again indicating that besides TNF-α, the onset of this survival pathway during cholestasis can be explained by IL-1β and IFNγ.[29;30] A protective effect of NF-κB activation in cholestasis is supported by our observations that *in vitro* overexpression of cIAP2 inhibited GCDCA-induced apoptosis. Furthermore, cytokine-induced activation of NF-κB in hepatocytes prior to GCDCA exposure significantly reduced GCDCA-induced apoptosis. Therefore, we postulate that cytokine-induced NF-κB-regulated A1/Bfl-1 and cIAP2 contribute to the protection of hepatocytes against bile acid-induced apoptosis during cholestasis.

We also observed a clear induction of the anti-apoptotic Bcl-2 in total liver homogenates. In contrast to studies describing Bcl-2 expression in hepatocytes[31], we found that this expression was restricted to bile duct epithelium and was not detected in hepatocytes in BDL livers. Our results are in accordance with other studies reporting absence of Bcl-2 expression in hepatocytes in a variety of liver diseases.[32;33] Therefore, we conclude that Bcl-2 is not involved in the protection of hepatocytes against bile acid-induced apoptosis.

The present report demonstrated that only GCDCA induces apoptosis in primary hepatocytes, whereas the taurine conjugates of CDCA and UDCA do not. An interesting observation is the FADD-independent activation of caspase-8 and caspase-3 in GCDCA-exposed primary hepatocytes. Although dominant-negative FADD did completely abolish cytokine-induced apoptosis, we did not detect inhibition of GCDCA-induced apoptosis. This is in contrast to results obtained in hepatocytes exposed to DCA. Blocking of FADD did inhibit apoptosis in these cells.[8] However, in this study, apoptosis is induced by another bile acid in the presence of a MAPK inhibitor. Not all bile acids induce apoptosis in hepatocytes: the glycine-conjugated bile acid GCDCA induces apoptosis, whereas the taurine-conjugated bile acids, TUDCA and TCDCA do not. Therefore, it is likely that DCA behaves differently compared to glycine-conjugated CDCA. Contrary to our data using primary cultures of rat hepatocytes, others observed FADD-dependent apoptosis in rat hepatoma cells.[7] However, it is known that the regulation of cell

survival and apoptosis in continuously proliferating hepatoma cells is different from non-proliferating primary non-transformed hepatocytes. In cancer cell lines like Jurkat and Burkitt's lymphoma, caspase-8 is activated in a FADD-independent manner.[35;36] Besides FADD, another adaptor protein could be involved in caspase-8 activation, e.g. FLASH, a protein that interacts with a dead-effector domain of caspase-8. Whether this protein is also present and active in hepatocytes has to be clarified.[36] A role for Fas in GCDCA-induced apoptosis has been proposed using hepatocytes from Fas-deficient mice.[7] These and other authors also demonstrated GCDCA-induced Fas trafficking and aggregation in rat hepatoma cells.[7;34] Our data demonstrating FADD-independent apoptosis of GCDCA appear to rule out an important role of Fas in GCDCA-induced apoptosis in primary cultures of rat hepatocytes. At present, apart from differences in species and cell type, we have no explanation for these discrepant results.

Another explanation for FADD-independent activation of caspase-8 after exposure to GCDCA is postmitochondrial processing of caspase-8. Indeed, one of the key findings of our study is that caspase-8 inhibition had no effect on GCDCA-induced caspase-3 activation. Hepatocytes are type II cells in which formation of the death inducing signaling complex (DISC) is strongly reduced.[37;38] In type II cells, apoptosis is dependent on the release of pro-apoptotic factors from mitochondria, which activate caspase-9 and subsequent caspase-3 activation. Recently, it has been demonstrated that caspase-6 is the direct activator of caspase-8 in the cytochrome c-induced apoptosis pathway.[39] We demonstrated GCDCA-induced caspase-9 activation, indicating the release of pro-apoptotic factors from mitochondria. Therefore, postmitochondrial activation of caspase-8 is very plausible and in accordance with existing literature.[39-41] This hypothesis is supported by two facts: mitochondria play a key role in bile acid-induced apoptosis[42] and GCDCA-induced apoptosis is inhibited by blockers of the mitochondrial permeability transition.[11]

In summary, we postulate that in primary hepatocytes, GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a FADD-independent manner (Fig 10).

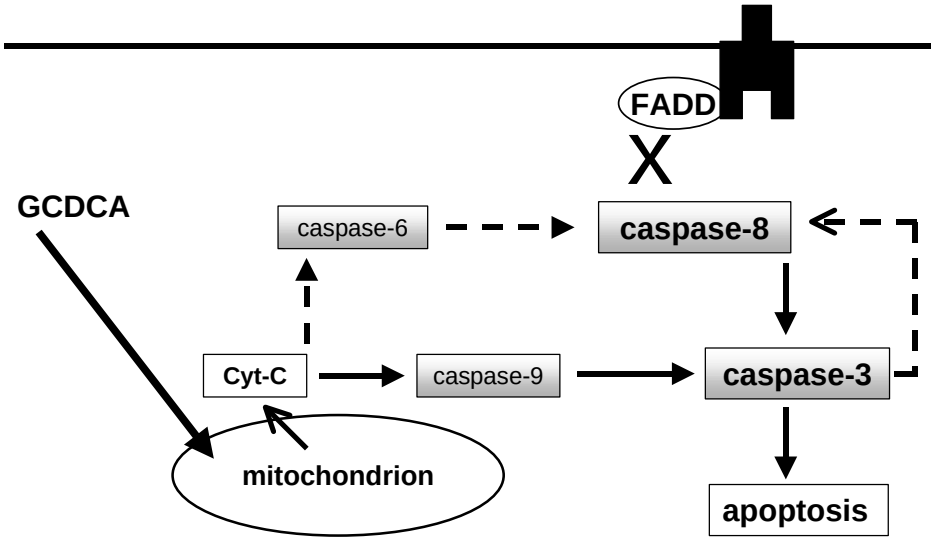


Figure 10. Mechanisms of glycochenodeoxycholic acid (GCDCA)-induced apoptosis in primary rat hepatocytes. GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a FADD-independent manner. See Discussion for details. Dotted arrows are postulated events in primary hepatocytes.

Overall, we conclude that hepatocytes are protected against bile acid-induced apoptosis during cholestatic liver injury due to induction of cytokine induced, but not bile acid-induced NF-κB-dependent anti-apoptotic genes like A1/Bfl-1 and cIAP2. These findings are relevant for the treatment of patients with inflammation inhibitors resulting in a-selective inhibition of NF-κB.

5.6 Acknowledgements

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5.7 References

1. Popper H. Cholestasis. Annu Rev Med 1968;19:39-56
2. Greim H, Trülzsch D, Roboz J, Dressler K, Czygan P, Hutterer F, Schaffner F. Mechanisms of cholestasis 5. Bile acids in normal rat livers and in those after bile duct ligation. Gastroenterology 1972;63:837-845.
3. Schölmerich J, Becher MS, Schmidt K, Schubert R, Kremer B, Feldhaus S, Gerok W. Influence of hydroxylation and conjugation of bile salts on their membrane-damaging properties-studies on isolated hepatocytes and lipid membrane vesicles. Hepatology 1984;4:661-666.
4. Rodrigues CMP, Fan G, Ma X, Kren BT, CJ Steer. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998;101:2790-2799.
5. Patel T, Bronk SF, Gores GJ. Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes. J Clin Invest 1994;94:2183-2192.
6. Gonzalez B, Fisher C, Rosser BG. Glycochenodeoxycholic acid (GCDC) induced hepatocyte apoptosis is associated with early modulation of intracellular PKC activity. Mol Cell Biochem 2000;207:19-27.
7. Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA, Kaufmann SH. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. J Clin Invest 1999;103:137-145.
8. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, Kukreja R et al. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and Fas receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase–signaling module enhanced DCA-induced apoptosis. Mol Biology of Cell 2001;12:2609-2645.

9. Botla R, Spivey JR, Aguilar H, Bronk SF, Gores GJ. Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. J Pharmacol Exp Therap 1995;272:930-938.
10. Rodrigues CMP, Ma X, Linehan-Stieers C, Fan G, Kren BT, Steer CJ. Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. Cell Death Diff 1999;6:842-854.
11. Yerushalmi B, Dahl R, Devereaux MWE, Gumpricht E, Sokol RJ. Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. Hepatology 2001;33:616-626.
12. Rust C, Karnitz LM, Paya CV, Moscat J, Simari RD, and Gores GJ. The bile acid taurochenodeoxycholate activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. J Biol Chem 2000;275:20210-20216
13. Rubin RA, Kowalski TE, Khadelwal M, Malet PF. Ursodiol for hepatobiliary disorders. Ann Intern Med 1994;121:207-218.
14. Benz C, Angermüller S, Otto G, Sauer P, Stremmel W and Stiehl A. Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. Eur J Clin Invest 2000;30:203-209.
15. Schoemaker MH, Ros JE, Homan M, Trautwein C, Liston P, Poelstra K, Van Goor H et al. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-κB-regulated Inhibitor of Apoptosis protein-2 (cIAP2) prevents apoptosis. J. Hepatol 2002;36:742-750.
16. Hatano E, Bennett BL, Manning AM, Qian T, Lemasters JJ, Brenner DA. NF-κB stimulates inducible nitric oxide synthase to protect mouse hepatocytes from TNF-α- and Fas-mediated apoptosis. Gastroenterology 2001;120:1251-1262.
17. Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. Br J Exp Pathol 1984;65:305-311.
18. Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990;12:511-518.
19. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW, Brenner DA. NF-κB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998;101:802-811.
20. Perrelet D, Ferri A, Mackenzie AE, Smith GM, Korneluk RG, Liston P, Sagot Y et al. IAP family proteins delay motoneuron cell death in vivo. Eur J Neuroscience 2000;12:2059-2067.
21. Hatano E, Bradham CA, Stark A, Iimuro Y, Lemasters JJ, Brenner DA. The mitochondrial permeability transition augments Fas-induced apoptosis in mouse hepatocytes. J Biol Chem 2000;275:11814-11823.
22. Vos TA, van Goor H, Tuyt LML, de Jager-Krikken A, Leuvenink R, Kuipers F, Jansen PLM et al. Expression of inducible nitric oxide synthase in endotoxemic rat hepatocytes is dependent on the cellular glutathione status. Hepatology 1999;29:421-426.

23. Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. J Immunol 1998;160:3480-3486.
24. Van der Woude CJ, Jansen PL, Tiebosch AT, Beuving A, Homan M, Kleibeuker JH and Moshage H. Expression of apoptosis-related proteins in Barrett's metaplasia-dysplasia-carinoma sequence: a switch to a more resistant phenotype. Hum Pathol 2002;33:686-92.
25. Miyoshi H, Rust C, Roberts PJ, Burgar LJ and Gores GJ. Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 1999;117:669-677.
26. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995;21:1465-1465.
27. Miyoshi H, Rust C, Guicciardi ME, Gores GJ. NF-kappaB is activated in cholestasis and functions to reduce liver injury. Am J Pathol 2001;158-967-975.
28. Graf D, Kurz AK, Reinehr R, Fischer R, Kircheis G and Häussinger D. Prevention of bile acid-induced apoptosis by betaine in rat liver. Hepatology 2002;36:829-839.
29. Grinko I, Geerts A, and Wisse E. Experimental biliary fibrosis correlates with increased numbers of fat-storing and Kupffer cells, and portal endotoxemia. J Hepatol 1995;23:449-458.
30. Sewnath ME, Van Der Poll T, Ten Kate FJ, Van Noorden CJ, and Gouma DJ. Interleukin-1 receptor type 1 gene-deficient bile duct-ligated mice are partially protected against endotoxin. Hepatology 2002;35:149-58
31. Kurosawa H, Que FG, Robert LR, Fesmier PJ, and Gores GJ. Hepatocytes in the bile duct-ligated rat express Bcl-2. Am J Physiol 1997;272:G1587-G1593.
32. Nakopoulou L, Stefanaki K, Vourlakou CH, Manolaki N, Gakiopoulou H, and Michalopoulos G. Bcl-2 protein expression in acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Pathol Res Pract 1999;195:19-24
33. Iwata M, Harada K, Kono N, Kaneko S, Kobayashi K, and Nakanuma Y. Expression of Bcl-2 familial proteins is reduced in small bile duct lesions of primary biliary cirrhosis. Hum Pathol 2000;31:179-184.
34. Takikawa Y, Miyoshi H, Rust C, Roberts P, Siegel R, Mandal PK, Millikan RE et al. The bile acid-activated phosphatidylinositol 3-kinase pathway inhibits Fas apoptosis upstream of Bid in rodent hepatocytes. Gastroenterology 2001;120:1810-1817
35. Schrantz N, Bourgeade M, Mouhamad S, Leca G, Sharma S, and Vazquez A. p38-mediated regulation of an Fas-associated Death Domain protein independent pathway leading to caspase-8 activation during TGF-β-induced apoptosis in human Burkitt Lymphoma B cells BL41. Mol Biol Cell 2001;12:3139-3151.
36. Varghese J, Sade H, Vandenabeele P, and Sarin A. Head Involution Defective (Hid)-triggered apoptosis requires caspase-8 but not FADD (Fas-associated Death Domain) and is regulated by Erk in mammalian cells. Journal Biol Chem 2002;277:35097-35104.

37. Imai Y, Kimura T, Murakami A, Yajima N, Sakamaki K, Yonehara S. The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis. Nature 1999;398:777-785.
38. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO journal 1998;17:1675-1687.
39. Cowling V and Downward J. Caspase-6 is the direct activator of caspase-8 in the cytochrome c-induced apoptosis pathway:absolute requirement for removal of caspase-6 prodomain. Cell Death and Diff 2002;9:1046-1056.
40. Bajt ML, Lawson JA, Vonderfecht SL, Gujral JS, Jaeschke H. Protection against Fas receptor-mediated apoptosis in hepatocytes and nonparenchymal cells by a caspase-8 inhibitor in vivo: evidence for a postmitochondrial processing of caspase-8. Toxicol Sci 2000;58:109-117
41. Slee EA, Adrain C, and Martin SJ. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 1999;6:1067-74
42. Rodrigues CM and Steer CJ. Mitochondrial membrane perturbations in cholestasis. J Hepatology 2000;32:135-141.

Chapter 6

TUDCA protects rat hepatocytes from bile acid-induced apoptosis via activation of survival pathways

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Hepatology, in press

6.1 Abstract

Ursodeoxycholic acid (UDCA) is used in the treatment of cholestatic liver diseases, but its mechanism of action is not well defined yet. The aim of this study is to explore the protective mechanisms of the taurine-conjugate of UDCA (tauroursodeoxycholic acid (TUDCA)) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis in primary cultures of rat hepatocytes. Hepatocytes were exposed to GCDCA, TUDCA, the glyco-conjugate of UDCA (GUDCA) and taurochenodeoxycholic acid (TCDCA). The PI3-kinase (PI3K) pathway and NF-κB were inhibited using LY 294002, and adenoviral overexpression of dominant negative IκB, respectively. The role of p38 and ERK MAP kinase pathways were investigated using the inhibitors SB 203580 and U0 126, and Western blot analysis. Transcription was blocked by actinomycin-D. Apoptosis was determined by measuring caspase-3, -9 and -8 activity using fluorimetric enzyme detection, Western blot analysis, immunocytochemistry and nuclear morphology. Our results demonstrated that uptake of GCDCA is needed for apoptosis induction. TUDCA, but not TCDCA and GUDCA, rapidly inhibited, but did not delay, apoptosis at all time points tested. However, the protective effect of TUDCA was independent of its inhibition of caspase-8. Up to six hours pre-incubation with TUDCA prior to GCDCA clearly decreased GCDCA-induced apoptosis. Up to 1.5 hours after exposure with GCDCA, addition of TUDCA was still protective. This protection was dependent on activation of p38, ERK MAP kinases and PI3-kinase pathways, but independent of competition on the cell membrane, NF-κB activation and transcription. In conclusion: TUDCA contributes to the protection against GCDCA-induced mitochondria-controlled apoptosis by activating survival pathways.

6.2 Introduction

Cholestatic liver diseases are characterized by accumulation of toxic bile acids, e.g. glycochenodeoxycholic acid (GCDCA), causing damage to hepatocytes and cholangiocytes. Ursodeoxycholic acid (UDCA) is used as a treatment for patients with chronic cholestatic liver diseases. In primary biliary cirrhosis, doses of 13 to 15 mg/kg/day of UDCA decrease serum liver enzymes, improve liver histology, and delay the time to liver transplantation or death for up to 4 years.[1;2] However, the mechanisms of the beneficial effect of UDCA in these conditions remain unclear. From *in vitro* and *in vivo* studies, it is postulated that UDCA protects cholangiocytes against membrane damage induced by hydrophobic bile acids.[3;4] Furthermore, it has been demonstrated that UDCA stimulates biliary secretion of bile acids and other toxic compounds.[5;6] In addition, anti-apoptotic effects of UDCA have been described, such as the inhibition of the mitochondrial membrane permeability transition in hepatocytes,[7;8] leading to prevention of the mitochondrial release of cytochrome c.[9] A recent study has suggested that TUDCA does inhibit apoptosis by preventing the binding of Bax to mitochondria.[10]

Hepatocytes are exposed to many pro-apoptotic compounds. Therefore, anti-apoptotic signalling pathways are important to limit programmed cell death. The anti-apoptotic action of UDCA may in part be due to activation of these anti-apoptotic pathways. A major survival pathway in hepatocytes is the activation of the transcription factor NF-κB. Activation of NF-κB-regulated survival genes causes inhibition of apoptosis.[11;12] Although we have shown that NF-κB is not activated by bile acids[13], its role in the protection of UDCA against bile acid-induced apoptosis is not clear. Furthermore, other cell survival pathways, like the activation of mitogen-activated protein kinases (MAPK), could be involved in the anti-apoptotic action of UDCA. These kinases are involved in regulation of cell proliferation, differentiation and apoptosis and are comprised of at least three different pathways: ERK, p38 and JNK.[14] Although it is postulated that inhibition of ERK

enhances UDCA-induced apoptosis,[15] little is known about the role of MAP kinases in the protection of taurine-conjugated UDCA against GCDCA-induced apoptosis. Another important survival pathway is the phosphatidylinositol-3 kinase (PI3K) pathway. This kinase cascade results in activation of a number of cellular intermediates of which Akt seems to be one of the most important survival factors.[16] The mechanisms by which PI3K/Akt promote cell survival are diverse and its role in the protection of UDCA against GCDCA-induced apoptosis has not been explored yet. In rats, the taurine conjugate of UDCA predominates compared to glycine-conjugated UDCA.[17;18] Furthermore, TUDCA may be of benefit for patients suffering from primary biliary cirrhosis.[19] Therefore, we have investigated the anti-apoptotic actions of TUDCA in primary rat hepatocytes.

6.3 Materials and methods

Animals

Specified pathogen-free male Wistar rats (220-250 g) were purchased from Harlan, Zeist, the Netherlands. They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local Committee for Care and Use of Laboratory Animals.

Hepatocyte isolation

Hepatocytes were isolated as described previously.[20] Cell viability was consistently more than 90 % as determined by trypan blue exclusion. Isolated hepatocytes were plated at a density of 150000 cells per cm^2 in William's medium E (Life Technologies Ltd., Breda, The Netherlands) supplemented with 50 μg/ml gentamycin (BioWhittaker, Verviers, Belgium) without the addition of hormones or growth factors. During the attachment period (4 hours) 50 nmol/L dexamethasone

(Sigma, St Louis, MO) and 5 % FCS (Life Technologies Ltd.) were added to the medium. Cells were cultured in a humidified incubator at 37 °C / 5 % CO_2.

Experimental design

Experiments were started twenty-four hours after isolation. Hepatocytes were exposed to 50 μmol/L glyco-chenodeoxycholic acid (GCDCA, Calbiochem, La Jolla, CA), 50 μmol/L tauro-ursodeoxycholic acid (TUDCA, Calbiochem), 50 μmol/L glyco-ursodeoxycholic acid (GUDCA, Calbiochem), or 50 μmol/L tauro-chenodeoxycholic acid (TCDCA, Calbiochem) for 4 hours or the indicated time period. In some experiments, hepatocytes were exposed to 20 ng/ml recombinant mouse tumor necrosis factor α (TNFα, R&D Systems, Abingdon, United Kingdom) or a mixture of cytokines as described previously.[11] The signal transduction or apoptosis pathways were specifically inhibited with the following compounds: 50 μmol/L of caspase-8 inhibitor Ac-IETD-CHO or the caspase-3 inhibitor Ac-DEVD-CHO (Biomol, Plymouth Meeting, USA), 10 μmol/L of the p38 inhibitor SB 203580 (Biomol), 10 μmol/L of the ERK1/2 inhibitor U0 126 (Promega, Madison, USA), 50 μmol/L PI3-kinase inhibitor LY 294002 (Sigma-Aldrich), and 200 ng/ml of the transcriptional inhibitor actinomycin-D (Roche Diagnostics, Almere, The Netherlands). All inhibitors were added 30 minutes prior to bile acids or cytokines. Hepatocytes received adenovirus (MOI of 10) fifteen hours prior to exposure of bile acids. Each experimental condition was performed in triplicate wells. Each experiment was performed at least three times, using hepatocytes from different isolations.

Cells were harvested at the indicated time-points after the addition of bile acids, and rinsed 2 times with ice-cold PBS prior to the addition of hypotonic cell lysis buffer.[11] For measurement of caspase-8 activity, cells were harvested in cell lysis buffer using a caspase-8 fluorometric Protease Assay Kit (BioVision, Mountain View, USA).

HepG2 cell experiments

The human hepatoma cell line HepG2 and a stable derivative expressing rNtcp were cultured as described before.[21;22] Cells were incubated with indicated amounts of GCDCA or 1 μg/ml anti-Fas antibody (clone nr. 7C11; Immunotech, Marseille, France) for 4 hours followed by harvesting in hypotonic cell lysis buffer.[11]

Adenoviral constructs

Recombinant, replication-deficient adenovirus Ad5IκBAA was used to inhibit NF-κB activation as described previously.[23] As a control virus Ad5LacZ was used which contains the *E. coli* β-galactosidase gene.

Caspase-3 and caspase-8 enzyme activity assay

Hepatocytes were scraped and cell lysates were obtained by three cycles of freezing (-80 °C) and thawing (37 °C) followed by centrifugation for 5 minutes at 13.000 rpm. Caspase-3 enzyme activity was assayed using a caspase-3 activity kit with fluorimetric detection (Promega) according to the manufacturer's instructions. Caspase-8 activity was measured using a Caspase-8 Fluorometric Protease Assay Kit (BioVision) according to the manufacturer's instructions. Cells were harvested directly in chilled cell lysis buffer supplied in the kit. Assays were performed with 20 μg of protein.

Nuclear staining

Morphology of apoptotic nuclei was demonstrated with acridine orange. Cells were seeded on glass coverslides and treated as indicated. These coverslides were fixed in methanol for 5 minutes, air-dried and rinsed two times in PBS before incubating in acridine orange (1:1000) for 15 minutes in the dark. Coverslides were rinsed two times with PBS and placed upside down on microscope slides using nail polish. Fluorescent nuclei were visualized using a Leica confocal laser-scanning microscope.

Immunocytochemistry and uptake of fluorescent bile acids

Analysis of active caspase-9 was performed on hepatocytes cultured on coverslips and exposed to 50 μmol/L bile acids for 4 hours. Coverslips were washed in PBS, fixed in 4 % paraformaldehyde for 10 minutes followed by incubation in 1 % Triton-X100 for 5 minutes. Before adding primary antibodies, cells were washed twice with PBS. Antibody against active caspase-9 was used at a dilution of 1:50 for 30-60 minutes. FITC-conjugated goat-anti-rabbit Ig (Molecular Probes, Eugene, Oregon, USA) was added at a dilution of 1:600 for 45 minutes.

The uptake of bile acids was demonstrated using the fluorescent bile acid cholyl lysyl fluorescein (CLF).[24] Cells were incubated with 2 μmol/L CLF at 37 °C for 15 minutes. All slides were evaluated on a Leica confocal laser-scanning microscope

Western blot analysis

Western blot analysis of cell lysates was performed using polyclonal rabbit antibodies against cleaved caspase-9 and phosphorylated p38 MAP, and monoclonal antibody against phosphorylated ERK1/2 (p44/42) MAP kinase (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. Hepatocytes exposed to 50 μmol/L deoxycholic acid (DCA) served as positive control for phophorylated ERK1/2.[25] In addition, activated neutrophil extracts (kindly provided by Dr. Gwenny Fuhler, Department of Haematology, University Hospital Groningen) were used to confirm detection of phosphorylated ERK1/2 MAP kinase. For the detection of caspase-9 and phospho-p38, horse radish-peroxidase conjugated swine-anti-rabbit Ig was used as a secondary antibody at a dilution of 1:2000. Phospho-ERK1/2 was detected with horse radish-peroxidase rabbit-anti-mouse Ig (1:2000). Each lane contained the lysate of 150000 cells. Equal loading was demonstrated by Ponceau-S staining. After Western blot analysis of phosphorylated p38 and ERK1/2 MAP kinases, blots were stripped using 0.1 % SDS at 65 °C for 30 minutes and incubated with 1:1000 antibody against total p38 MAP kinase or total

ERK1/2 MAP kinase (Santa Cruz Biotechnology, Santa Cruz, USA). Western blot analysis for iNOS was performed as described before.[26]

Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± standard deviation. A Mann-Whitney test was used to determine the significance of differences between two experimental groups. A P value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

6.4 Results

Ntcp is required for GCDCA-induced mitochondria-controlled apoptosis

To investigate the protective mechanisms of TUDCA against GCDCA-induced apoptosis, we first determined whether GCDCA needs to be taken up by cells to induce apoptosis. Previous studies have postulated death receptor activation by toxic bile acids.[27-29] Nonetheless strong distinction between the role of the bile acid uptake transporter Ntcp and death receptor-mediated apoptosis has not been made yet. Recently, we have shown that GCDCA induced apoptosis in a mitochondrial-controlled manner in primary rat hepatocytes, which was FADD-independent.[13] In the present study, we examined whether Ntcp is required for GCDCA-induced apoptosis. For this purpose, we exposed Ntcp-negative and Ntcp-positive HepG2 cells[21] to different concentrations of GCDCA. Both cell lines do express Fas death receptor. Anti-Fas antibody served as positive control. As shown in Figure 1a, only HepG2 cells expressing Ntcp on their cell membrane are sensitive to GCDCA-induced apoptosis, which increased with increasing amounts of GCDCA. In contrast, anti-Fas antibody induced apoptosis in both Ntcp-positive and Ntcp-negative cell lines to the same extent.

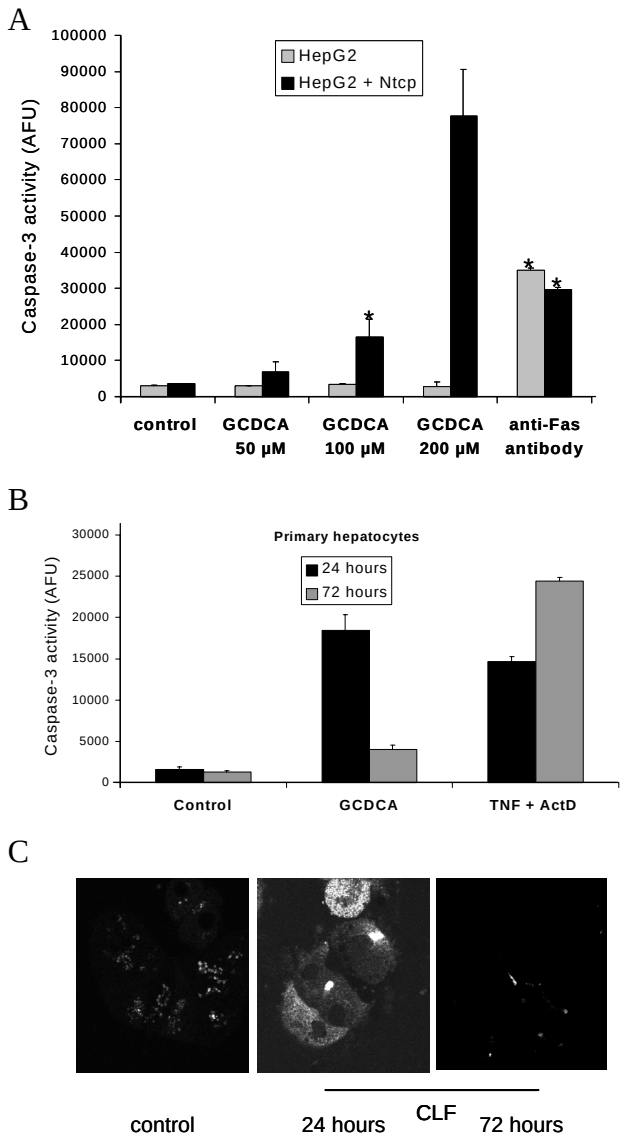


Figure 1. Ntcp is required for glycochenodeoxycholic acid (GCDCA)-induced apoptosis. (A) Ntcp positive and negative HepG2 cells were treated with different amounts of GCDCA and 1 µg/µl anti-Fas antibody (positive control) for 4 hours. Only in Ntcp-positive HepG2 cells, caspase-3 activity increased with increasing amounts of GCDCA. *P<0.05 for GCDCA 100 µM and anti-Fas antibody vs. control. (B) Caspase-3 activity in primary rat hepatocytes exposed to 50 µmol/L GCDCA or 20 ng/ml TNF-α in the presence of actinomycin-D (ActD) at 24 hours and 72 hours after isolation. (C) Uptake of bile acids demonstrated by fluorescence microscopy. Primary hepatocytes were exposed to 2 µmol/L of the fluorescent bile acid cholyl lysyl fluorescein (CLF) for 15 minutes. Accumulation is observed in the bile canaliculus between adjacent hepatocytes at 24 hours but not 72 hours after isolation. Original magnification is 400x.

These data indicate that GCDCA needs to be taken up first by Ntcp before the onset of apoptosis and that ligand-dependent death receptor activation is not involved.

To exclude HepG2-specific artefacts, primary hepatocytes were exposed to GCDCA at 24 hours and 72 hours after isolation. Ntcp was expressed at high levels 24 hours after isolation (data not shown) but decreased in hepatocytes maintained in primary cultures for 72 hours, thereby confirming previous data.[30] In addition, uptake of bile acids in these hepatocytes decreased in time, as shown with the fluorescent bile acid CLF (Fig 1c). Accumulation of CLF was only detected in bile canaliculi of hepatocytes cultured for 24 hours, as previously reported.[24] At this time point, GCDCA strongly induced caspase-3 activity. In contrast, GCDCA did not induce caspase-3 activity anymore in hepatocytes cultured for 72 hours (Fig 1b). Induction of caspase-3 activity by TNF-α in the presence of actinomycin-D remained unaffected. All together, these data provide evidence that Ntcp is required for GCDCA-induced apoptosis in primary hepatocytes.

TUDCA inhibits but does not delay GCDCA-induced caspase-3 activity and apoptotic nuclear morphology

GCDCA-induced caspase-3 activity in hepatocytes peaks around 4 hours.[13] Therefore, at this time-point, we investigated the effect of TUDCA on GCDCA-induced caspase-3 activity in primary hepatocytes. TUDCA itself did not induce caspase-3 activation (Fig 2a) confirming previous data.[13] TUDCA, but not TCDCA and GUDCA, inhibited GCDCA-induced caspase-3 activity for 70 % as shown in Figure 1a. A concentration-dependent curve displayed that the minimal concentration exerting the maximal protective effect is 50 μM of TUDCA (Fig 2b). To demonstrate that TUDCA inhibits but does not delay GCDCA-induced caspase-3 activity, a time course study was performed. Two to 15 hours after addition of GCDCA + TUDCA, caspase-3 activity was inhibited significantly at all time-points (Fig 2c). Nuclei staining was performed with acridine orange confirming that GCDCA-induced activation of caspase-3 activity results in apoptosis. Nuclear

fragmentation and condensation were observed 4 hours after the addition of GCDCA, which increased after 8 hours and persisted up to 15 hours. After 8 hours, 30 to 40 percent of hepatocytes displayed apoptotic nuclei, which was inhibited in the presence of TUDCA to 5 percent (Fig 2d). Cytokine-exposed hepatocytes in which transcription was blocked with actinomycin-D served as positive control.

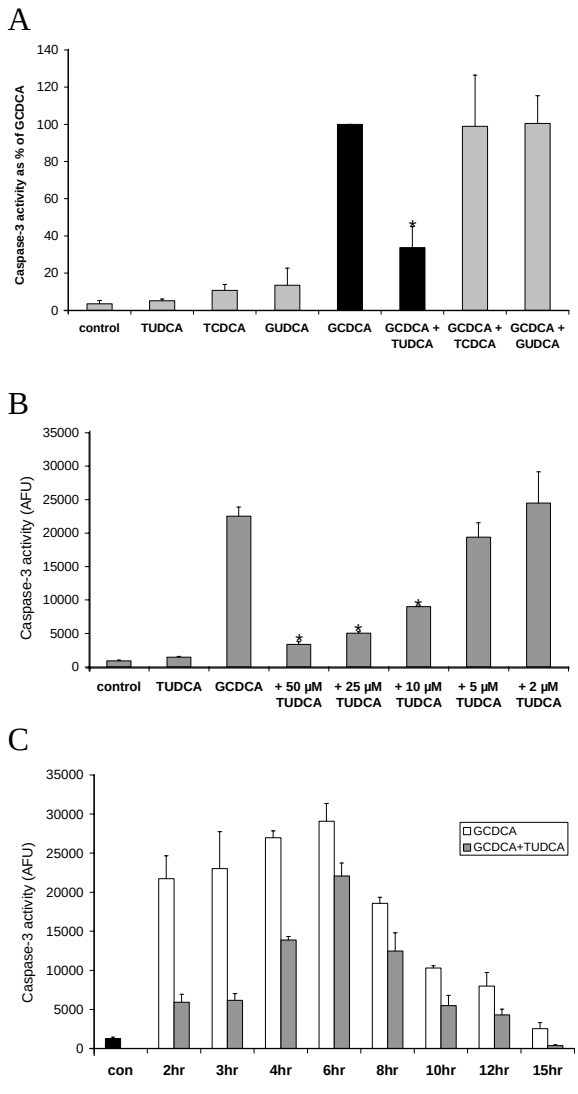


Figure 2. TUDCA, but not TCDCA and GUDCA, inhibits GCDCA-induced caspase-3 activity. (A) Primary rat hepatocytes were stimulated for 4 hours with 50 µmol/L glycochenodeoxycholic acid (GCDCA) and/or 50 µmol/L tauroursodeoxycholic acid (TUDCA), 50 µmol/L taurochenodeoxycholic acid (TCDCA), and/or 50 µmol/L glycoursodeoxycholic acid (GUDCA). Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of at least 3 independent experiments with n = 3 per condition. *P < 0.05 for GCDCA + TUDCA vs. GCDCA alone. (B) Caspase-3 activity. Primary rat hepatocytes were stimulated for 4 hours with 50 µmol/L glycochenodeoxycholic acid and different amounts of TUDCA, as indicated in the figure. *P<0.05 for GCDCA + 50, 25, and 10 µM TUDCA vs. GCDCA alone. (C) Time course study. TUDCA (50 µmol/L) significantly inhibits GCDCA-induced caspase-3 activity at all indicated time-points. Representative data of 3 independent experiments are shown.

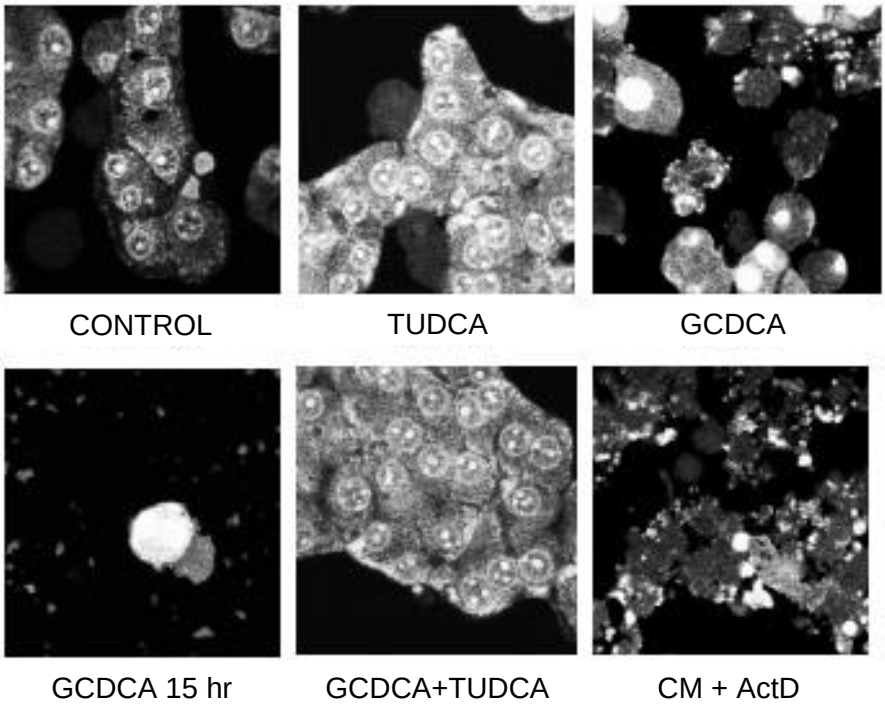


Figure 2. TUDCA, but not TCDCA and GUDCA, inhibits GCDCA-induced nuclear fragmentation. (D) Nuclear morphology in hepatocytes as determined by acridine orange staining. Cells were treated for 8 hours as indicated in the figure. 50 µmol/L GCDCA induces nuclear condensation and fragmentation, which persists for at least 15 hours and is blocked with 50 µmol/L TUDCA. Hepatocytes treated with cytokinemix (CM) + actinomycin-D (ActD) for 15 hours served as positive controls. Orignial magnification is 1000x.

The protective action of TUDCA depends on the inhibition of caspase-9 activation

Since we have previously noticed that GCDCA induces apoptosis in a mitochondria-controlled manner[13], the effects of TUDCA on caspase-9 and caspase-8 were investigated. As shown in Figure 3, TUDCA also prevented

GCDCA-induced activation of caspase-9. Both Western blot (Fig 3a) and immunocytochemistry (Fig 3b) demonstrated this effect.

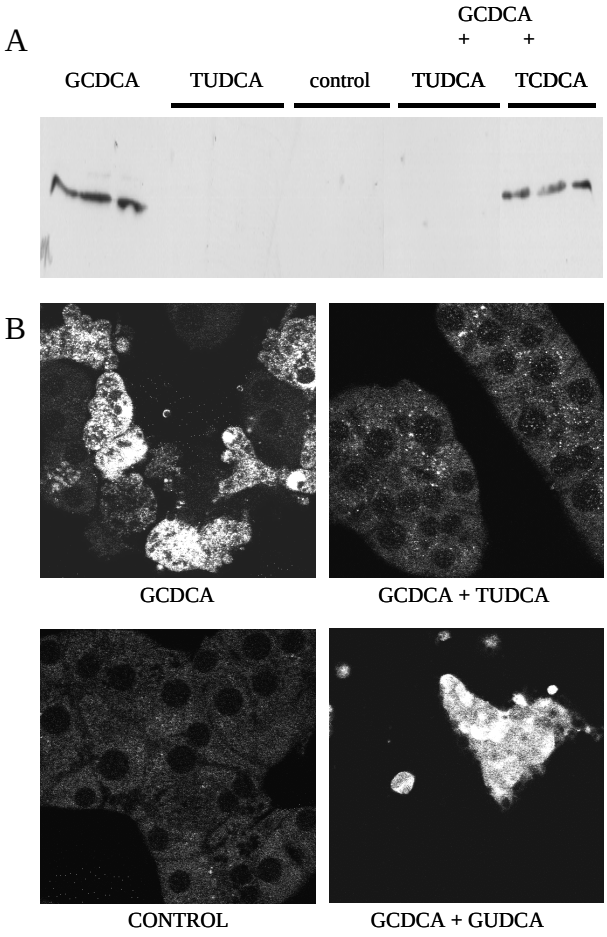


Figure 3. Tauroursodeoxycholic acid (TUDCA), but not taurochenodeoxycholic acid (TCDCA) or glycoursodeoxycholic acid (GUDCA), prevents glycochenodeoxycholic acid (GCDCA)-induced activation of caspase-9. Primary rat hepatocytes were exposed for 4 hours to 50 µmol/L of GCDCA and/or TUDCA, plus TCDCA or GUDCA. (A) Western blot analysis on cell lysates for active caspase-9. (B) Immunocytochemistry for active caspase-9 (original magnification is 400x).

Although TUDCA inhibited GCDCA-induced caspase-8 activity for 50 % (Fig 4), we have previously shown that caspase-8 inhibition does not inhibit GCDCA-induced caspase-3 activity.[13] In contrast, the caspase-3 inhibitor Ac-DEVD-CHO and the caspase-9 inhibitor Ac-LEHD-CHO inhibited GCDCA-induced activation of both caspase-3 (Fig 4a) and caspase-8 (Fig 4b). These data demonstrate that inhibition

of caspase-9 is more important in the TUDCA-mediated protection against GCDCA-induced apoptosis than caspase-8 inhibition.

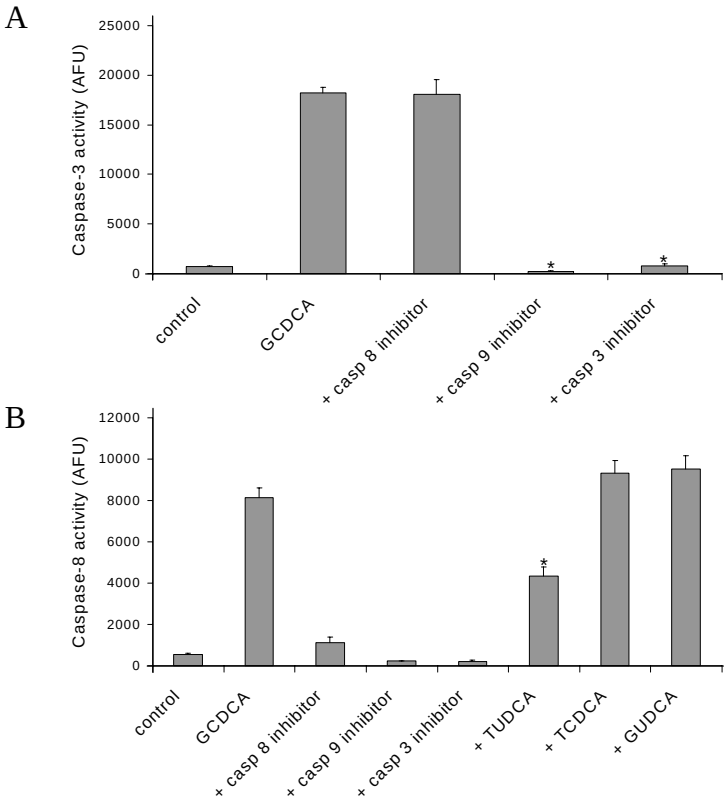


Figure 4. Caspase-3 and caspase-8 activation in glycochenodeoxycholic acid (GCDCA)-exposed primary rat hepatocytes in the presence of bile acids and caspase inhibitors. Representative data of 3 independent experiments are shown with n = 3 per condition. (A) Caspase-3 activity. Cells were treated for 4 hours with 50 µmol/L of (GCDCA) plus 50 µmol/L caspase-8 inhibitor, 50 µmol/L caspase-9 inhibitor, or 50 µmol/L caspase-3 inhibitor. *P<0.05 for GCDCA + caspase-9 inhibitor and GCDCA + caspase-3 inhibitor vs. GCDCA. (B) Caspase-8 activity. Cells were treated for 4 hours with 50 µmol/L of GCDCA plus 50 µmol/L tauroursodeoxycholic acid (TUDCA), 50 µmol/L taurochenodeoxycholic acid (TCDCA), or 50 µmol/L glycoursodeoxycholic acid (GUDCA), 50 µmol/L caspase-8 inhibitor, 50 µmol/L caspase-9 inhibitor, or 50 µmol/L caspase-3 inhibitor. *P<0.05 for GCDCA + TUDCA vs. GCDCA

In contrast to TUDCA, TCDCA and GUDCA did not inhibit GCDCA-induced caspase-9 (Fig 3) and caspase-8 activity (Fig 4b).

TUDCA does not compete with GCDCA for uptake at the cell membrane

Next, we investigated whether TUDCA needs to be present at the same time with GCDCA, prior to GCDCA or after the addition of GCDCA, to exert its protective effect. For this purpose, we pre-incubated hepatocytes with TUDCA for 9, 6 and 3 hours, washed these cells and exposed them to GCDCA in fresh medium for 4 hours. Six and 3 hours pre-incubation with TUDCA significantly inhibited GCDCA-induced caspase-3 activity for 50 % (Fig 5a). Pre-incubation with TUDCA for 3 hours also inhibited GCDCA-induced caspase-9 activation (Fig 5b).

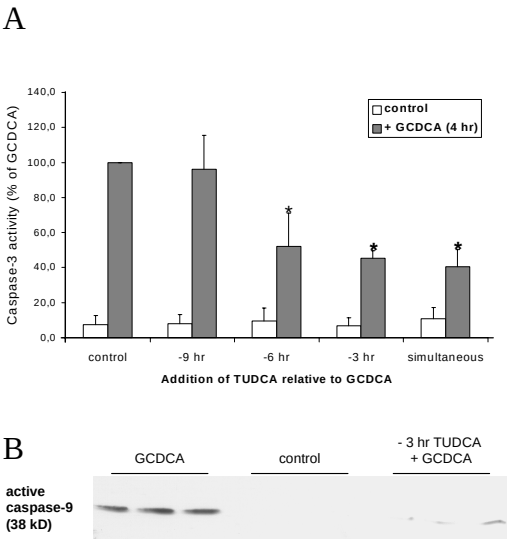
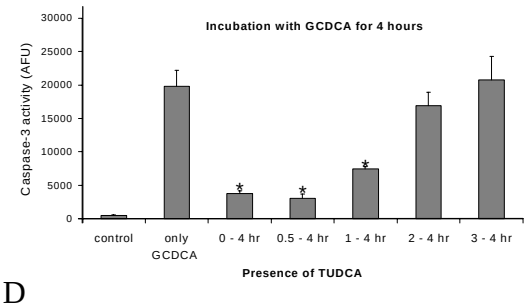


Figure 5 (A+B). Tauroursodeoxycholic acid (TUDCA) does not compete with glycochenodeoxycholic acid (GCDCA) for uptake at the cell membrane. (A) Pre-incubation with TUDCA inhibits GCDCA-induced apoptosis. Primary rat hepatocytes were exposed to 50 µmol/L of TUDCA for 9 (-9 hr), 6 (-6 hr) and 3 (-3 hr) hours after which cells were washed and exposed to 50 µmol/L of GCDCA for 4 hours. Cells were also incubated for 4 hours with GCDCA alone or with simultaneous addition of TUDCA (simultaneous). Caspase-3 activity is presented as percentage of GCDCA alone. *P<0.05 for -6 hours, -3 hours and simultaneous incubation of TUDCA + GCDCA vs. GCDCA alone (control). (B) Western blot analysis for active caspase-9 on cell lysates of hepatocytes pre-incubated for 3 hours with TUDCA followed by addition of GCDCA in refreshed medium for 4 hours.

C



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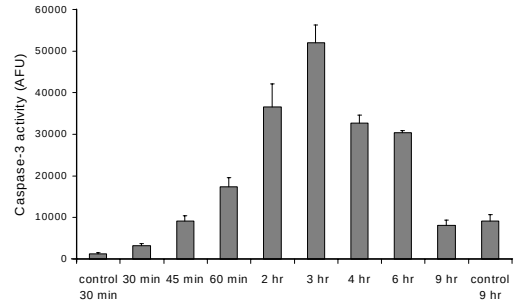

Figure 5 (C+D). Tauroursodeoxycholic acid (TUDCA) does not compete with glycochenodeoxycholic acid (GCDCA) for uptake at the cell membrane. (C) Caspase-3 activity. Primary rat hepatocytes were incubated with 50 µmol/L of GCDCA for 4 hours (only GCDCA). Simultaneously (0-4 hr) or half an hour (0.5-4 hr), 1 hour (1-4 hr), 2 hours (2-4 hr), or 3 hours (3-4 hr) after the addition of GCDCA, 50 µmol/L TUDCA was added. All cells were harvested 4 hours after the addition of GCDCA. Representative data of 3 independent experiments are shown with n = 3 per condition. *P<0.05 for (0-4 hr) of TUDCA, (0.5-4 hr) of TUDCA, (1-4 hr) of TUDCA vs. only GCDCA. (D) Time course study of (50 µmol/L) GCDCA-induced caspase-3 activity. Representative data of 3 independent experiments are shown with n = 3 per condition.

Furthermore, exposure to TUDCA up to 90 min after the addition of GCDCA still exerted protection against GCDCA-induced apoptosis (Fig 5c). At this time point, GCDCA-induced apoptosis had not reached its maximum yet (Fig 5d), confirming previous results.[13] These data indicate that the protective effect of TUDCA is not due to competition with GCDCA for uptake at the cell membrane. In addition, these data imply that the anti-apoptotic actions of TUDCA are very rapidly induced since simultaneous addition or addition of TUDCA after GCDCA is still able to prevent apoptosis in these cells. This could mean that signalling cascades are activated by TUDCA.

NF-κB is not involved in the protection of TUDCA against GCDCA-induced apoptosis

Previous results demonstrated that activation of the transcription factor NF-κB resulted in the transcription of survival genes protecting hepatocytes against apoptosis.[11;12] However, TUDCA does not activate NF-κB and does not induce the expression of NF-κB-regulated anti-apoptotic genes.[13] Since NF-κB can be activated indirectly, we investigated the role of NF-κB activation in relation to the anti-apoptotic mechanisms of TUDCA. Therefore, primary hepatocytes were infected with recombinant adenovirus expressing dominant negative IκB preventing NF-κB activation. Functionality of this virus was demonstrated by EMSA and sensitizing hepatocytes to cytokine-induced apoptosis (data not shown). As presented in Figure 6, inhibition of the NF-κB survival pathway does not

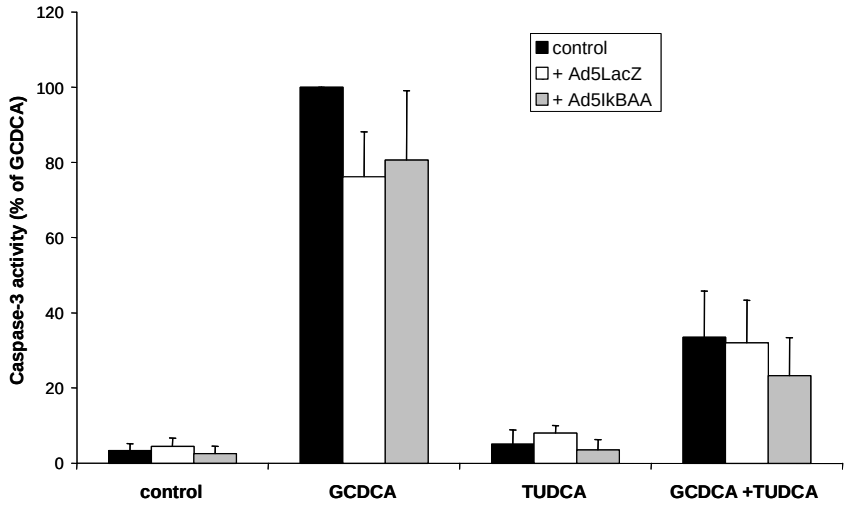

Figure 6. NF-κB is not involved in the protection of TUDCA against GCDCA-induced apoptosis. Primary rat hepatocytes were exposed for 4 hours to 50 µmol/L of glycochenodeoxycholic acd (GCDCA) or tauroursodeoxycholic acd (TUDCA) or both, 15 hours after receiving recombinant adenovirus inhibiting NF-κB activation (Ad5IkBAA). Ad5LacZ served as control virus. Protection of TUDCA against GCDCA-induced caspase-3 activity did not change significantly. Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of at least 3 independent experiments with n = 3 per condition.

significantly reduce the protective effect of TUDCA against GCDCA-induced caspase-3 activity.

Anti-apoptotic action of TUDCA depends on the activation of p38 MAP kinase, ERK MAP kinase, and PI3 kinase, whereas gene transcription is not involved

To investigate whether PI3 kinase and MAP kinases pathways are involved in the anti-apoptotic effects of TUDCA, specific inhibitors of PI3 kinase (LY 294002), p38 MAP kinase (SB 203580) and ERK1/2 MAP kinase (U0 126) were used. DMSO was used as a solvent for inhibitors but did not have an effect itself (data not shown). Experiments with inhibitors in control hepatocytes and in hepatocytes exposed to TUDCA or GCDCA were included as well. Hepatocytes exposed to inhibitors of MAP kinase pathways in the absence of bile acids demonstrated caspase-3 values around control level (Fig 7a). Caspase-3 activity increased slightly when PI3 kinase was blocked in control hepatocytes, and this effect was enhanced in combination with MAP kinase inhibitors. This pattern was similar for hepatocytes exposed to inhibitors in the presence of TUDCA (Fig 7a).

GCDCA-induced apoptosis was slightly, but not significantly, enhanced with inhibitors of p38 or ERK MAP kinases (Fig 7a). In contrast, inhibition of PI3 kinase pathway aggravated GCDCA-induced caspase-3 activity significantly (Fig 7a). In the presence of both MAP kinase and PI3 kinase inhibitors, exposure to GCDCA significantly increased caspase-3 values compared to GCDCA alone.

The protective effect of TUDCA against GCDCA-induced apoptosis was partially, but significantly abolished by inhibition of p38 MAP kinase pathway (Fig 7a).

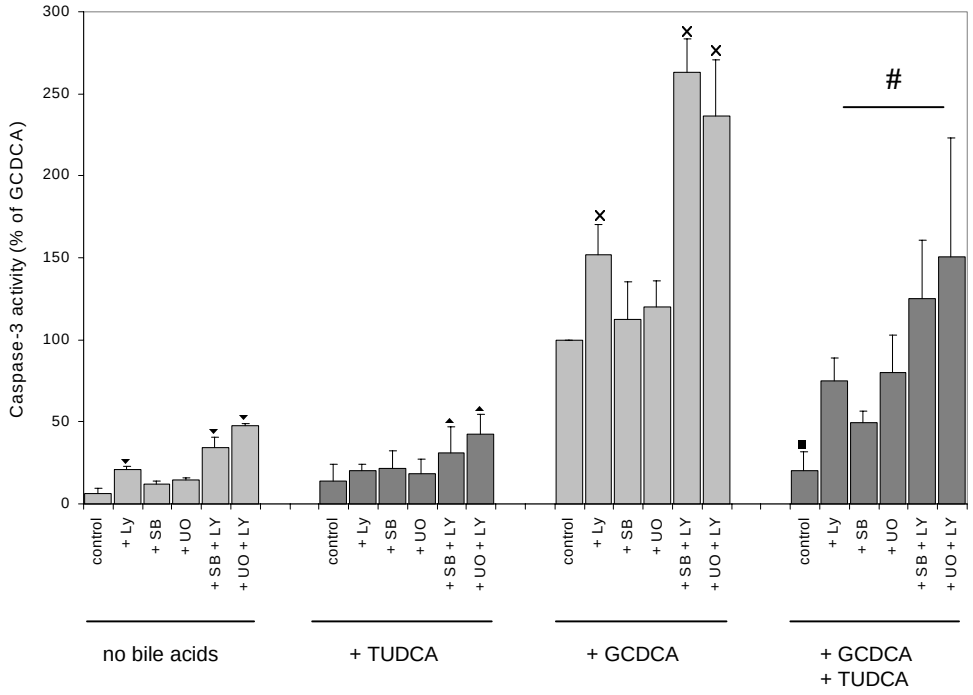

Figure 7 (A). TUDCA protects against GCDCA-induced apoptosis by activation of the p38 MAP kinase, ERK1/2 MAP kinase and the PI3 kinase pathway. (A) Caspase-3 activity in primary rat hepatocytes treated as indicated in the figure. 50 µmol/L of glycochenodeoxycholic acd (GCDCA) and/or tauroursodeoxycholic acid (TUDCA) were used plus or minus inhibitors of ERK MAP kinase (U0 126; U0), p38 MAP kinase (SB 203580; SB), and/or PI3 kinase (LY 294002; LY). Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of at least 3 independent experiments with n = 3 per condition. ▼P<0.05 for control + LY, control + SB + LY, and for control + UO + LY vs. control. ▲P<0.05 for TUDCA + SB+LY vs TUDCA and for TUDCA + UO+LY vs TUDCA. ✕P<0.05 for GCDCA + LY vs GCDCA and for GCDCA + SB+LY vs. GCDCA and for GCDCA + UO+LY vs. GCDCA. ■P<0.05 for GCDCA+TUDCA vs. GCDCA. #P<0.05 for GCDCA+TUDCA + inhibitors vs. GCDCA+TUDCA.

In support, TUDCA activated p38, which was blocked by SB 203580 as shown in Figure 7b. Western blot demonstrated equal presence of total p38 MAP kinase in all lanes (Fig 7b). Blocking of the PI3 kinase survival cascade resulted in abrogation of TUDCA protection against apoptosis as well (Fig 7a). The protective

effect of TUDCA against GCDCA-induced apoptosis was completely abolished when both p38 MAP kinase and PI3 kinase pathways were blocked (Fig 7b).

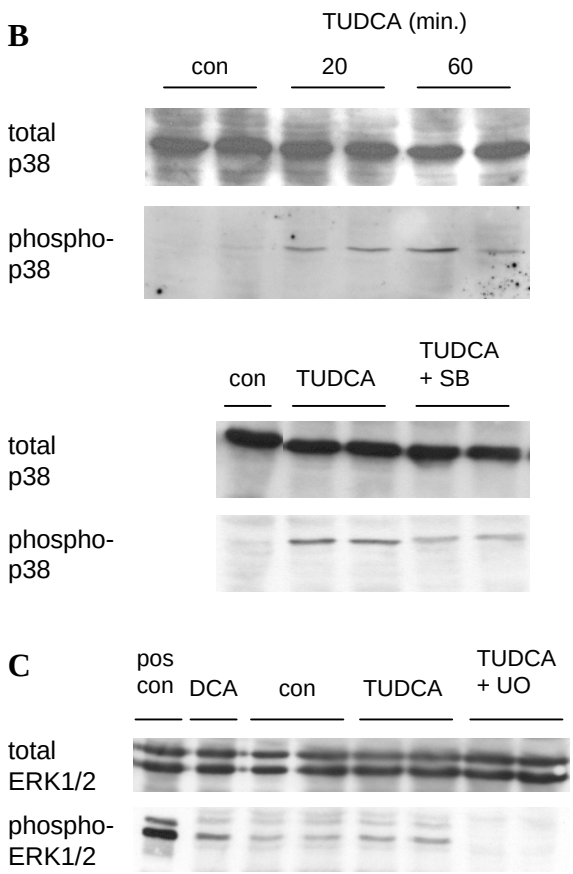

Figure 7 (B+C). TUDCA protects against GCDCA-induced apoptosis by activation of the p38 MAP kinase, ERK1/2 MAP kinase and the PI3 kinase pathway. (B) Western blot analysis for phosphorylated p38 MAP kinase in control lysates (con) and cell lysates of TUDCA-exposed hepatocytes (20 and/or 60 minutes) with or without p38 MAP kinase inhibitor (SB). The same blots for total p38 MAP kinase are presented. (C) Western blot analysis for phosphorylated ERK1/2 MAP kinase in cell lysates of TUDCA-exposed hepatocytes (30 minutes) with and without ERK1/2 inhibitor U0 126 (U0), and control lysates (con). Deoxycholic acid (DCA) served as positive control for ERK1/2 phosphorylation in hepatocytes, whereas an activated neutrophil extract was included for Western blot detection of ERK1/2 (pos. con). Upper panel presents same blot for total ERK1/2 MAP kinase.

Next, the ERK1/2 MAP kinase was investigated. As shown in Figure 7a, specific inhibition of ERK1/2 MAP kinase using U0 126 significantly prevented the protective effect of TUDCA against GCDCA-induced apoptosis. In addition, Western blot demonstrated that TUDCA activates ERK1/2, which can be blocked with U0 126 (Fig 7c). The bile acid deoxycholic acid (DCA) was included as positive control for hepatocytes, which was confirmed with a neutrophil extract

displaying high level of phopho-ERK1/2. U0 126 also inhibited DCA-mediated ERK1/2 phosphorylation (data not shown). Total ERK1/2 MAP kinase was equally present in all lanes (Fig 7c). Inhibition of both ERK1/2 MAP kinase and PI3 kinase abolished the protective effect of TUDCA against GCDCA-induced apoptosis completely (Fig 7a).

Finally, the role of trancription in the protection of TUDCA against bile acid-induced apoptosis was studied. Inhibition of transcription using actinomycin-D, at a dose sensitizing hepatocytes to cytokine-induced apoptosis (Fig 2d) had no influence on the protective effect of TUDCA (Fig 8). Blocking of transcription was confirmed with Western blot for cytokine-induced transcription of inducible nitric oxide synthase (iNOS) (Fig 8 inset).[11]

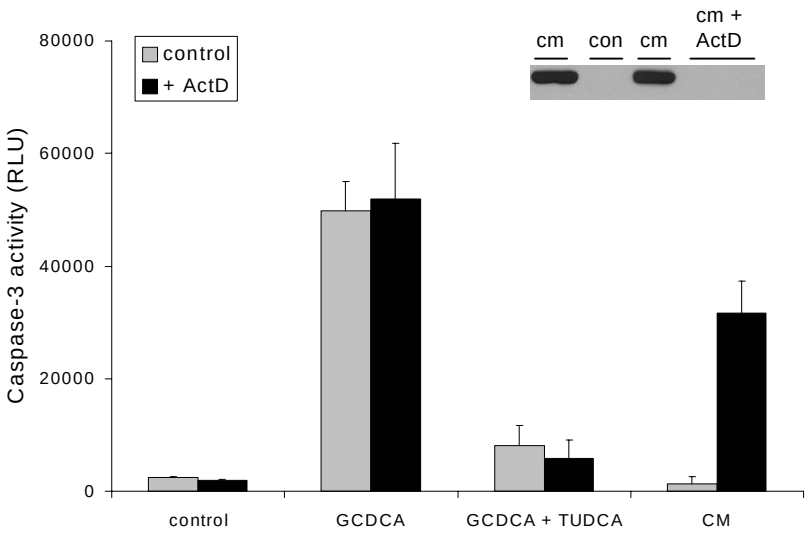

Figure 8. Transcription-independent protection of tauroursodeoxycholic acid (TUDCA) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis. Caspase-3 activity in rat hepatocytes treated with 50 µmol/L GCDCA and/or TUDCA with or without 200 ng/ml of transcription inhibitor actinomycin-D (ActD) for 4 hours. Actinomycin-D did not significantly change the protective effect of TUDCA. Cytokine-exposed hepatocytes (CM) for 6 hours served as positive control for ActD. Inset: actinomycin-D blocks transcription. Western blot for cytokine (CM)-induced expression of inducible nitric oxide synthase (iNOS), 11 hours after exposure.

6.5 Discussion

In this study, we investigated the protective mechanisms of taurine-conjugated UDCA against GCDCA-induced apoptosis in primary rat hepatocytes. We have previously shown that GCDCA induces apoptosis in a mitochondria-dependent manner, in which FADD is not involved and caspase-8 activation is not initially required.[13] In the present study, we investigated this further by blocking caspase-9. Indeed, peptide inhibitors of caspase-9 and caspase-3 blocked caspase-8 activation. Although these caspase inhibitors could have overlapping inhibitory effects, previous results with the human homologue of Inhibitor of Apoptosis protein1 (HIAP1) demonstrated that overexpression of HIAP1 inhibited GCDCA-induced apoptosis. HIAP1 exclusively inhibits caspase-3 and caspase-9 activation, but not caspase-8.[31] These data indicate that GCDCA induces apoptosis in a mitochondria-controlled manner.

In the present study, we have demonstrated that TUDCA inhibits, but does not delay GCDCA-induced caspase-9 and caspase-3 activity, and the formation of apoptotic nuclei. Furthermore, we have shown that Ntcp is required for GCDCA-induced apoptosis in primary hepatocytes, indicating that ligand-dependent death receptor activation is not likely to occur. This report demonstrates that the anti-apoptotic action of TUDCA against GCDCA-induced apoptosis is not due to a direct competitive effect on the cell membrane. E.g. TUDCA could compete with GCDCA for uptake by the bile acid importer Ntcp, thus preventing the uptake of pro-apoptotic GCDCA into hepatocytes. Pre-incubation for several hours with TUDCA followed by removal of TUDCA inhibits GCDCA-induced caspase-3 and caspase-9 activity, indicating the activation of survival pathways. Nine hour pre-incubation with TUDCA does not protect cells against GCDCA, likely because of quenching of survival signals.

Overall, TUDCA appears to protect mitochondria from GCDCA-induced injury by preventing GCDCA-induced caspase-9 activation rather than by preventing death receptor-mediated apoptosis.

The inhibitory effect on GCDCA-induced apoptosis is exclusively exerted by TUDCA. Our results demonstrate that taurine-conjugated CDCA does not inhibit GCDCA-induced caspase-3, caspase-9 and caspase-8 activity. This is in contrast to others showing inhibition of Fas-mediated caspase-8 activation by TCDCA-induced PI3-kinase.[32] However, the regulation of cell survival and apoptosis in a hepatoma cell line and primary non-transformed hepatocytes differs and this may explain the different results.

GUDCA could be clinically relevant since in humans, contrary to rats, glycine-conjugated UDCA is much more abundant compared to taurine-conjugated UDCA.[17;18] On the other hand, TUDCA has been reported to be beneficial in humans.[19] Nevertheless, TUDCA abundance in rats could explain why GUDCA does not inhibit GCDCA-induced apoptosis in rat hepatocytes in vitro. Primary human hepatocytes should give more information about the protective effect of taurine- and glycine-conjugated UDCA against bile acid-induced apoptosis.

Our data demonstrate that the p38 and ERK MAP kinase pathways and the PI3 kinase pathway are involved in the protection of TUDCA against GCDCA-induced apoptosis. Since blocking of both PI3 and MAP kinases exaggerates caspase-3 activity compared to blocking a single pathway, these pathways are partly redundant. Moreover, inhibition of the PI3 kinase pathway alone or in combination with MAP kinase inhibitors aggravates GCDCA-induced apoptosis and sensitizes hepatocytes slightly to TUDCA. Indeed, we have demonstrated that in addition to TUDCA (Fig 7b), GCDCA activates p38 and ERK MAP kinases (data not shown). These results are important for the interpretation of the protective effects of TUDCA. Blocking of protective kinases induces higher caspase-3 activity in GCDCA-exposed hepatocytes compared to GCDCA + TUDCA-exposed hepatocytes, implying additional protective effects of TUDCA, e.g. direct alterations of the mitochondrial membrane environment. Evidence for this was given recently by showing that TUDCA stabilizes the lipid and protein structure of mitochondrial outer membranes, thus inhibiting Bax binding to the outer membrane.[10]

Since simultaneous addition of TUDCA and GCDCA, or addition of TUDCA after GCDCA blocks apoptosis in hepatocytes, TUDCA very rapidly exerts its protective effect. Indeed, no transcription or NF-κB activation is needed for protection, indicating that survival signaling involves post-translational mechanisms exerted within 30 minutes as shown by Western blot (Fig 7b and c). TUDCA protection against GCDCA-induced apoptosis can only be exerted up to 1,5 hours after GCDCA addition. An explanation could be that in this time frame, GCDCA-induced apoptosis has not reached its maximum yet, whereas after 2 hours, apoptosis has proceeded beyond the point that TUDCA can be protective. Further evidence for rapid anti-apoptotic mechanisms of TUDCA is inferred from our previous results using cytokine-mediated protection against GCDCA. Cytokines do not protect against bile acid-induced apoptosis after the addition of GCDCA, since their protective mechanism depends on NF-κB-mediated transcription with a time frame of hours instead of minutes.[13]

Our PI3 kinase data supports other reports, although in these studies unconjugated UDCA was found to be apoptotic in itself.[15] Activation of Akt protects against apoptosis via several mechanisms, including the phosphorylation of the pro-apoptotic Bcl-2 family member Bad, which can no longer associate with and inhibit anti-apoptotic Bcl-XL.[33;34] Akt activation also results in the phosphorylation and inactivation of caspase-9[35] and may suppress pro-apoptotic Bax translocation to the mitochondria.[36] It is reported that bile acids activate the ERK1/2 MAP kinase pathway via activation of the EGF receptor.[15;25;37;38] The exact mechanism is still unclear, but mitochondrial-derived reactive oxygen species may be involved as recently suggested[37;39], and TUDCA prevents the generation of ROS.[10] The inhibition of Bax relocation to the mitochondria could also be mediated by ERK1/2 MAP kinase.[40] Since TUDCA prevents Bax-induced membrane perturbation[10], TUDCA-activation of ERK1/2 MAP kinase and PI3 kinase could be a mechanism to act on Bax. All together, our data fit very well with mitochondria-controlled bile acid-induced apoptosis in primary hepatocytes and provide more information about the link between TUDCA-activated survival pathways and mitochondria.

Interestingly, our data are in contrast to a recent study describing that the protection of TUDCA against TLCS-induced apoptosis is p38 MAP kinase-, ERK1/2 MAP kinase- and PI3 kinase-independent, and depends on inhibition of Fas trafficking and caspase-8 activation.[41] The authors suggest that TUDCA does inhibit TLCS-induced apoptosis upstream of caspase-8 activation. However, they also suggest that TUDCA inhibits TLCS-triggered mitochondrial ROS formation[41], which is needed for Fas trafficking.[39] Therefore, TUDCA may inhibit TLCS-induced apoptosis in a mitochondria-controlled manner. Alternatively, GCDCA and TLCS may induce apoptosis via different mechanisms, which could explain the discrepancies between the protective mechanisms of TUDCA against these bile acids.

The activation of the p38 MAP kinase pathway by TUDCA in order to inhibit GCDCA-induced stress in rat hepatocytes is in line with the activation of p38 MAP kinases during other forms of environmental stress.[14] Several reports describe the involvement of these kinases in mRNA stabilization.[42] Since we demonstrated that the protective effect of TUDCA is at a post-transcriptional level, this mechanism could be present in primary rat hepatocytes as well.

Although, the NF-κB pathway is involved in protection against cytokine-induced stress in primary rat hepatocytes[11;12], we did not find evidence that the NF-κB pathway is involved in the protective action of TUDCA against GCDCA-induced apoptosis. Bile acids do not directly activate NF-κB.[13] Moreover, inhibition of the NF-κB pathway did not change the protective action of TUDCA.

In summary, we have shown that the anti-apoptotic effect of TUDCA against GCDCA-induced apoptosis in primary rat hepatocytes is independent of caspase-8 inhibition, but is due to activation of p38, ERK MAP kinases and PI3 kinase survival pathways. Furthermore, we have demonstrated that TUDCA protects against GCDCA-induced mitochondrial injury in primary rat hepatocytes. Our data provide more information about the mechanism of action of UDCA in cholestatic liver diseases.

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6.7 References

1. Poupon RE, Lindor KD, Cauch-Dudek K, Dickson ER, Poupon R, Heathcote EJ. Combined analysis of randomized controlled trials of ursodeoxycholic acid in primary biliary cirrhosis. Gastroenterology 1997; 113(3):884-890.
2. Paumgartner G, Beuers U. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. Hepatology 2002; 36(3):525-531.
3. Guldutuna S, Zimmer G, Imhof M, Bhatti S, You T, Leuschner U. Molecular aspects of membrane stabilization by ursodeoxycholate [see comment]. Gastroenterology 1993; 104(6):1736-1744.
4. Van Nieuwkerk CM, Elferink RP, Groen AK, Ottenhoff R, Tytgat GN, Dingemans KP et al. Effects of Ursodeoxycholate and cholate feeding on liver disease in FVB mice with a disrupted mdr2 P-glycoprotein gene. Gastroenterology 1996; 111(1):165-171.
5. Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G et al. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. Hepatology 2001; 33(5):1206-1216.
6. Kurz AK, Graf D, Schmitt M, Vom DS, Haussinger D. Tauroursodesoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. Gastroenterology 2001; 121(2):407-419.
7. Botla R, Spivey JR, Aguilar H, Bronk SF, Gores GJ. Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. J Pharmacol Exp Ther 1995; 272(2):930-938.

8. Rodrigues CM, Fan G, Ma X, Kren BT, Steer CJ. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998; 101(12):2790-2799.
9. Rodrigues CM, Ma X, Linehan-Stieers C, Fan G, Kren BT, Steer CJ. Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. Cell Death Differ 1999; 6(9):842-854.
10. Rodrigues CM, Sola S, Sharpe JC, Moura JJ, Steer CJ. Tauroursodeoxycholic acid prevents bax-induced membrane perturbation and cytochrome C release in isolated mitochondria. Biochemistry 2003; 42(10):3070-3080.
11. Schoemaker MH, Ros JE, Homan M, Trautwein C, Liston P, Poelstra K et al. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. J Hepatol 2002; 36(6):742-750.
12. Hatano E, Bennett BL, Manning AM, Qian T, Lemasters JJ, Brenner DA. NF-kappaB stimulates inducible nitric oxide synthase to protect mouse hepatocytes from TNF-alpha- and Fas-mediated apoptosis. Gastroenterology 2001; 120(5):1251-1262.
13. Schoemaker MH, Gommans WM, Conde de la Rosa L, Homan M, Trautwein C, Van Goor H et al. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to NF-κB activation. J.Hepatol. 39(2), 153-161. 2003.
14. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298(5600):1911-1912.
15. Qiao L, Yacoub A, Studer E, Gupta S, Pei XY, Grant S et al. Inhibition of the MAPK and PI3K pathways enhances UDCA-induced apoptosis in primary rodent hepatocytes. Hepatology 2002; 35(4):779-789.
16. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999; 13(22):2905-2927.
17. Perwaiz S, Tuchweber B, Mignault D, Gilat T, Yousef IM. Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry. J Lipid Res 2001; 42(1):114-119.
18. Combes B, Carithers RL, Jr., Maddrey WC, Munoz S, Garcia-Tsao G, Bonner GF et al. Biliary bile acids in primary biliary cirrhosis: effect of ursodeoxycholic acid. Hepatology 1999; 29(6):1649-1654.
19. Invernizzi P, Setchell KD, Crosignani A, Battezzati PM, Larghi A, O'Connell NC et al. Differences in the metabolism and disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis. Hepatology 1999; 29(2):320-327.
20. Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990; 12(3 Pt 1):511-518.
21. Plass JR, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL et al. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. Hepatology 2002; 35(3):589-596.

22. Kullak-Ublick GA, Ismair MG, Kubitz R, Schmitt M, Haussinger D, Stieger B et al. Stable expression and functional characterization of a Na+-taurocholate cotransporting green fluorescent protein in human hepatoblastoma HepG2 cells. Cytotechnology 34, 1-9. 2000.
23. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW et al. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration [published erratum appears in J Clin Invest 1998 Apr 1;101(7):1541]. J Clin Invest 1998; 101(4):802-811.
24. Mills CO, Milkiewicz P, Muller M, Roma MG, Havinga R, Coleman R et al. Different pathways of canalicular secretion of sulfated and non-sulfated fluorescent bile acids: a study in isolated hepatocyte couplets and TR- rats. J Hepatol 1999; 31(4):678-684.
25. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R et al. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. Mol Biol Cell 2001; 12(9):2629-2645.
26. Vos TA, Gouw AS, Klok PA, Havinga R, Van Goor H, Huitema S et al. Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. Gastroenterology 1997; 113(4):1323-1333.
27. Miyoshi H, Rust C, Roberts PJ, Burgart LJ, Gores GJ. Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 1999; 117(3):669-677.
28. Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA et al. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. J Clin Invest 1999; 103(1):137-145.
29. Higuchi H, Bronk SF, Takikawa Y, Werneburg N, Takimoto R, El Deiry W et al. The bile acid glycochenodeoxycholate induces trail-receptor 2/DR5 expression and apoptosis. J Biol Chem 2001; 276(42):38610-38618.
30. Rippin SJ, Hagenbuch B, Meier PJ, Stieger B. Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. Hepatology 2001; 33(4):776-782.
31. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998; 17(8):2215-2223.
32. Takikawa Y, Miyoshi H, Rust C, Roberts P, Siegel R, Mandal PK et al. The bile acid-activated phosphatidylinositol 3-kinase pathway inhibits Fas apoptosis upstream of bid in rodent hepatocytes. Gastroenterology 2001; 120(7):1810-1817.
33. Zha J, Harada H, Osipov K, Jockel J, Waksman G, Korsmeyer SJ. BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J Biol Chem 1997; 272(39):24101-24104.
34. Muslin AJ, Tanner JW, Allen PM, Shaw AS. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell 1996; 84(6):889-897.
35. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998; 282(5392):1318-1321.

36. Tsuruta F, Masuyama N, Gotoh Y. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. J Biol Chem 2002; 277(16):14040-14047.
37. Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P et al. Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. Hepatology 2002; 35(2):307-314.
38. Yoon JH, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. Gastroenterology 2002; 122(4):985-993.
39. Reinehr R, Graf D, Haussinger D. Bile salt-induced hepatocyte apoptosis involves epidermal growth factor receptor-dependent CD95 tyrosine phosphorylation. Gastroenterology 2003; 125(3):839-853.
40. Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. Oncogene 2003; 22(19):2869-2881.
41. Graf D, Kurz AK, Fischer R, Reinehr R, Haussinger D. Taurolithocholic acid-3 sulfate induces CD95 trafficking and apoptosis in a c-Jun N-terminal kinase-dependent manner. Gastroenterology 2002; 122(5):1411-1427.
42. Reunanen N, Li SP, Ahonen M, Foschi M, Han J, Kahari VM. Activation of p38 alpha MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. J Biol Chem 2002; 277(35):32360-32368.

Chapter 7
Specific and enhanced gene transfer: redirection of PDGF-receptor β re-targeted adenovirus from hepatocytes to activated stellate cells

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7.1 Abstract

Chronic liver damage may lead to liver fibrosis and eventually liver failure. Hepatic stellate cells are the key players in hepatic fibrogenesis. Therefore, hepatic stellate cells are attractive targets for anti-fibrotic gene therapy. Recombinant adenovirus is a promising vehicle for delivering therapeutic genes to liver cells. However, this vector has considerable tropism for hepatocytes and Kupffer cells, which makes it necessary to retarget the adenovirus to the activated stellate cells, meanwhile reducing its affinity for hepatocytes. We developed a selective gene delivery tool by constructing a fusion protein recognising both the adenovirus and the Platelet Derived Growth Factor-receptor β (PDGF-Rβ) which is highly expressed on activated stellate cells. The targeting moiety consists of a PDGF peptide cloned in front of the single-chain antibody fragment (S11) directed against the adenoviral knob. Both 3T3 fibroblasts (expressing the PDGF-Rβ) and primary isolated activated rat stellate cells showed enhanced gene transfer by the PDGF-Rβ-retargeted adenovirus compared to control virus or negative control virus (scrambled protein). Compared to control adenovirus the PDGF-Rβ-retargeted adenovirus showed a dramatically decreased tropism for primary rat hepatocytes. Our novel approach demonstrates that therapeutic genes can be selectively directed to stellate cells, which opens new possibilities for the treatment of liver fibrosis.

7.2 Introduction

Chronic liver injury results in the accumulation of toxic metabolites, reactive oxygen species and bile acids that cause cell damage in the liver. This may induce liver fibrosis leading to organ dysfunction.[1] Liver fibrosis is characterised by excessive deposition of scar tissue due to a disturbed balance between extracellular matrix breakdown and deposition.[2] The central effector cell during liver fibrosis is the hepatic stellate cell, which is the major producer of extracellular matrix components. Stellate cells are localised in the space of Disse and in normal conditions they store vitamin A. During liver injury, stellate cells are exposed to paracrine stimuli from injured hepatocytes, endothelial cells and Kupffer cells.[3] This process initiates the activation and proliferation of stellate cells, which is considered as a key event in fibrosis. Activated stellate cells markedly increase matrix production through the action of Transforming Growth Factor-β (TGF-β). In addition, the number of stellate cells strongly increases by enhanced expression of the Platelet Derived Growth Factor-receptor (PDGF-R) on their cell membranes and the concomitant production of its ligand PDGF.[4]

Current pharmacological approaches for the treatment of liver fibrosis are not effective enough. A way to enhance effectiveness is to develop new anti-fibrotic therapies that specifically act on activated stellate cells. Targeting of therapeutic genes may represent such a novel therapy. Previous studies have shown that apoptosis of activated stellate cells may contribute to the resolution of fibrosis.[5;6] Induction of apoptosis seems therefore a promising therapeutic strategy. Apoptosis can be accomplished by inhibiting the activation of the transcription factor NF-κB using adenoviruses expressing an IκB dominant negative protein.[7] However, a therapeutic effect can only be achieved when apoptosis is induced exclusively in activated stellate cells. Induction of apoptosis in hepatocytes would most likely be deleterious in fibrotic livers. Therefore, in the present study, a novel tool has been developed to deliver therapeutic genes specifically to activated stellate cells. For

this purpose, we have taken advantage of the increased expression of the PDGF-receptorβ (PDGF-Rβ) on activated stellate cells after the onset of fibrosis. PDGF is a dimeric molecule composed of A and/or B chains and induces proliferation of stellate cells. PDGF-Rβ only binds the B-chain of PDGF.[8] In addition, activated stellate cells predominantly express PDGF-Rβ on their cell membranes, whereas liver endothelial cells only contain PDGF-Rα.[9] Thus, activated stellate cells specifically bind PDGF-BB.[8;9] In addition, the concentration of the PDGF-Rβ on activated stellate cells is much higher during liver fibrosis compared to other PDGF-Rβ-positive cells. This knowledge can be used to develop gene targeting strategies that discriminate between activated stellate cells and other (liver) cells.

We have created a fusion protein, which consists of a PDGF-Rβ-recognising peptide[10] coupled to a single-chain antibody fragment, which binds to the knob of recombinant adenovirus.[11] Homing of this PDGF-R-recognising peptide to the PDGF-Rβ has previously been demonstrated in another study using PDGF peptide-modified albumin as a drug carrier.[10] The arginine (R) and isoleucine (I) of the peptide are responsible for binding to the PDGF-Rβ.[12] Selective binding of the peptide to the PDGF-Rβ on activated stellate cells has been demonstrated *in vivo*. Additionally, it has been shown that this PDGF-peptide does not initiate a signalling cascade upon binding to PDGF-R.[10]

In general, adenovirus attachment to cells takes place by binding of the knob of the fiber coat protein to the coxsackie virus and adenovirus receptor (CAR).[13] Cell entry is subsequently mediated by binding of the penton base coat protein to integrins on the cell membrane.[14] In the present study, retargeting of recombinant adenoviruses to the PDGF-Rβ on activated stellate cells has been investigated. Previously, this ligand-directed strategy has been used successfully by selective targeting to the epidermal growth factor receptor.[11;15] Also the use of the adenoviral vector is appropriate because comparative evaluation of gene delivery devices in hepatic stellate cells revealed that these cells are more susceptible to adenoviral-mediated gene transfer as compared to non-viral vectors.[16] In a normal liver,

adenovirus predominantly enters hepatocytes and Kupffer cells. After fibrotic injury, adenoviral transduction efficiency is much higher in non-parenchymal cells than in hepatocytes as shown by detection of X-gal.[17] In this situation, also hepatocytes are readily infected.[18] As indicated above, prevention of adenovirus uptake by hepatocytes is important for anti-fibrotic therapies. Therefore, retargeting of the recombinant adenoviral vectors to activated stellate cells is crucial to achieve safe and efficient gene transfer in fibrotic livers. We present here the first tool to achieve that goal.

7.3 Materials and methods

Cell culture

NIH/3T3 fibroblasts were cultured in DMEM (Gibco™, Praisley, Scotland), 5% fetal bovine serum (BIO Whittaker Europe, Verviers, Belgium) containing glutamine, penicillin and streptomycin (Gibco). HCS-T6 cells (immortalised rat liver stellate cells) were kindly provided by Dr. SL Friedman (Mount Sinai School of Medicine, NY, USA) and maintained as described before.[19] Primary rat hepatocytes and stellate cells were isolated and cultured as described previously.[20;21] Rat hepatic stellate cells (HSCs) were cultured for at least 10 days. At this stage, these cells have an activated phenotype. All cells were cultured in a humidified atmosphere at 37 $^{\circ}$C / 5% CO_2.

Adenovirus AdTL

Cesium chloride-purified E1- and E3-deleted Adenovirus type 5 was obtained by standard techniques.[22] This recombinant adenovirus (AdTL) contains expression cassettes for Green Fluorescent Protein (GFP) and firefly Luciferase under the control of the CMV promotor.[23]

Construction of CSRNLIDC-S11 and CIDNLSRC-S11 fusion proteins

For the construction of fusion proteins, pUC119-pelB-S11-mychis vector was used (a generous gift of Dr. RE Hawkins, Bristol University, UK). Downstream of the coding sequence for the single-chain antibody fragment (scFv) against adenoviral fiber knob (S11), a *Myc* tag, and a histidine-tag (His6) sequence were present. The PelB leader was located upstream of the coding sequence for the fusion protein and allowed the isolation of the protein out of the periplasmic space of JM109 *E.coli* bacteria.

DNA encoding the PDGF-BB peptide (amino acids CSRNLIDC)[10] was introduced between the PelB leader and the S11 sequence using oligonucleotides. The sense oligo

5'-CATGCCC**TGCTCGCGGAACCTCATCGATTGT**GGCGGCGGCAGCTC-3'

was annealed to anti-sense oligo

5'-CATGGAGCTGCCGCCGCC**ACAATCGATGAGGTTCCGCGAGCA**GGG-3'.

This results in a DNA fragment with overlap extensions similar to the restriction sequence of *Nco*I. The bold nucleotides represent the DNA sequences encoding the PDGF-BB peptide CSRNLIDC. The glycine linker is presented as an underlined sequence and separates the CSRNLIDC-peptide from S11. The DNA fragment was phosphorylated and cloned into dephosphorylated, *Nco*I restricted pUC119-pelB-S11-mychis vector.

DNA encoding for a scrambled peptide (amino acids CIDNLSRC) was introduced upstream of the S11 sequence. Using pUC119-pelB-S11-mychis as a template, PCR was performed with the following primers, forward 5'-

TTAACCATG**GCCTGCATAGACAACCTCTCGAGATGT**GGCGGCGGCAGCT

CTATGGCCCAGGTGCAACTGCAGC-3',

and reverse 5'-TTAATCT AGATTATTAATGGTGATGATGG-3'. *E. coli* JM109 were transformed with pUC119pelBS11-mychis, which included the sequence CSRNLIDC or CIDNLSRC.

Expression and purification of fusion proteins and adenovirus knob domain

JM 109 *E. coli*, containing plasmids encoding for CSRNLIDC-S11 or CIDNLSRC-S11, were grown overnight at 37 °C (with shaking at 200 rpm) in 2TY medium which contained 100 µg/ml ampicillin and 1% glucose. The overnight culture was diluted 1:100 in 1 liter 2TY medium containing 100 µg/ml ampicillin and 0.1% glucose. Bacterial suspensions were grown at 37 °C (200 rpm) to an optical density of 0.8 – 1.0 at $O.D._{600\ nm}$. Protein expression was induced by addition of 1 mM isopropyl B-D-thiogalactoside (IPTG; Invitrogen, Breda, Netherlands) followed by an incubation for 4 hours at 30 °C (200 rpm).

Bacteria were harvested by centrifugation at 6,000 g (4 °C) for 10 min and resuspended in 20 ml of ice cold TES buffer (0.2 M Tris-HCL, 0.5 mM EDTA, 0.5 M Sucrose, pH 8.0). This suspension was incubated for 15 minutes on ice and subsequently centrifuged at 38,000 g (4 °C) for 30 min. The pellet was resuspended in 2-5 volumes ice-cold sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.8) followed by addition of 1 mg/ml lysozyme (ICN biomedicals, Aurora, USA) and an incubation step on ice for 30 min. This suspension was sonicated 3 times for 10 sec and centrifuged for 20 min at 10,000 g (4 °C) after which the supernatant was collected.

Purification of His-tagged proteins was performed by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. For this purpose, the supernatant was mixed overnight with 8 ml Probond Resin Ni-NTA beads (Invitrogen) at 4 °C. This mixture was washed with sonication buffer until $O.D._{280nm}$ was 0.02, after which another wash step was performed with 20 mM Imidazole (diluted in sonication buffer). Protein was eluted with 250 mM Imidazole. Fractions with maximum protein amounts (determined with $O.D._{280nm}$ and anti-myc staining on a dotblot loaded with equal volume of protein elutions) were pooled and dialysed against phosphate buffer saline (PBS) at 4 °C overnight.

Expression of the knob domain of adenovirus serotype 5 was induced in E. coli M15(pREP4)(PQE30-Ad5knob) (kindly provided by Dr. J. Douglas, University of Alabama, Birmingham, USA) using LB medium with 100 µg/ml ampicillin and 25

µg/ml kanamycin and a final concentration of 2 mM IPTG. Cells were harvested by centrifugation at 4,000g for 10 min followed by resuspension in sonication buffer and treated as described above.

Western blot analysis

SDS-PAGE was performed with 5 µg protein (under denaturing conditions) on a 10 % gel followed by Western blot analysis with anti-*myc* monoclonal antibody at a dilution of 1:10. This antibody was collected from supernatant of hybridoma cells 1729 culture medium after centrifugation at 200g. Rabbit-anti-mouse horseradish peroxidase (DAKO A/S, Denmark) was used at a dilution of 1:3000. Protein was visualised with an AEC-kit according to manufacturer's instructions (Sigma, Steinheim, Germany). Kaleidoscope pre-stained standards were used (Bio-rad Laboratories, Hercules, USA) to detect molecular weight.

Analysis of S11 binding to the adenoviral fiber knob

The S11 binding activity of CSRNLIDC-S11 and CIDNLSRC-S11 was tested by enzyme-linked immunosorbent assay (ELISA). A fusion protein directed against the epidermal growth factor receptor (425-S11) and S11 alone were used as positive control.[11] Proteins were added to a 96-wells plate pre-coated with 1 µg per well of our generated adenoviral type 5 knob. As a negative control, only immunostaining on adenoviral knob pre-coated wells was performed. ELISA was performed with anti-*myc* monoclonal antibody (diluted 1:10). After that, a secondary antibody was added (rabbit anti-mouse HRP conjugate (1:3000)), and incubated for 1 hour, followed by addition of 1,2-orthophenylenediamine dihydrochloride (OPD) substrate (DAKO A/S, Denmark). After washing the wells, binding of the fusion proteins was determined by measuring the absorbance at 490 nm.

PDGF-receptor binding analysis of CSRNLIDC-S11

Binding of CSRNLIDC-S11 to the PDGF-R-positive HSC-T6 cell line was evaluated with fluorescence-activated cell sorting analysis (FACS). Cells were incubated with

10 µg of CSRNLIDC-S11 for one hour at 4 °C. Successively, anti-myc antibody (1:10) and PE (phycoerythrin) conjugated goat-anti-mouse (GAMPE, 1:100; DAKO) were used. In between, washing steps were performed by centrifugation for 3 min at 1500 rpm in serum free medium.

Targeting and untargeting assays

NIH/3T3 fibroblasts were used as a model system as they express high levels of PDGFR and very low levels of CAR.[24;25] These cells were plated in 96-well plates (NUNC™, Denmark) at a density of 10.000 cells per well and incubated overnight at 37 °C. Primary isolated culture-activated rat stellate cells (HSCs) were plated in 12-well plates until monolayers were subconfluent. Rat hepatocytes were used to investigate un-targeting of PDGF-R-retargeted virus. After isolation, hepatocytes were plated on 12-well plates for 5 hours as described previously.[26]

Virus was pre-incubated with CSRNLIDC-S11 or CIDNLSRC-S11 at a ratio of 1.0 x 10^9 viral particles per 1 µg or 10 µg protein for 30 min at 37 °C. NIH/3T3 fibroblasts and HSCs were infected in triplo (in medium containing 2% FBS) with 500 plaque forming units (pfu) and 50 pfu per cell of pre-incubated AdTL, respectively. Hepatocytes were infected in serum free medium using 50 pfu per cell of pre-incubated AdTL. To study the involvement of CAR, cells received 20 µg/ml adenoviral type 5 knob for 1 hour at 37 °C before adding (pre-incubated) virus. Cells were incubated for 24 hours in a humidified atmosphere at 37 °C / 5% CO_2, after which they were evaluated for GFP expression using a Zeiss Axiovert 25 fluorescence microscope. After another 24 hours, cells were harvested in 1x cell lysis buffer (Promega, Madioson, USA). Gene transfer was determined by measuring luciferase enzyme activity according to manufacturer's instructions (Promega) using the Lumicount™ (Packard Bioscience Benelux NV, Groningen, Netherlands).

Immunohistochemical analysis

The PDGF-receptorβ was detected as described before.[10]

Statistical Analysis

Results are presented as the mean of at least 3 independent experiments ± standard deviation. A Mann-Whitney test was used to determine the significance of differences between two experimental groups. A P-value of less than 0.05 ($P<0.05$) was considered to be statistically significant.

7.4 Results

Functionality of fusion protein CSRNLIDC-S11

To develop a selective gene delivery tool for activated stellate cells, we constructed a fusion protein. This fusion protein consists of a single-chain antibody fragment (S11), which binds to the fiber knob of adenovirus type 5 (Fig 1).

In front of the coding sequence of S11, we cloned DNA encoding a peptide (CSRNLIDC) which recognises the PDGF-Rβ. A glycine linker separates this sequence from the coding sequence of S11. A control construct was made in which a scrambled DNA sequence encoding CSRNLIDC was cloned in front of the DNA sequence encoding S11. This scrambled fusion protein should not bind to the PDGF-Rβ.

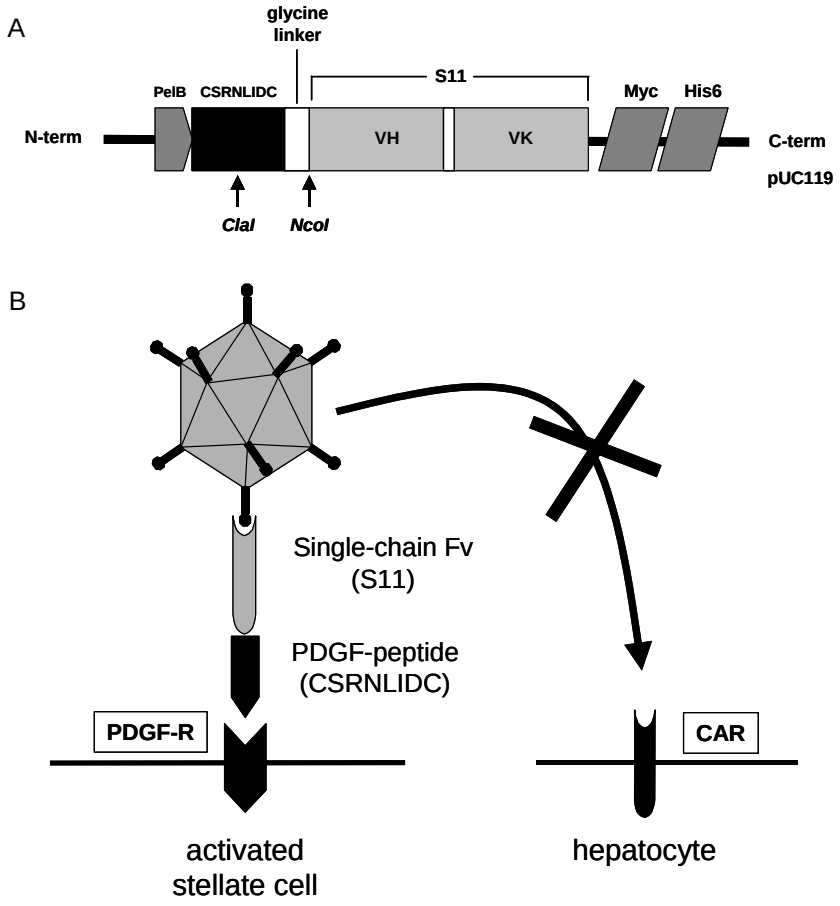

Figure 1. Construction of a fusion protein recognising both adenoviral type 5 fiber knob and the PDGFβ-receptor. The PDGFβ-receptor is highly expressed on activated stellate cells. (A) In front of the single-chain antibody fragment (scFv) S11, the coding sequence of the PDGF-BB-derived peptide CSRNLIDC was cloned. In between, a glycine linker was located. The PelB leader allows the fusion protein to enter bacterial periplasmic space, which is followed by cleavage of the leader signal. At the C-terminus of the fusion protein, a histidine- and Myc-tag are situated allowing purification and detection of the fusion protein, respectively. (B) Schematic representation of our gene targeting approach to activated stellate cells. Coating of adenovirus with the fusion protein CSRNLIDC-S11 will block binding to its native receptor CAR. CSRNLIDC binds to the PDGF-β receptor but does not initiate a signalling cascade.

After induction of both proteins in *E.coli* JM109, correct protein expression was detected at 30 kDa for CSRNLIDC-S11 and CIDNLSRC-S11. (Fig 2a) The binding of these proteins to adenoviral fiber knob was analysed with ELISA. As shown in Figure 2b, both proteins bound to the adenoviral fiber knob similar to S11 itself, demonstrating functionality of the S11-single-chain antibody fragment even in the context of a fusion protein. Binding of CSRNLIDC-S11 to a PDGF-Rβ positive stellate cell line was subsequently determined with FACS analysis. Addition of CSRNLIDC-S11 to the stellate cell line HSC-T6 resulted in a clear shift of the PE signal compared to the control situation (only antibodies) and S11 alone (data not shown) (Fig 2c). These results confirm previous data that demonstrated binding of the CSRNLIDC peptide to the PDGF-Rβ.[10]

CSRNLIDC-S11 directed gene transfer in fibroblasts

Functionality of the CSRNLIDC-S11 fusion protein and our targeting approach was further investigated using NIH/3T3 fibroblasts as a model system. These cells express very high levels of PDGF-Rβ and low levels of CAR on their cell membranes. Adenoviral gene transfer was measured in terms of luciferase expression. Unmodified recombinant adenovirus (AdTL) hardly resulted in gene transfer in these fibroblasts (Fig 3). In addition, the scrambled fusion protein did not result in adenoviral gene transfer. In contrast, modification of AdTL with CSRNLIDC-S11 dramatically increased luciferase gene transfer in a dose-dependent manner (Fig 3).

A ratio of 10 µg CSRNLIDC-S11 per 10^9 viral particles resulted in the maximal enhancement of transfection (57x). In the presence of higher amounts of protein, the infection rate started to decline (data not shown). This could be explained by an excess of fusion protein competing with the targeted virus for binding to the PDGF-Rβ. Besides luciferase gene expression, expression of adenovirus-encoding GFP was monitored in these cell cultures. GFP expression correlated with luciferase gene expression (data not shown).

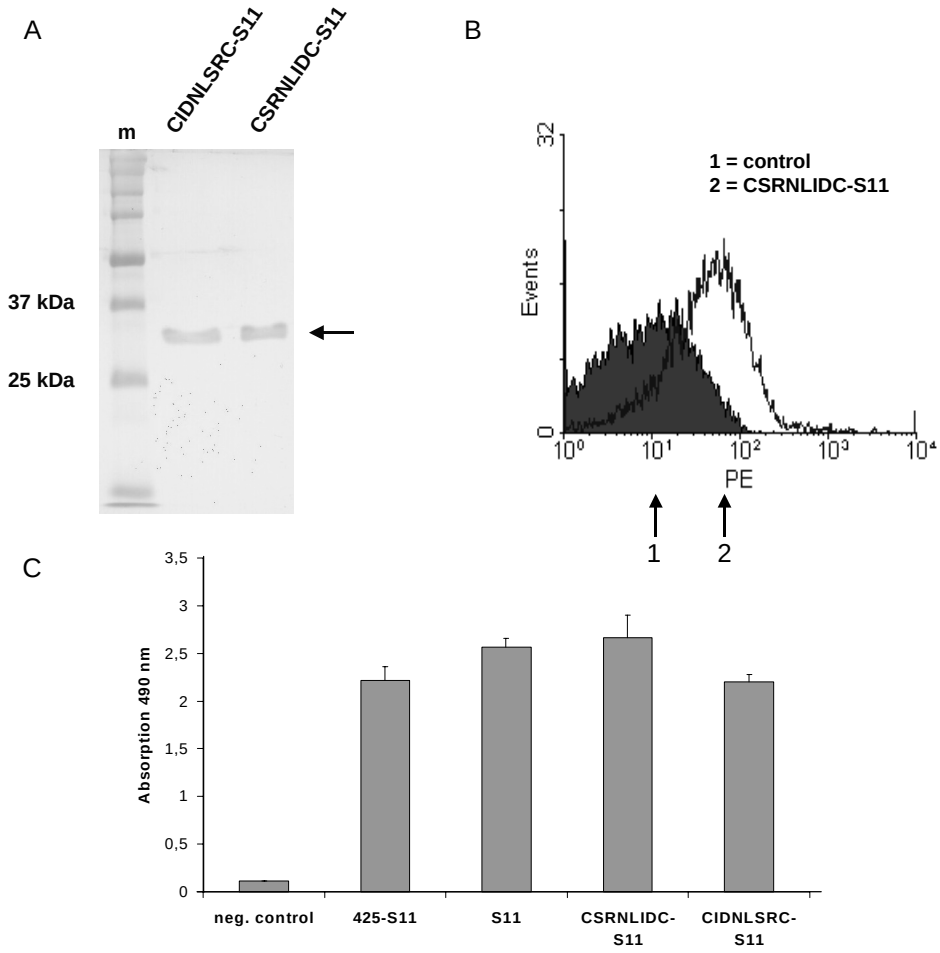

Figure 2. Characterisation of CSRNLIDC-S11 and CIDNLSRC-S11 fusion proteins. (A) Western blot of Ni-NTA-purified proteins, that were produced in JM109 *E. coli.* Five µg of protein was loaded on a 10 % gel, which was detected with anti-Myc antibody. (MW of approximately 30 kDa). (B) The binding of CSRNLIDC-S11 to a PDGFβ-receptor positive hepatic stellate cell line (HSC-T6) was evaluated by fluorescence-activated cell sorting. Anti-Myc and PE (phycoerythrin)-conjugated goat-anti-mouse antibodies were used for detection. (C) Binding activity of the S11-single-chain antibody fragment against adenoviral fiber knob using ELISA. S11 alone and a bi-specific single-chain antibody directed against the epidermal growth factor receptor (425-S11) served as positive control. Wells that were solely pre-coated with adenoviral knob served as negative control.

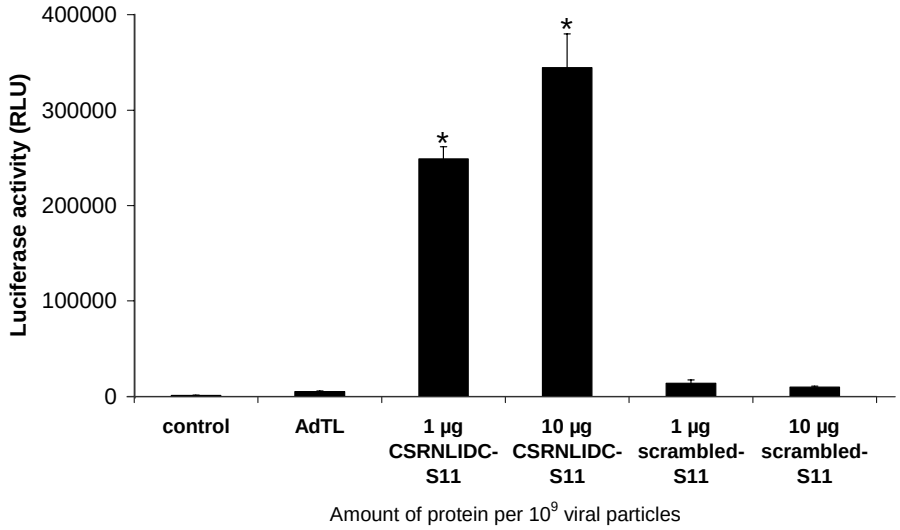

Figure 3. Specific transfection of fibroblasts by PDGFβ-receptor targeted adenovirus. NIH/3T3 fibroblasts express high levels of PDGFβ-receptor and low levels of CAR. These cells were used as a model system. 10^9 viral particles of AdTL were pre-incubated with or without 1 µg or 10 µg of CSRNLIDC-S11 or CIDNLSRC-S11 (scrambled-S11) for 30 min at 37 °C. NIH/3T3 fibroblasts were infected with 500 pfu/cell. Gene transfer is presented as luciferase activity (relative light units) which was measured 48 hours after infection. Representative data of 5 independent experiments are shown with n = 3 per condition. *P<0.05 for CSRNLIDC-S11 vs AdTL.

CSRNLIDC-S11 directed gene transfer in activated stellate cells

The results of our model system allowed us to examine the transfection efficiency of PDGF-Rβ-retargeted adenovirus in primary isolated culture-activated stellate cells. Besides high levels of PDGF-Rβ on their cell membrane[27], activated stellate cells do express CAR.[17] These cells were very efficiently transfected with non-modified virus (Fig 4). This gene transfer was blocked by addition of adenoviral knob in order to block CAR. However, modification of AdTL with CSRNLIDC-S11 resulted in an enhancement of the adenoviral gene transfer compared to non-modified virus in activated stellate cells. This transfection was not inhibited by CAR

blockade (Fig 4), suggesting that PDGF-Rβ-mediated infection by CSRNLIDC-S11-modified virus is more efficient than CAR-mediated infection.

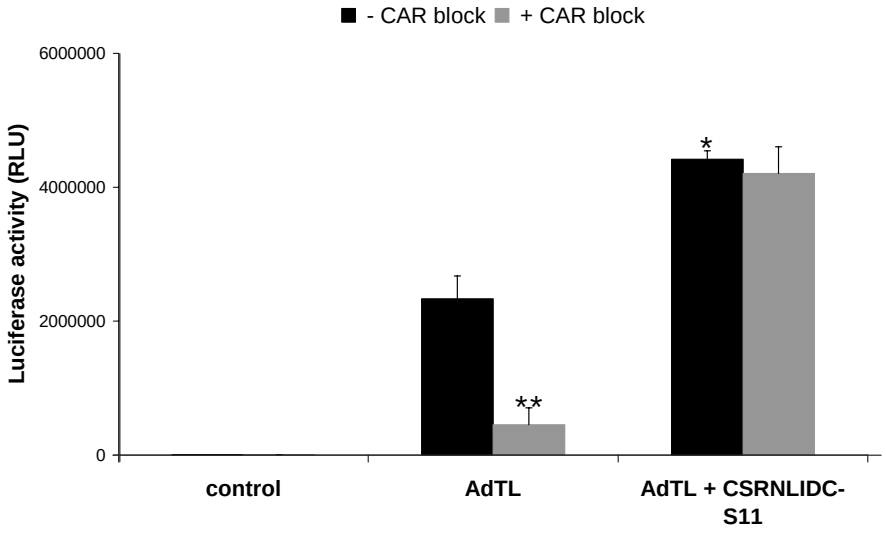

Figure 4. CSRNLIDC-S11 increases adenoviral gene transfer in culture-activated rat stellate cells. Culture-activated stellate cells were infected with PDGFβ-receptor retargeted adenovirus. A ratio of 10 µg of CSRNLIDC-S11 per 10^9 viral particles (AdTL) was used. Activated stellate cells were infected with 50 pfu/cell in the presence or absence of 20 µg/ml fiber knob. Soluble fiber knob was used to block CAR. Gene transfer is presented as luciferase activity (relative light units) which was measured 48 hours after infection. Representative data of 3 independent stellate cell isolations are shown with the mean of n = 3 per condition. *P<0.05 for CSRNLIDC-S11 vs AdTL. **P<0.05 for AdTL + CAR block vs AdTL - CAR block.

Untargeting of primary hepatocytes

To achieve specific gene transfer in activated stellate cells, a reduction of targeting to hepatocytes is essential. Therefore, primary cultures of isolated hepatocytes were used to examine our targeting strategy. Hepatocytes express high levels of CAR on their cell membranes. As expected, non-modified virus efficiently transfected hepatocytes (Fig 5). Addition of the PDGF-Rβ-specific fusion protein

CSRNLIDC-S11 to the recombinant adenovirus blocked the adenoviral gene transfer in primary hepatocytes. This demonstrates a successful blockade of the CAR-mediated interaction between adenovirus and hepatocytes.

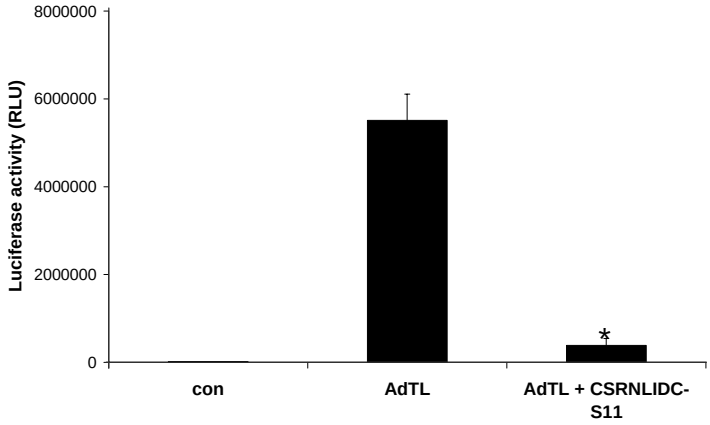

Figure 5. Untargeting of primary rat hepatocytes using PDGFβ-receptor targeted adenoviral gene transfer. (A) Primary cultures of hepatocytes were infected with 50 pfu/cell after pre-incubation of AdTL virus with CSRNLIDC-S11 for 30 min. A ratio of 10 µg conjugate per 10^9 viral particles was used. Gene transfer is presented as luciferase activity (relative light units) measured 48 hours after infection. Representative data of at least 3 independent hepatocyte isolations are shown with the mean of n = 3 per condition. *P<0.05 for CSRNLIDC-S11 vs AdTL.

7.5 Discussion

We developed a new tool to specifically deliver genes to activated stellate cells. Stellate cells play a central role in liver fibrosis. Since current pharmaco-therapeutical strategies to treat liver fibrosis are not satisfactory, a search for new therapies is needed. In this study, we investigated whether selective adenoviral gene targeting to activated stellate cells is an option. We combined a PDGFβ-receptor-specific peptide[10] with a single-chain antibody fragment recognising adenovirus type 5 fiber.[11;15] In this way, we were able to construct a fusion protein

(CSRNLIDC-S11) which targets recombinant adenovirus type 5 specifically to activated stellate cells, whereas gene transfer in hepatocytes is dramatically reduced *in vitro* and *ex vivo.*

Evidence for the specificity of our approach was provided by competition studies on PDGF-Rβ positive fibroblasts and activated stellate cells, using CSRNLIDC-peptide-modified albumin and PDGF-BB.[10] We now demonstrate the feasibility of this approach in gene transfection. The present manuscript demonstrates that the scrambled variant of our fusion protein did not mediate adenoviral gene transfer in NIH/3T3 fibroblasts. Moreover, transfection of activated stellate cells is enhanced by our modified virus, which can not be blocked by CAR block. We clearly show un-targeting of hepatocytes. These data indicate that PDGF-Rβ-mediated gene transfer in stellate cells using CSRNLIDC-S11-modified recombinant virus is more efficient than CAR-mediated infection.

A few adenoviral gene therapy studies have previously been performed in cirrhotic rats using non-modified virus expressing a gene coding for hepatocyte growth factor, interferon alpha or urokinase-type plasminogen activator.[28-30] Results of these studies were obtained in carbon tetrachloride- and dimethylnitrosamine-induced liver cirrhotic models. Attenuation of liver fibrosis by adenoviral delivery of matrix metalloproteinases has also been described in a thioacetamide-induced model.[31] Although fibrosis was reduced in these studies, normal liver histology was not completely restored because of the a-specificity of these unmodified vectors. In addition, a high amount of viral particles was needed to overcome low gene transfer in fibrotic livers[17] and virus degradation in Kupffer cells. In fibrotic livers, this may cause a strong immune response. Furthermore, discrimination between activated and non-activated stellate cells, endothelial cells, Kupffer cells and hepatocytes, was not accomplished in fibrotic livers.[18] Targeted vectors are likely to be less immunogenic[32] because of a rapid uptake in the target cell[10] thus avoiding the immune system. Therefore, our PDGF-Rβ-directed adenovirus may improve the safety and efficacy of gene transfer to the activated stellate cell.

The PelB leader in our construct allows the fusion protein to enter the bacterial periplasmic space where an S-S bridge can be formed and a cyclic CSRNLIDC peptide will be generated. We did not demonstrate this cyclic structure. However, a different fusion peptide without two cysteine amino acids in the PDGF-Rβ-binding part (SRNLID-S11), hardly retargeted the adenovirus to NIH/3T3 fibroblasts (data not shown). These results confirm other data demonstrating that cyclic peptides display stronger binding activity to their receptor.[33]

Besides monitoring GFP expression, the AdTL virus allowed us to measure luciferase expression, which resulted in data that can be quantified. This is preferred over a LacZ-staining. The GFP observations are informative since they demonstrate that enhanced gene expression by PDGFβ-R-retargeted virus results from a higher number of transduced cells and not from a few cells expressing the genes more abundantly.

An interesting strategy to resolve liver fibrosis would be the induction of apoptotic cell death in activated stellate cells.[6] Although it has been suggested that matrix degradation by metalloproteinases may be beneficial[31;34], the activated stellate cells are still present in this approach. In addition, patients suffering from liver fibrosis, often suffer from hepatocarcinoma as well and matrix degradation may interfere with this disease.[35] Selective induction of apoptosis could remove the activated stellate cells. Apoptosis can be induced by using adenovirus expressing a dominant negative IκB protein. In this way, NF-κB activation will be inhibited as shown for hepatocytes.[26;36;37] This can also be performed in stellate cells,[7] thus reducing the amount of matrix producing cells during fibrosis. Furthermore, it has been shown that adenoviral delivery of antisense mRNA is able to suppress the synthesis of TGF-β1 in culture-activated stellate cells.[38] TGF-β1 is an important pro-fibrogenic cytokine, and selective reduction of this cytokine may be a powerful tool to reduce fibrogenesis.

Reduction of fibrosis should be achieved by specifically delivering of the dnIκB gene or antisense TGF-β1 mRNA to activated stellate cells. This could be

accomplished using our PDGF-Rβ-retargeted adenovirus. To further increase activated stellate cell specificity, transcriptional retargeting can be used. A suitable candidate could be the promoter of the *CSRP2* gene, which is exclusively expressed by stellate cells.[39] *CSRP2* encoding protein has been reported to be a new factor in the JAK/STAT signalling pathway. Taken these improvements together, induction of apoptosis in activated stellate cells may result in a safe reduction of liver fibrosis.

A disadvantage of our two-component system, which consists of a fusion protein coupled to a virus, might be difficult to transfer to a clinical setting. Therefore, it would be preferable to incorporate the targeting peptide into the HI-loop of the adenoviral fiber knob. This has been performed successfully in previous studies.[40-42] The question arises whether insertion of the CSRNLIDC peptide in the HI-loop of the adenoviral fiber knob will also result in improved gene transfer in activated stellate cells. Our hypothesis is that two peptides are necessary for mimicking the binding sites present in the dimeric PDGF molecule.[10] Incorporation in the HI-loop could result in more copies of the PDGF-Rβ-specific peptide, thereby enhancing efficiency and safety of adenoviral gene transfer to the activated stellate cells.

Overall, our PDGF-Rβ-retargeted recombinant adenovirus allows selective gene transfer in activated stellate cells and prevents gene uptake by other cells like hepatocytes. Hepatocytes are generally responsible for most of the uptake of unmodified adenovirus, which seriously hamper efficiency of anti-fibrotic therapies based on adenoviral gene transfer. Using our new approach, a therapeutic gene, e.g. dominant negative IκB, can be transferred in activated stellate cells, thereby sensitising only these cells to apoptosis. This strategy may be beneficial in fibrosis. Furthermore, specific gene targeting to activated stellate cells allows reduced administration of adenoviral particles and decreases vector-related toxicity. The modified adenovirus described in this paper is the first viral vector targeted to this important cell-type, which opens new possibilities for the treatment of liver fibrosis.

7.6 Acknowledgements

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7.7 References

1. Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 2000; 275(4):2247-2250.
2. Rojkind M, Giambrone MA, Biempica L. Collagen types in normal and cirrhotic liver. Gastroenterology 1979; 76(4):710-719.
3. Kawada N. The hepatic perisinusoidal stellate cell. Histol Histopathol 1997; 12(4):1069-1080.
4. Friedman SL. Liver fibrosis - from bench to bedside. J Hepatol 2003; 38 Suppl 1:S38-53.:S38-S53.
5. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S et al. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 1998; 102(3):538-549.
6. Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN et al. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. Gastroenterology 2001; 121(3):685-698.
7. Lang A, Schoonhoven R, Tuvia S, Brenner DA, Rippe RA. Nuclear factor kappaB in proliferation, activation, and apoptosis in rat hepatic stellate cells. J Hepatol 2000; 33(1):49-58.
8. Pinzani M, Marra F. Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis 2001; 21(3):397-416.
9. Heldin P, Pertoft H, Nordlinder H, Heldin CH, Laurent TC. Differential expression of platelet-derived growth factor alpha- and beta- receptors on fat-storing cells and endothelial cells of rat liver. Exp Cell Res 1991; 193(2):364-369.

10. Beljaars L, Weert B, Geerts A, Meijer DK, Poelstra K. The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue. Biochem Pharmacol 2003; 66(7):1307-1317.
11. Haisma HJ, Grill J, Curiel DT, Hoogeland S, van Beusechem VW, Pinedo HM et al. Targeting of adenoviral vectors through a bispecific single-chain antibody. Cancer Gene Ther 2000; 7(6):901-904.
12. Clements JM, Bawden LJ, Bloxidge RE, Catlin G, Cook AL, Craig S et al. Two PDGF-B chain residues, arginine 27 and isoleucine 30, mediate receptor binding and activation. EMBO J 1991; 10(13):4113-4120.
13. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997; 275(5304):1320-1323.
14. Einfeld DA, Schroeder R, Roelvink PW, Lizonova A, King CR, Kovesdi I et al. Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. J Virol 2001; 75(23):11284-11291.
15. Watkins SJ, Mesyanzhinov VV, Kurochkina LP, Hawkins RE. The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. Gene Ther 1997; 4(10):1004-1012.
16. Weiskirchen R, Kneifel J, Weiskirchen S, van de LE, Kunz D, Gressner AM. Comparative evaluation of gene delivery devices in primary cultures of rat hepatic stellate cells and rat myofibroblasts. BMC Cell Biol 2000; 1(1):4.
17. Yu Q, Que LG, Rockey DC. Adenovirus-mediated gene transfer to nonparenchymal cells in normal and injured liver. Am J Physiol Gastrointest Liver Physiol 2002; 282(3):G565-G572.
18. Nakamura T, Akiyoshi H, Saito I, Sato K. Adenovirus-mediated gene expression in the septal cells of cirrhotic rat livers. J Hepatol 1999; 30(1):101-106.
19. Vogel S, Piantedosi R, Frank J, Lalazar A, Rockey DC, Friedman SL et al. An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. J Lipid Res 2000; 41(6):882-893.
20. Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990; 12(3 Pt 1):511-518.
21. Schoemaker MH, Gommans WM, Conde de la Rosa L, Homan M, Trautwein C, Van Goor H et al. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to NF-kB activation. J.Hepatol. 39(2), 153-161. 2003.
22. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 1998; 95(5):2509-2514.
23. Alemany R, Curiel DT. CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther 2001; 8(17):1347-1353.
24. Somasundaram R, Schuppan D. Type I, II, III, IV, V, and VI collagens serve as extracellular ligands for the isoforms of platelet-derived growth factor (AA, BB, and AB). J Biol Chem 1996; 271(43):26884-26891.

25. Hidaka C, Milano E, Leopold PL, Bergelson JM, Hackett NR, Finberg RW et al. CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J Clin Invest 1999; 103(4):579-587.
26. Schoemaker MH, Ros JE, Homan M, Trautwein C, Liston P, Poelstra K et al. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. J Hepatol 2002; 36(6):742-750.
27. Pinzani M, Gentilini A, Caligiuri A, De Franco R, Pellegrini G, Milani S et al. Transforming growth factor-beta 1 regulates platelet-derived growth factor receptor beta subunit in human liver fat-storing cells. Hepatology 1995; 21(1):232-239.
28. Salgado S, Garcia J, Vera J, Siller F, Bueno M, Miranda A et al. Liver cirrhosis is reverted by urokinase-type plasminogen activator gene therapy. Mol Ther 2000; 2(6):545-551.
29. Suzuki K, Aoki K, Ohnami S, Yoshida K, Kazui T, Kato N et al. Adenovirus-mediated gene transfer of interferon alpha improves dimethylnitrosamine-induced liver cirrhosis in rat model. Gene Ther 2003; 10(9):765-773.
30. Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita R et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med 1999; 5(2):226-230.
31. Iimuro Y, Nishio T, Morimoto T, Nitta T, Stefanovic B, Choi SK et al. Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat. Gastroenterology 2003; 124(2):445-458.
32. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. J Virol 1998; 72(5):4212-4223.
33. Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. Biotechnology (N Y) 1995; 13(3):265-270.
34. Garcia-Banuelos J, Siller-Lopez F, Miranda A, Aguilar LK, Aguilar-Cordova E, Armendariz-Borunda J. Cirrhotic rat livers with extensive fibrosis can be safely transduced with clinical-grade adenoviral vectors. Evidence of cirrhosis reversion. Gene Ther 2002; 9(2):127-134.
35. Frommel TO, Yong S, Zarling EJ. Immunohistochemical evaluation of Bcl-2 gene family expression in liver of hepatitis C and cirrhotic patients: a novel mechanism to explain the high incidence of hepatocarcinoma in cirrhotics. Am J Gastroenterol 1999; 94(1):178-182.
36. Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA, Lemasters JJ. The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. Mol Cell Biol 1998; 18(11):6353-6364.
37. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW et al. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration [published erratum appears in J Clin Invest 1998 Apr 1;101(7):1541]. J Clin Invest 1998; 101(4):802-811.
38. Arias M, Lahme B, van de LE, Gressner AM, Weiskirchen R. Adenoviral delivery of an antisense RNA complementary to the 3' coding sequence of transforming growth factor-beta1 inhibits fibrogenic activities of hepatic stellate cells. Cell Growth Differ 2002; 13(6):265-273.

39. Weiskirchen R, Moser M, Weiskirchen S, Erdel M, Dahmen S, Buettner R et al. LIM-domain protein cysteine- and glycine-rich protein 2 (CRP2) is a novel marker of hepatic stellate cells and binding partner of the protein inhibitor of activated STAT1. Biochem J 2001; 359(Pt 3):485-496.
40. Michael SI, Hong JS, Curiel DT, Engler JA. Addition of a short peptide ligand to the adenovirus fiber protein. Gene Ther 1995; 2(9):660-668.
41. Krasnykh V, Dmitriev I, Mikheeva G, Miller CR, Belousova N, Curiel DT. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. J Virol 1998; 72(3):1844-1852.
42. Wesseling JG, Bosma PJ, Krasnykh V, Kashentseva EA, Blackwell JL, Reynolds PN et al. Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors. Gene Ther 2001; 8(13):969-976.

Chapter 8
Summary, general discussion and perspectives

During acute and chronic liver injury, liver cells are exposed to increased levels of cytokines (protein hormones of the immune system), bile acids and oxidative stress (disturbed balance of oxidative and reductive capacity in the cell). This may result in death of hepatocytes, whereas stellate cells become active and proliferative. Eventually, acute and chronic liver injury is followed by loss of liver function for which no effective therapies are available. Therefore, understanding of the cellular mechanisms controlling death of liver cells is of clinical and scientific importance.

In this thesis, cell death and protective mechanisms of hepatocytes were explored in acute and chronic liver injury. Besides programmed cell death (apoptosis), which follows a tightly controlled cascade of events, necrotic cell death (necrosis) can occur. The strict regulation of apoptosis may allow therapeutic intervention strategies.

8.1 Acute liver injury

During acute liver injury, hepatocytes are exposed to increased levels of inflammatory mediators, of which TNF-α, IL-1β, IFN-γ and LPS are prominent components. Binding of these agents to receptors on the cell membrane induces cell-signalling pathways. Eventually, this leads to apoptosis in hepatocytes, which can be accompanied by necrosis, and ultimately will result in loss of liver function. We have used TNF-α, IL-1β, IFN-γ and LPS simultaneously to investigate cell death in hepatocytes *in vitro*.

Chapter 3 describes how hepatocytes are able to cope with exposure to inflammation. We have demonstrated that the activation of the transcription factor NF-κB is crucial for the protection of hepatocytes against cytokine-induced apoptotic cell death. In conditions where NF-κB activation is inhibited, cytokines induce receptor-mediated apoptosis, which is FADD-dependent and peaks 6 hours after the addition of cytokines. As long as NF-kB is able to regulate the transcription of anti-apoptotic genes cIAP2 and A1/Bfl-1, cytokine-induced

apoptosis is blocked in hepatocytes. (Fig 1)

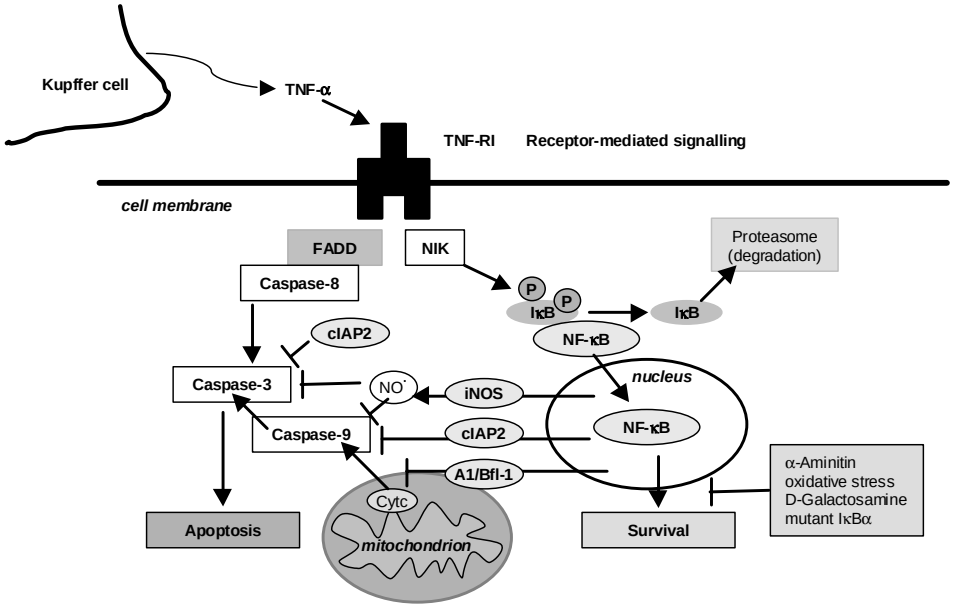


Figure 1. Schematic summary of cytokine-induced signalling in hepatocytes. The activation of the transcription factor NF-κB protects against cytokine-induced apoptotic cell death through the upregulation of anti-apoptotic genes, like cIAP2, A1/Bfl-1 and iNOS.

In chapter 4, a mouse model has been used for the study of acute liver failure *in vivo*. In this model, LPS injection results in cytokine release by Kupffer cells, which induces cell signalling in hepatocytes. Only in the presence of a hepatocyte specific transcription inhibitor, like D-galactosamine, hepatocytes are sensitive to LPS-induced cytokines. Since NF-κB is essential in the protection of hepatocytes against cytokine-induced apoptosis, our *in vitro* study (chapter 3) correlates with this model and provides another clue that NF-κB is a major regulator of cell survival.

NF-κB-regulated cIAP2 and A1/Bfl-1 belong to the IAP and Bcl-2 family, respectively, which are important sensors of intra- and extracellular stress and act to inhibit apoptosis. Other anti-apoptotic genes belonging to these families are cIAP1, XIAP1 and Bcl-XL. They are all expressed in hepatocytes, but are not induced upon cytokine stimulation.

Interestingly, NF-κB is also involved in the regulation of pro-apoptotic Bcl-2 family members, namely Bid and Bak. This is in line with previous data obtained in other cell types demonstrating that NF-κB has both pro-apoptotic and anti-apoptotic functions within a single cell.[1] Bid and Bak are both important in the release of pro-apoptotic mitochondrial factors, like cytochrome c and Smac/DIABLO (chapter 2: fig 5). Their importance in hepatocytes is demonstrated in Bid-deficient mice.[2]. In addition, Bid is a requisite for Bak-induced cytochrome c release.[3] In spite of this, blocking of NF-κB activation (and thus, Bid and Bak transcription) does not inhibit cytokine-induced apoptosis in hepatocytes. One explanation can be the post-translational modification of Bid and Bak, often needed to become fully pro-apoptotic, and this may still occur when NF-κB-regulated transcription is blocked.

Overall, NF-κB inhibition sensitises hepatocytes towards apoptosis and this shows that NF-κB-regulated transcription of anti-apoptotic cIAP2 and A1/Bfl-1 is dominant over the NF-κB-dependent activation of pro-apoptotic Bak and Bid (chapter 3). Therefore, in hepatocytes, NF-κB functions as an anti-apoptotic factor by regulating cIAP2 and A1/Bfl-1.

There is accumulating evidence that $NO^{\bullet}$ plays a role in apoptosis. A protective role of NF-κB-regulated inducible nitric oxide synthase (iNOS) has therefore been investigated in chapter 3. However, the contribution of iNOS to the protection against apoptosis is not fully elucidated yet. Endogenous $NO^{\bullet}$ is derived from iNOS, which can inhibit apoptosis by inactivation of caspases[4] (Fig 1). On the other hand, inhibition of iNOS by L-NAME does not sensitise hepatocytes to cytokine-induced apoptosis *in vitro* (chapter 3). This can be explained by the fact that L-

NAME does not affect NF-κB-regulated transcription of cIAP2 and A1/Bfl. In this situation, the lack of iNOS-derived $NO^{\bullet}$ is compensated by the presence of active cIAP2, again indicating that cIAP2 is essential to protect hepatocytes against cytokine-induced apoptosis *in vitro*. Moreover, adenovirus-mediated iNOS gene transfer inhibits hepatocyte apoptosis *in vitro.*[7] All these results support the idea that iNOS is protective against cytokine-induced apoptosis in hepatocytes.

Intriguingly, we have found that exogenous $NO^{\bullet}$, provided by an $NO^{\bullet}$-donor, also inhibits apoptosis in cytokine-exposed hepatocytes *in vitro*. This result suggests that $NO^{\bullet}$-donors could serve as therapeutic agents. Indeed, it has been shown that $NO^{\bullet}$-donors, like V-PYRRO/NO and NOC18, block TNF-α-induced apoptosis and toxicity in the liver.[5; 66; 8] Furthermore, in acetaminophen-induced hepatoxicity, another model of acute liver injury, V-PYRRO/NO reduced liver damage.[9] Nevertheless, one must be cautious with iNOS and its product $NO^{\bullet}$ as a therapeutic agent since $NO^{\bullet}$ is very reactive. Harmful effects of enhanced $NO^{\bullet}$ production are reported in several pathophysiological processes such as endotoxemia.[10] For example, large amounts of $NO^{\bullet}$ can also rapidly interact with superoxide anions yielding peroxynitrite and tissue damage. Especially during acute liver injury when liver cells are already sensitive, $NO^{\bullet}$ may react unpredictable. Therefore, we did not further explore $NO^{\bullet}$ or iNOS as a potential therapeutic agent for the threatment of acute liver injury. Instead, we decided to investigate the protective effect of cIAP2 in our *in vivo* model (chapter 4) since cIAP2 is a major anti-apoptotic player *in vitro*.

During acute liver injury, massive cell death of hepatocytes occurs due to breakdown of the NF-κB defence (chapter 4). In patients suffering from acute liver injury, NF-κB activation may be inhibited in various ways, depending on the type of injury. The mushroom-derived toxin α-amanitin for instance inhibits transcription. (Fig.1) It is also likely that in conditions accompanied with prolonged oxidative stress, NF-κB activation is prevented by massive consumption of reduced glutathione. This alters the intracellular redox status. Oxidative stress may result in

the oxidation of essential sulfhydryl groups of NF-κB (Fig 1), which then is not able to regulate the transcription of anti-apoptotic genes, as recently discussed.[11; 12]

In this thesis, LPS/GalN injection is used as a model for acute liver injury. Although adenoviral expression of the human homologue of cIAP2 (AdHIAP1) clearly prevents cytokine-induced apoptosis *in vitro*, we were not able to demonstrate this in the LPS/GalN model (chapter 4). Pre-treatment of these mice with AdHIAP1 aggravated LPS/GalN-induced liver injury. In addition, AdHIAP1 did not inhibit apoptotic cell death of hepatocytes. Various explanations can be given for these results, like sub-optimal transgene expression, liver priming by the adenoviral vector, the severity of the LPS/GalN model, or the HIAP1 transgene itself. In chapter 4, we have discussed these issues extensively. Because results from XIAP transgenic mice are similar to data obtained from the adenoviral overexpression of HIAP1, it appears that IAP overexpression is detrimental in the LPS/GalN model. This unexpected finding remains to be elucidated yet.

Our studies underline the complexity of what seemed to be a simple and promising approach, namely the expression of a therapeutic transgene in a diseased environment. In a controlled setting like our *in vitro* study on hepatocytes, apoptosis is completely blocked by cIAP2 (chapter 3), whereas in a complex setting with many interacting effects, like acute liver injury, it is much more difficult to influence signalling pathways and thereby cell fate. Compensatory mechanisms may exist that circumvent the therapeutic effect, like alternate caspase activation after inhibition of caspase-9 or caspase-3 in hepatocytes.[13] In addition, caspase-inhibition may increase oxidative-stress, thereby counteracting the anti-apoptotic effect of caspase-inhibition. Evidence for this hypothesis has been achieved recently.[14; 15] These studies have demonstrated that caspase activation in response to TNF-α has anti-necrotic effects as well. Removal of damaged and reactive oxygen species (ROS)-overproducing 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 acute liver injury.

Anti-inflammatory agents may help to reduce liver damage during acute liver injury. 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.[16]

Although we have not achieved promising results with cIAP2 as a therapeutic target *in vivo*, our studies described in chapter 3 and 4 are relevant for developing gene therapy which aims to interfere in cell signalling during liver diseases.

An alternative therapeutic strategy for acute liver injury might be *ex vivo* gene therapy.[17] The viral transduction of a therapeutic gene into hepatocytes before transplanting these cells into the liver may be beneficial. The effectiveness of this strategy depends on the capacity of the transplanted cells to survive and proliferate.[18] A pilot-study in humans demonstrated the feasibility of this *ex vivo* gene therapy for homozygous familial hypercholesterolaemia.[19] A recent study reported the transplantation of adenoviral-transduced Kupffer cells encoding a superoxide dismutase in acute liver injury. This strategy decreased liver injury after ischemia-reperfusion in rats.[20] Given the severe shortage of human hepatocytes, the application of porcine hepatocytes could be considered.[21] Overexpression of an anti-apoptotic gene in hepatocytes *ex vivo*, followed by cell transplantation, may be beneficical during acute liver injury.

Anti-apoptotic genes are often over-expressed in cancer cells resulting in inhibition of apoptosis and subsequent chemotherapeutic drug resistance.[22] For example in human hepatocelluar carcinoma, the IAP family member XIAP, and the Bcl-2 family member Bcl-2 become highly over-expressed.[23-25] Given the functional importance of anti-apoptotic proteins in apoptosis control, gene therapy with anti-apoptotic genes may induce carcinogenesis.[50-51] Therefore, anti-apoptotic gene therapy will only be safe when a tightly controlled expression pattern of anti-apoptotic genes can be assured.

8.2 Chronic liver injury

Cholestatic liver disorders (cholestasis) are characterised by the accumulation of bile acids in the liver causing damage to liver cells. Cholestasis could eventually result in liver fibrosis, which can be considered as an excessive wound healing. In chapter 5 and 6, cell death in chronic cholestatic liver injury has been investigated. For this purpose, a rat model has been used in which the bile duct has been ligated. This bile duct ligation ensures the accumulation of bile acids in the liver, thereby triggering cell damage and liver fibrosis.

We have shown that during chronic cholestatic liver injury, the predominant mode of hepatocyte cell death is necrosis and not apoptosis (chapter 5). This unexpected finding is contrary to existing data,[26] but recently supported by other expert investigators, stating that necrosis, and not apoptosis, is the principal mode of cell death during cholestatic liver injury.[27; 28]

This finding has important consequences for therapeutic intervention strategies in cholestatic liver diseases. Not apoptotic, but necrotic signalling must be tackled. Recently, neutrophil-induced oxidative stress in response to chemokines has been shown to contribute substantially to the injury process. Therefore, neutrophils could be an important therapeutic target to improve cholestatic liver injury.[29] In addition, adenoviral delivery of a superoxide dismutase gene may be useful in the treatment of oxidative stress-related injury. Superoxide dismutases are potent detoxifying enzymes of superoxide radicals and have been shown to reduce liver injury.[30; 31]

Another important finding is the absence of the anti-apoptotic protein Bcl-2 in hepatocytes. The expression of Bcl-2 during chronic liver injury is induced in cholangiocytes only. This sharply contrasts to previous reported data.[26] Our results imply that Bcl-2 is not involved in the protection of hepatocytes against bile acid-induced liver injury. Although Bcl-2 seems to be a potential therapeutic candidate, it is questionable whether over-expression of Bcl-2 in hepatocytes would prevent necrotic cell damage during chronic liver injury.[32]

In chapter 5, we have investigated the role of the transcription factor NF-κB in chronic liver injury. Indeed, NF-κB is activated during chronic cholestatic liver injury and we have found evidence that the NF-κB-regulated genes cIAP2 and A1/Bfl-1 are induced. However, the activation of these anti-apoptotic proteins is not triggered by bile acids, which has previoulsy been proposed by others as they accumulate in the liver during cholestasis. In contrast, NF-κB has been shown to be activated in response to cytokine-mediated signalling (chapter 5). In the cholestatic rat livers, mRNA levels of cytokines, like TNF-α, IL-1β, and IFN-γ, are increased. In addition, we have found that the bile acid glycochenodeoxycholic acid (GCDCA), but not taurochenodeoxycholic acid (TCDCA) or tauroursodeoxycholic acid (TUDCA), induce apoptosis in hepatocytes *in vitro* (Fig 2). This GCDCA-induced apoptosis is inhibited when hepatocytes are pre-incubated with cytokines for 1 to 3 hours. Recently, it has been reported that cytokine-induced A1/Bfl-1 sequesters and inhibits pro-apoptotic Bid.[33] Bid is activated during GCDCA-induced apoptosis.[34] These data suggest a protective mechanism of cytokines against bile acid-induced cell damage during chronic liver injury. Further support for the protective role of NF-κB during cholestasis comes from our data demonstrating that adenoviral overexpression of cIAP2 inhibits GCDCA-induced apoptosis *in vitro* (chapter 5).

All together, our data imply that during chronic cholestatic liver injury, hepatoyctes are resistant against bile acid-induced apoptosis due to the cytokine-induced activation of anti-apoptotic NF-κB (Fig 2). This conclusion is relevant for the treatment of patients with inflammation inhibitors, like dexamethasone and anti-TNF-α antibodies, or Kupffer cell depletion. These anti-inflammatory strategies will attenuate cytokine production and NF-κB activation and thus stimulate apoptosis in hepatocytes. In particular, the question raises whether these anti-inflammatory strategies are favourable in chronic liver diseases. The therapeutic implications of these strategies have been addressed in animal models of chronic liver injury.[35; 36]

Although Kupffer cell depletion resulted in improved survival rates in the case of LPS-exposed bile duct ligated (BDL) rats[35], the consequence of a disruption of the cellular environment has not been elucidated yet. In addition, a-selective inhibition of NF-κB by immune modulators could be harmful to hepatocytes as NF-κB inactivation sensitises these cells to apoptosis. A Kupffer cell-specific carrier can solve this problem, namely dexamethasone coupled to mannosylated albumin.[36] However, treatment of BDL rats with this carrier resulted in a pro-fibrotic effect. Since Kupffer cells are much more sensitive to activating stimuli in a diseased liver compared to normal circumstances, unpredictable effects may be generated. Additionally, inhibition of cytokine release from Kupffer cells during chronic liver injury sensitises hepatocytes as well, by removing the NF-κB stimulus. Moreover, it has been shown that elevated levels of TNF-α and IL-1β are protective because they downregulate the bile acid-uptake transporter Ntcp in hepatocytes. This reduces the import of toxic bile acids.[37] Furthermore, Kupffer cell-derived cytokines enable hepatocyte proliferation by activating protective pathways during liver regeneration.[38]

In conclusion, increased levels of cytokines protect hepatocytes against bile acid-induced apoptosis during chronic liver injury. Therefore, therapeutic interventions reducing the effects of these cytokines in chronic liver inflammation will not be beneficial per se. For this purpose, a better understanding of all the components involved in this process is needed.

Bile acid-induced cell damage

In chapter 5 and 6, we unravelled the mechanisms of bile acid-induced damage to hepatocytes. Although bile acids do not induce apoptotic cell death in hepatocytes *in vivo*, thorough understanding of (protection against) cell death may lead to novel treatments of cholestatic liver diseases.

We have shown that exposure to glycochenodeoxycholic acid (GCDCA) for 4 hours induces apoptosis in hepatocytes *in vitro*. Not all bile acids are cytotoxic. Neither the taurine-conjugated bile acid chenodeoxycholic acid (TCDCA) nor

taurine-conjugated ursodeoxycholic acid (TUDCA) induces apoptosis in hepatocytes. Instead, TUDCA, but not TCDCA, is an anti-apoptotic bile acid, which blocks GCDCA-induced apoptosis (Fig 2). The protective mechanisms of TUDCA are relevant, since un-conjugated UDCA is used as a treatment of patients with chronic liver diseases. Because the protective actions of UDCA are not completely understood yet, we explored its anti-apoptotic mechanisms in chapter 6. We have used TUDCA, since in rats, UDCA will be conjugated predominantly with taurine (TUDCA) compared to glycine (GUDCA). In humans, it is the other way around. Nevertheless, TUDCA treatment of patients suffering from primary biliary cirrhosis may even be beneficial over UDCA treatment, which can not be fully explained by re-conjugation to GUDCA.[39]

We have demonstrated in chapter 5 that GCDCA induces apoptosis in a FADD-independent manner (Fig 2). Blocking of receptor-mediated signalling, by adenoviral overexpression of dominant negative FADD, did not inhibit GCDCA-induced apoptosis. Likewise, caspase-8 inhibition did not block GCDCA-induced apoptosis. This is in contrast to cytokines that induce apoptosis in hepatocytes via receptor-mediated signalling (chapter 3). This discrepancy can be explained by the fact that GCDCA-induced apoptosis depends on mitochondria-released pro-apoptotic factors. Thus, GCDCA needs to be taken up by hepatocytes in order to induce its effects via mitochondria-controlled apoptosis. In this way, mitochondria-mediated activation of caspase-3 results in a feedback activation loop of caspase-8 (Fig 2).

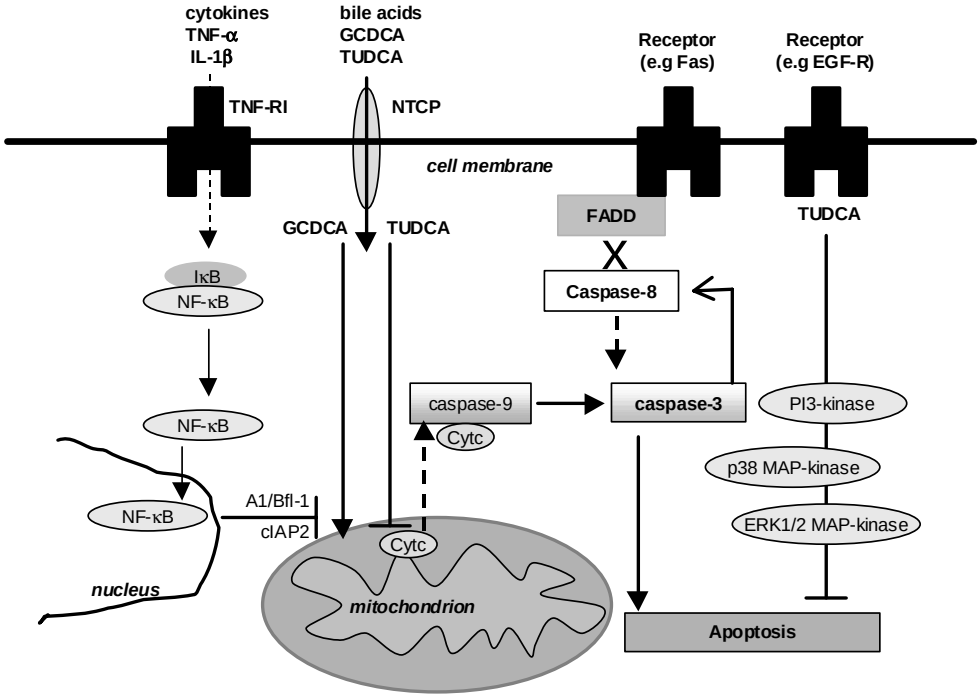


Figure 2. Schematic overview of protective mechanisms against GCDCA-induced apoptosis in hepatocytes, like tauroursodeoxycholic acid (TUDCA) and cytokine-mediated activation of NF-κB (—┤= inhibition).

In contrast to the more slow induction of apoptosis by GCDCA, TUDCA rapidly induces protective mechanisms against GCDCA, like the activation of MAP kinases and PI3-kinases (Fig 2). The transcription factor NF-κB is not involved in the protective actions of TUDCA (chapter 6). One to three hour pre-incubation of hepatocytes with TUDCA blocks GCDCA-induced apoptosis. In addition, TUDCA can be added 1 hour after GCDCA without losing the ability to inhibit apoptosis. These results indicate that a competitive effect of TUDCA for GCDCA-uptake by Ntcp on the cell membrane is not likely.

In contrast to TUDCA, cytokines do not protect against bile acid-induced apoptosis when added áfter bile acids (chapter 5). The reason for this difference is that the protective mechanism of cytokines depends on NF-κB-mediated transcription with a time frame of hours, whereas the protective action of TUDCA depends on post-transcriptional phosphorylation of proteins, which only takes minutes.

Finally, direct effects of TUDCA on the mitochondrial membrane can not be excluded. Recently, it has been reported that TUDCA prevents binding of pro-apoptotic Bax to the mitochondria.[40] Additionally, TUDCA has an inhibitory effect on the permeability of the mitochondrial membrane, indicating that TUDCA has an important role in the stabilisation of the mitochondrial membrane during bile acid-induced apoptosis (Fig 2).

In conclusion, GCDCA is a toxic bile acid inducing mitochondria-controlled apoptotic cell death, which can be prevented and inhibited by TUDCA through signalling pathways and direct actions on the mitochondrial membrane.

Gene targeting

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. To interfere with cellular processes in these cells, we have developed a novel gene delivery tool, which is described in chapter 7. Recombinant adenovirus has been modified in such a way that it selectively targets to the PDGFβ-receptor, which is highly induced on activated stellate cells during liver fibrosis. The modification consists of a fusion-protein, which recognises a knob on the outside of the adenoviral vector, whereas the other part of the fusion protein binds to the PDGFβ-receptor. The fusion protein prevents binding of the adenoviral knob to its preferential receptor CAR and mediates retargeting of the virus to the PDGFβ-receptor. Unmodified adenovirus is rapidly taken up by hepatocytes. In contrast, an optimal ratio of protein amount per viral particles demonstrates that our modified virus un-targets

primary hepatocytes, whereas gene transfer in activated stellate cells is achieved very efficiently.

With this tool, a therapeutic gene can be transferred into activated stellate cells during liver fibrosis. Induction of apoptotic cell death may be a promising therapeutic strategy, since it has been shown that apoptosis of activated stellate cells reduces liver fibrosis.[45;46] This strategy has also been investigated with Gliotoxin, a fungal metabolite.[47] Gliotoxin induces apoptosis of activated stellate cells which is followed by reduced liver fibrosis. However, it does not improve liver functions since hepatocytes are affected as well. These results confirm the need for specific therapies. With our strategy described in chapter 7, a therapeutic gene could be selectively targeted to the activated stellate cells. Such target gene could be the transcription factor NF-κB, since NF-κB protects activated stellate cells against apoptotic cell death.[48] Activation of NF-κB requires the phosphorylation of IκBα. Specific delivery of a mutant form of IκBα to the activated stellate cells may be beneficial to reduce the amount of activated stellate cells. Obviously, activation of NF-κB in hepatocytes should not be prevented because this will induce apoptosis in hepatocytes, as outlined in this thesis. Apoptosis-inducing strategies will therefore only be beneficial if cell-selective delivery is achieved.

It has been suggested that over-expression of metalloproteinases effectively attenuates fibrosis.[41;42] Metalloproteinases are enzymes degradating the excessive extracellular matrix, which is disturbed during liver fibrosis. An alternative therapy could be over-expression of a dominant-negative mutant of an inhibitor of metalloproteinases, TIMP-1, thereby ensuring metalloproteinase activity.[43] In contrast to the beneficial role in liver fibrosis, metalloproteinases favour carcinogenesis, whereas for TIMP-1, this is the other way around. A clinical application must be considered critically since patients suffering from liver cirrhosis very often suffer from hepatocarcinoma as well.[44]

Although the target cell population is much smaller compared to the population of hepatocytes, the fibrotic process in the liver favours uptake of the transgene in

the cells around the space of Disse that line the sinusoids.[49] In addition to this, our retargeting strategy aims at transduction of the therapeutic transgene in the activated stellate cells only. It has been reported that the liver can be safely transduced with clinical-grade adenoviral vectors, in spite of the injured liver during fibrosis.[41] In combination with a cell type-specific promotor driving the expression of the therapeutic gene, the application of a cell-specific targeting approach would represent a second line of safety. Finally, inclusion of our PDGF-Rβ-targeting peptide in the HI-loop of the fiber knobs of the recombinant adenoviral gene vector will further diminish the hurdles regarding gene therapy.

8.3 Conclusions

Understanding of the cellular mechanisms controlling death of liver cells is of clinical and scientific importance to develop novel therapies. This thesis describes protective mechanisms of hepatocytes against cell death during acute and chronic liver injury. Our studies have resulted in the identification of new intervention strategies to prevent apoptotic cell death of hepatocytes during acute liver diseases. In addition, we have developed a novel gene delivery tool specifically targeting to activated stellate cells. In this way, apoptosis can be induced selectively in activated stellate cells, which may be a relevant therapeutic strategy during chronic liver diseases.

8.4 References

1. Lin B, Williams-Skipp C, Tao Y, Schleicher MS, Cano LL, Duke RC, Scheinman RI. NF-kappaB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type. Cell Death Differ 1999;6:570-582.

2. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 1999;400:886-891.
3. Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 2000;14:2060-2071.
4. Li J, Billiar TR. The anti-apoptotic actions of nitric oxide in hepatocytes. Cell Death Differ 1999;6:952-955.
5. Ou J, Carlos TM, Watkins SC, Saavedra JE, Keefer LK, Kim YM, Harbrecht BG, Billiar TR. Differential effects of nonselective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. Nitric Oxide 1997;1:404-416.
6. Akahori M, Yamada S, Takeyama N, Tanaka T. Nitric oxide ameliorates actinomycin D/endotoxin-induced apoptotic liver failure in mice. J Surg Res 1999;85:286-293.
7. Tzeng E, Billiar TR, Williams DL, Li J, Lizonova A, Kovesdi I, Kim YM. Adenovirus-mediated inducible nitric oxide synthase gene transfer inhibits hepatocyte apoptosis. Surgery 1998;124:278-283.
8. Saavedra JE, Billiar TR, Williams DL, Kim YM, Watkins SC, Keefer LK. Targeting nitric oxide (NO) delivery in vivo. Design of a liver-selective NO donor prodrug that blocks tumor necrosis factor-alpha-induced apoptosis and toxicity in the liver. J Med Chem 1997;40:1947-1954.
9. Liu J, Li C, Waalkes MP, Clark J, Myers P, Saavedra JE, Keefer LK. The nitric oxide donor, V-PYRRO/NO, protects against acetaminophen-induced hepatotoxicity in mice. Hepatology 2003;37:324-333.
10. Harbrecht BG, Billiar TR. The role of nitric oxide in Kupffer cell-hepatocyte interactions. Shock 1995;3:79-87.
11. Day CP. Apoptosis in alcoholic hepatitis: a novel therapeutic target? J Hepatol 2001;34:330-333.
12. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K. Evidence that reactive oxygen species do not mediate NF-kappaB activation. EMBO J 2003;22:3356-3366.
13. Zheng TS, Hunot S, Kuida K, Momoi T, Srinivasan A, Nicholson DW, Lazebnik Y, Flavell RA. Deficiency in caspase-9 or caspase-3 induces compensatory caspase activation. Nat Med 2000;6:1241-1247.
14. Cauwels A, Janssen B, Waeytens A, Cuvelier C, Brouckaert P. Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. Nat Immunol 2003;4:387-393.
15. Khwaja A, Tatton L. Resistance to the cytotoxic effects of tumor necrosis factor alpha can be overcome by inhibition of a FADD/caspase-dependent signaling pathway. J Biol Chem 1999;274:36817-36823.
16. Moore TA, Lau HY, Cogen AL, Monteleon CL, Standiford TJ. Anti-tumor necrosis factor-alpha therapy during murine Klebsiella pneumoniae bacteremia: increased mortality in the absence of liver injury. Shock 2003;20:309-315.

17. Di Campli C, Gasbarrini G, Gasbarrini A. Review article: a medicine based on cell transplantation -- is there a future for treating liver diseases? Aliment Pharmacol Ther 2003;18:473-480.
18. Fausto N. Genetic engineering of liver cells for survival and proliferation. J.Hepatol. 36, 858-859. 2002.
Ref Type: Abstract
19. Grossman M, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ, III, Stein EA, Lupien PJ, Brewer HB, Jr., Raper SE, . A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. Nat Med 1995;1:1148-1154.
20. Froh M, Wheeler MD, Smutney O, Zhong Z, Bradford BU, Thurman RG. New method of delivering gene-altered Kupffer cells to rat liver: studies in an ischemia-reperfusion model. Gastroenterology 2003;124:172-183.
21. Maruyama M, Totsugawa T, Kunieda T, Okitsu T, Shibata N, Takesue M, Kurabayashi Y, Oshita M, Nakaji S, Kodama M, Tanaka N, Kobayashi N. Hepatocyte isolation and transplantation in the pig. Cell Transplant 2003;12:593-598.
22. Schmitt CA, Lowe SW. Apoptosis and therapy. J Pathol 1999;187:127-137.
23. Shiraki K, Sugimoto K, Yamanaka Y, Yamaguchi Y, Saitou Y, Ito K, Yamamoto N, Yamanaka T, Fujikawa K, Murata K, Nakano T. Overexpression of X-linked inhibitor of apoptosis in human hepatocellular carcinoma. Int J Mol Med 2003;12:705-708.
24. Takahashi M, Saito H, Atsukawa K, Ebinuma H, Okuyama T, Ishii H. Bcl-2 prevents doxorubicin-induced apoptosis of human liver cancer cells. Hepatol Res 2003;25:192-201.
25. Chiu CT, Yeh TS, Hsu JC, Chen MF. Expression of Bcl-2 family modulated through p53-dependent pathway in human hepatocellular carcinoma. Dig Dis Sci 2003;48:670-676.
26. Miyoshi H, Rust C, Roberts PJ, Burgart LJ, Gores GJ. Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 1999;117:669-677.
27. Fickert P, Trauner M, Fuchsbichler A, Zollner G, Wagner M. Inhibition of poly(ADP-ribose) polymerase (PARP) protects mice from cholestatic liver injury. Hepatology 38[No.4, suppl 1],pp.1A-818A.2003. Ref Type: Abstract
28. Gujral J, Jaeschke H. Apoptosis and oncotic necrosis during acute liver injury after bile duct ligation in mice. Hepatology 38[No.4, suppl 1], pp. 1A-818A. 2003. Ref Type: Abstract
29. Gujral JS, Farhood A, Bajt ML, Jaeschke H. Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. Hepatology 2003;38:355-363.
30. Wheeler MD, Kono H, Yin M, Rusyn I, Froh M, Connor HD, Mason RP, Samulski RJ, Thurman RG. Delivery of the Cu/Zn-superoxide dismutase gene with adenovirus reduces early alcohol-induced liver injury in rats. Gastroenterology 2001;120:1241-1250.
31. Zhong Z, Froh M, Wheeler MD, Smutney O, Lehmann TG, Thurman RG. Viral gene delivery of superoxide dismutase attenuates experimental cholestasis-induced liver fibrosis in the rat. Gene Ther 2002;9:183-191.
32. Kane DJ, Ord T, Anton R, Bredesen DE. Expression of bcl-2 inhibits necrotic neural cell death. J Neurosci Res 1995;40:269-275.

33. Werner AB, de Vries E, Tait SW, Bontjer I, Borst J. Bcl-2 family member Bfl-1/A1 sequesters truncated bid to inhibit is collaboration with pro-apoptotic Bak or Bax. J Biol Chem 2002;277:22781-22788.
34. Higuchi H, Yoon JH, Grambihler A, Werneburg N, Bronk SF, Gores GJ. Bile acids stimulate cFLIP phosphorylation enhancing TRAIL-mediated apoptosis. J Biol Chem 2003;278:454-461.
35. Kennedy JA, Lewis H, Clements WD, Kirk SJ, Campbell G, Halliday MI, Rowlands BJ. Kupffer cell blockade, tumour necrosis factor secretion and survival following endotoxin challenge in experimental biliary obstruction. Br J Surg 1999;86:1410-1414.
36. Melgert BN, Olinga P, Van Der Laan JM, Weert B, Cho J, Schuppan D, Groothuis GM, Meijer DK, Poelstra K. Targeting dexamethasone to Kupffer cells: effects on liver inflammation and fibrosis in rats. Hepatology 2001;34:719-728.
37. Geier A, Dietrich CG, Voigt S, Kim SK, Gerloff T, Kullak-Ublick GA, Lorenzen J, Matern S, Gartung C. Effects of proinflammatory cytokines on rat organic anion transporters during toxic liver injury and cholestasis. Hepatology 2003;38:345-354.
38. Wuestefeld T, Klein C, Streetz KL, Betz U, Lauber J, Buer J, Manns MP, Muller W, Trautwein C. Interleukin-6/glycoprotein 130-dependent pathways are protective during liver regeneration. J Biol Chem 2003;278:11281-11288.
39. Invernizzi P, Setchell KD, Crosignani A, Battezzati PM, Larghi A, O'Connell NC, Podda M. Differences in the metabolism and disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis. Hepatology 1999;29:320-327.
40. Rodrigues CM, Sola S, Sharpe JC, Moura JJ, Steer CJ. Tauroursodeoxycholic acid prevents bax-induced membrane perturbation and cytochrome C release in isolated mitochondria. Biochemistry 2003;42:3070-3080.
41. Garcia-Banuelos J, Siller-Lopez F, Miranda A, Aguilar LK, Aguilar-Cordova E, Armendariz-Borunda J. Cirrhotic rat livers with extensive fibrosis can be safely transduced with clinical-grade adenoviral vectors. Evidence of cirrhosis reversion. Gene Ther 2002;9:127-134.
42. Iimuro Y, Nishio T, Morimoto T, Nitta T, Stefanovic B, Choi SK, Brenner DA, Yamaoka Y. Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat. Gastroenterology 2003;124:445-458.
43. Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, Benyon C, Iredale JP. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. J Biol Chem 2002;277:11069-11076.
44. Frommel TO, Yong S, Zarling EJ. Immunohistochemical evaluation of Bcl-2 gene family expression in liver of hepatitis C and cirrhotic patients: a novel mechanism to explain the high incidence of hepatocarcinoma in cirrhotics. Am J Gastroenterol 1999;94:178-182.
45. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 1998;102:538-549.

46. Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN, Arthur MJ, Iredale JP, Mann DA. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. Gastroenterology 2001;121:685-698.
47. Dekel R, Zvibel I, Brill S, Brazovsky E, Halpern Z, Oren R. Gliotoxin ameliorates development of fibrosis and cirrhosis in a thioacetamide rat model. Dig Dis Sci 2003;48:1642-1647.
48. Lang A, Schoonhoven R, Tuvia S, Brenner DA, Rippe RA. Nuclear factor kappaB in proliferation, activation, and apoptosis in rat hepatic stellate cells. J Hepatol 2000;33:49-58.
49. Nakamura T, Akiyoshi H, Saito I, Sato K. Adenovirus-mediated gene expression in the septal cells of cirrhotic rat livers. J Hepatol 1999;30:101-106.
50. D'Sa-Eipper C, Subramanian T, Chinnadurai G. Bfl-1, a bcl-2 homologue, suppresses p53-induced apoptosis and exhibits potent cooperative transforming activity. Cancer Res 1996;56:3879-82.
51. Roy HK, Olusola BF, Clemens DL, Karolski WJ, Ratashak A, Lynch HT, and Smyrk TC. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogensis. Carcinogenesis;23:201-205.

Chapter 9
Samenvatting

(Dutch Summary)

9.1 Achtergrond (Hoofdstuk 2)

De lever

Een heel belangrijke functie van de lever is het ontgiften van het bloed. De lever staat dagelijks bloot aan een grote verscheidenheid aan lichaamseigen (bijv. ammonia, bilirubine) en lichaamsvreemde stoffen (bijv. medicijnen, alcohol), die continu met het bloed worden aangevoerd en potentieel schadelijk zijn. Het onschadelijk maken van deze verbindingen gebeurt voornamelijk in hepatocyten, de parenchymcellen van de lever. Via deze cellen worden verbindingen opgenomen, afgebroken en verwijderd. De onschadelijk gemaakte verbindingen worden afgevoerd via het bloed of via uitscheiding in de gal. Vanuit het bloed verlaten in water oplosbare stoffen het lichaam via de nieren en de urine, terwijl andere verbindingen in de gal verdwijnen via de darm met de ontlasting. Gal bestaat voor 80 % uit water en voor 20 % uit opgeloste stoffen zoals galzouten.

In een normale situatie zijn levercellen goed bestand tegen potentieel schadelijke stoffen door beschermende mechanismen en een groot vermogen tot het repareren van schade. Echter, acute of chronische blootstelling aan bepaalde verbindingen kan leiden tot dusdanige celschade, dat beschermingsmechanismen wegvallen en celdood optreedt. Dit kan uiteindelijk resulteren in een verstoorde leverfunctie.

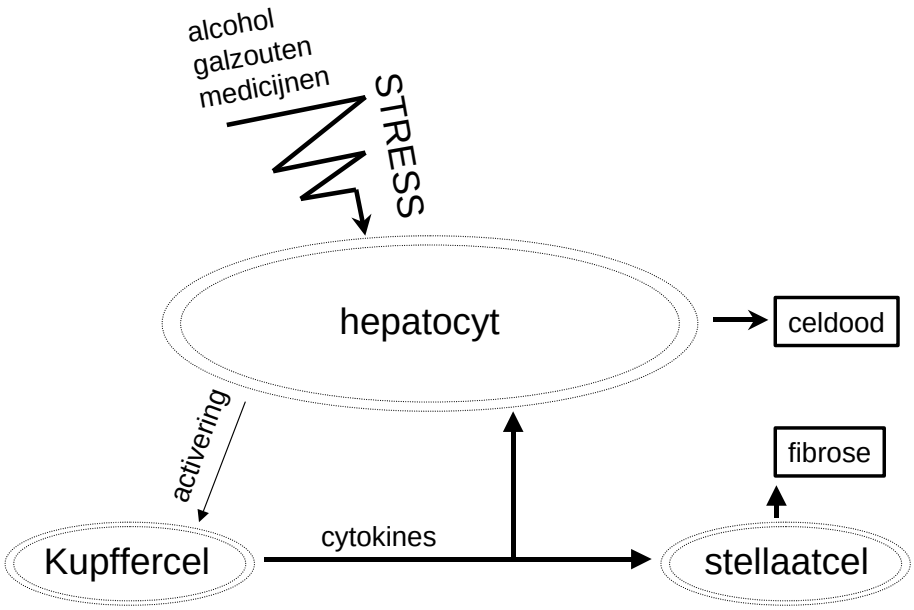
Acute en chronische leverschade

Acute leverschade ontwikkelt zich in een korte tijd in een tevoren gezond individu en komt voor bij virusinfecties (zoals hepatitis A, B, D and E), vergiftiging (paracetamol, alcohol, de paddestoel *Amanita phalloides*), ziekten die betrekking hebben op het afweersysteem (auto-immuunziekten) en bij afstoting na transplantatie. Tijdens acute leverschade worden voornamelijk de hepatocyten getroffen omdat de lever voor het grootste gedeelte uit deze cellen bestaat en de toxische stoffen vaak in deze cellen ontstaan of alleen in deze cellen effect hebben (Figuur 1). De functie van de hepatocyten is onmisbaar.

Chronische leverschade kan ontstaan door stofwisselingsziekten, virale hepatitis, of na langdurige blootstelling aan bijvoorbeeld alcohol, drugs, en geneesmiddelen. Dit kan leiden tot leverfibrose en uiteindelijk lever falen. Leverfibrose wordt gekenmerkt door een versterkt wondhelingsproces waarbij veel littekenweefsel ontstaat (fibrose) (Figuur 1). Chronische leverschade kan ook veroorzaakt worden door of gepaard gaan met cholestase. Cholestase is een verstoring in de vorming, uitscheiding of afvoer van galzouten (componenten van gal). Deze verstoring kan leiden tot ophoping van galzouten in de lever wat schade veroorzaakt aan levercellen.

Beschadiging van een cel kan leiden tot de vorming van reactieve zuurstofdeeltjes (radicalen). Deze radicalen verstoren de redox-balans in een cel waardoor allerlei verbindingen geoxideerd worden en de cel nog verder beschadigd raakt. Daarnaast worden tijdens acute en chronische leverschade cellen van het afweersysteem geactiveerd, zoals Kupffer-cellen (macrofagen in de lever), leukocyten en lymfocyten (witte bloedcellen), wat leidt tot een ontsteking (Figuur 1). Dit ontstekingsproces heeft als doel de schade te repareren. Daarbij spelen componenten van het afweersysteem (cytokinen) een rol. Echter, een verhoogde blootstelling aan cytokinen kan schadelijk zijn voor cellen. Cytokinen kunnen aan de buitenkant van een cel (bijvoorbeeld de hepatocyt) binden aan receptoren. Deze binding schakelt signaleringsroutes aan die beschermende mechanismen activeren in de cel maar ook kunnen leiden tot celdood (Figuur 2).

Cytokinen zetten ook aan tot de activering van de stellaatcellen (fibroblast-achtige cellen). De stellaatcellen zijn de belangrijkste spelers in de ontwikkeling van leverfibrose (Figuur 1). Geactiveerde stellaatcellen produceren een verhoogde hoeveelheid littekenweefsel, zoals collagenen. Deze reactie is bedoeld om de lever na beschadiging te herstellen. Echter, tijdens een langdurige beschadiging van de lever raakt de productie van littekenweefsel verstoord en ontstaat er leverfibrose (Figuur 1).



Figuur 1. Acute of chronische leverschade leidt tot beschadiging van de hepatocyten waardoor een ontstekingsreactie optreedt in de lever. Deze reactie wordt onder andere gekenmerkt door de activering van de zogenaamde Kupffer-cellen en een hoge productie van cytokinen (stoffen van het afweersyteem). Deze reacties zijn in eerste instantie bedoeld om de schade te repareren. Wanneer echter de beschermingsmechanismen van de hepatocyt het af laten weten, kan dit uiteindelijk leiden tot celdood van de hepatocyt. Daarnaast leidt een chronische leverschade tot de activering en celdeling van stellaatcellen, met als gevolg leverfibrose. Celdood van de hepatocyten en de ontwikkeling van leverfibrose kunnen resulteren in het verlies van leverfuncties en uiteindelijk fataal zijn.

Celdood in leverziekten

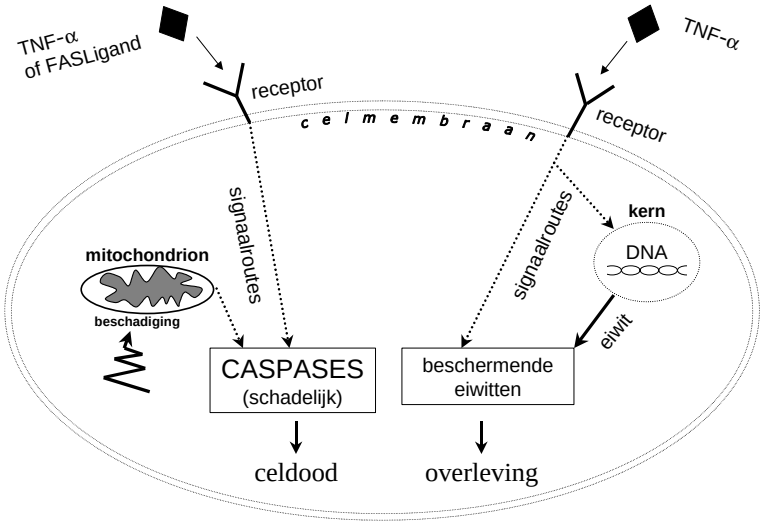
Tijdens acute en chronische leverziekten staan de hepatocyten bloot aan verhoogde concentraties van cytokinen en galzouten (Figuur 1). Wanneer de beschermingsmechanismen van de hepatocyt wegvallen, zal de cel dood gaan. Dit resulteert in een verstoorde leverfunctie.

Celdood kan plaatsvinden in verschillende vormen; necrose en apoptose. Apoptose is een sterk gecontroleerde vorm van celdood (geprogrammeerde celdood) waarbij de dode cellen netjes worden opgeruimd door de omgeving van de cel. Necrotische celdood is veel chaotischer, waarbij de inhoud van de cel vrijkomt in haar omgeving met ontstekingsreacties tot gevolg. Apoptotische celdood is onder andere van belang bij het opruimen van beschadigde cellen en bij de regulatie van de hoeveelheid cellen in een bepaald weefsel. Behalve in die gevallen dat apoptotische celdood dus gewenst is, kan apoptose ook levensbedreigend worden, zoals bijvoorbeeld bij acute leverschade, wanneer er grote aantallen hepatocyten wegvallen.

Effectieve therapieën voor acute en chronische leverziekten zijn niet voorhanden. Om deze ziekten goed te kunnen begrijpen is het daarom belangrijk om in kaart te brengen welke cellulaire mechanismen tot celdood leiden in hepatocyten.

Apoptotische celdood versus celoverleving

Het strikt gereguleerde proces van apoptotische celdood maakt het mogelijk om therapeutisch in te grijpen en daarmee celdood te remmen of te voorkomen. Daarom is de aandacht in dit proefschrift gericht op apoptotische celdood. Een cel kan op verschillende manieren tot apoptose worden aangezet. Het netwerk van interacties dat nodig is om apoptotische celdood te bewerkstelligen in een cel is te vergelijken met de binnenkant van een transistorradio. Wanneer er een essentieel onderdeeltje mist, doet de radio (lees: het proces dat apoptotische celdood bewerkstelligt) het niet meer.



Figuur 2. Overzicht van een cel met een aantal factoren dat betrokken is bij celoverleving en apoptotische celdood, zoals activering van signalen via receptoren op de celmembraan, of via de mitochondriën (energiefabriekjes in de cel). Apoptotische celdood is het gevolg van de activering van caspases. Beschermende stoffen (eiwitten) kunnen celdood remmen. Deze eiwitten worden direct geactiveerd of aangemaakt in de cel door het aflezen van de genetische informatie. Deze informatie ligt opgeslagen in zogenaamde genen in het DNA in de celkern. Sommige factoren zetten beide signaalroutes in werking (zoals TNF-α).

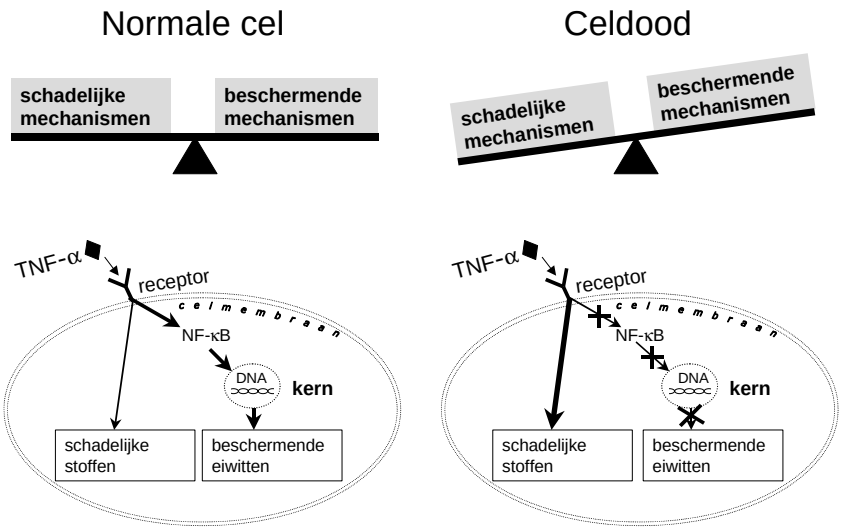
Apoptose kan worden aangeschakeld via binding van stoffen aan specifieke receptoren op de buitenkant van de cel (celmembraan) (Figuur 2). Voorbeelden van deze stoffen zijn Fas-ligand of cytokinen van het afweersysteem zoals TNF-α, die respectievelijk binden aan de receptoren Fas en TNF-R1. Binding aan de receptor leidt tot het aanschakelen van signaalroutes. In deze routes binden bepaalde enzymen (caspases genaamd) in een bepaalde volgorde aan elkaar waardoor deze geactiveerd raken. Caspases zijn onder andere verantwoordelijk voor het afbreken van bouwstenen (eiwitten, genetisch materiaal (DNA)) van de

cel. Op deze manier ontstaat een cascade die erop gericht is de cel op een gecontroleerde manier weg te werken (Figuur 2).

Een andere manier om apoptotische celdood te bewerkstelligen is via verstoring van een bepaald type organel (orgaan in een cel), namelijk het mitochondrion (Figuur 2). De mitochondriën zijn de energiefabriekjes van een cel en daarmee essentieel voor het normaal functioneren van de cel. Tijdens een apoptotische stimulus verandert de buitenmembraan van de mitochondriën waardoor stoffen (zogenaamde pro-apoptotische eiwitten) uit de mitochondriën in de cel terechtkomen. Deze stoffen bevorderen het apoptoseproces onder andere via de activering van caspases.

Een cel kan zich op verschillende manieren beschermen tegen apoptotische celdood (Figuur 2). Van nature bevat een cel anti-apoptotische verbindingen die bijvoorbeeld op de buitenmembraan van de mitochondriën werken en voorkomen dat schadelijke stoffen uit de mitochondriën in de cel komen. Een ander beschermingsmechanisme is het aanschakelen van overlevingsroutes, via de binding van stoffen aan receptoren op de celmembraan (Figuur 2). Voorbeelden van deze stoffen zijn de cytokines TNF-α en IL-1β. Hiermee is TNF-α dus een cytokine, die zowel routes aanschakelt die tot celdood leiden, als routes die bij celoverleving betrokken zijn. (Figuur 2). De balans zal uiteindelijk bepalen wat er gebeurt.

Samenvattend: zolang hepatocyten in de lever hun beschermende mechanismen kunnen activeren, zal de balans tussen celdood en celoverleving ten gunste van celoverleving doorslaan (Figuur 3). Tijdens leverschade kan deze balans verstoord raken en wordt apoptotische celdood op een gegeven moment niet meer geremd. Dit kan leiden tot verlies van leverfuncties en uiteindelijk fataal zijn.



Figuur 3. De balans tussen dood en overleving is sterk gereguleerd in een cel. Tijdens acute en chronische leverschade slaat de balans door naar celdood, soms met fatale gevolgen. Een van de belangrijkste beschermingsmechanismen in hepatocyten is de activering van de factor NF-κB onder invloed van cytokinen, zoals TNF-α (Hoofdstuk 3 en 5). TNF-α speelt dus een tweevoudige rol: het activeert beschermende stoffen via signaleringroutes maar kan ook schadelijke stoffen activeren. Wanneer NF-κB niet geactiveerd kan worden zal de activering van schadelijke stoffen de overhand krijgen en tot celdood leiden.

Nieuwe behandelmethoden: gentherapie

Tijdens acute en chronische leverschade zijn er twee belangrijke gebeurtenissen die leiden tot leverfalen: de celdood van hepatocyten in de lever en de activering en proliferatie (celdeling) van stellaatcellen (Figuur 1). Beide gebeurtenissen zijn ongewenst indien ze overmatig optreden. Daarom is in patiënten het remmen van celdood in de hepatocyt therapeutisch, terwijl juist een stimulering van apoptotische celdood gewenst is in geactiveerde stellaatcellen. Met andere woorden, de doelcel voor het ontwikkelen van een anti-apoptotische therapie is de

hepatocyt. Daarentegen is de geactiveerde stellaatcel de doelcel voor het ontwikkelen van een pro-apoptotische therapie.

Om onderscheid te maken tussen beide celtypen en in te grijpen in het sterk gereguleerde systeem van celdood en celoverleving, is selectiviteit cruciaal. Gentherapie is hiervoor een veelbelovende benadering. Gentherapie betekent het inbrengen van een therapeutisch gen in een specifiek celtype door middel van een transportmiddel. Op deze manier kan het eiwitproduct, dat afkomstig is van het ingebrachte gen en in grote hoeveelheden wordt aangemaakt, therapeutisch zijn voor de cel. In hepatocyten zou het dan kunnen gaan om een beschermend eiwit dat celdood blokkeert, terwijl in geactiveerde stellaatcellen een beschermende route wordt geblokkeerd, zodat celdood optreedt. Een goed transportmiddel voor een therapeutisch gen is een virus. Het adenovirus bijvoorbeeld is een transportmiddel dat van nature graag naar de lever gaat. Het adenovirus bestaat uit verschillende subtypen die geassocieerd worden met verschillende ziekten in mensen. Daarom wordt voor gentherapie-studies een recombinant adenovirus gebruikt, wat wil zeggen dat het virus dusdanig is verbouwd dat het relatief onschuldig is geworden.

9.2 Doel van het onderzoek (Hoofdstuk 1)

Het onderzoek dat in dit proefschrift is beschreven had als doel om tijdens acute en chronische leverschade mechanismen in kaart te brengen die tot celdood leiden en die celdood remmen in hepatocyten. Hierbij zijn nieuwe strategieën onderzocht om hepatocyten onder deze omstandigheden te beschermen tegen apoptotische celdood. In tegenstelling tot het beschermen van hepatocyten tegen celdood, is het stimuleren van celdood in geactiveerde stellaatcellen een mogelijke therapeutische strategie. Om dat te bereiken hebben wij een transportmiddel ontwikkeld en getest dat selectief een gen in de geactiveerde stellaatcel kan afleveren.

9.3 Resultaten en conclusies

In **Hoofdstuk 3** is in vers geïsoleerde rat hepatocyten onderzocht hoe deze zich beschermen tegen apoptotische celdood wanneer ze blootstaan aan grote hoeveelheden cytokinen, zoals tijdens acute leverschade. Uit eerder onderzoek was al gebleken dat een bepaalde factor erg belangrijk is voor de bescherming van hepatocyten en geactiveerd wordt door cytokinen. Deze factor heet NF-κB en is betrokken bij de regulatie van het aflezen van een bepaalde groep genen (ook wel transcriptiefactor genoemd). Wanneer NF-κB niet geactiveerd kan worden en de cellen blootstaan aan cytokinen zoals TNF-α, gaan de cellen in apoptose als gevolg van TNF-α gereguleerde celdood (Figuur 3).

Onze studie bevestigt deze resultaten en toont aan dat NF-κB betrokken is bij de regulatie van anti-apoptotische genen. Deze genen behoren tot de Bcl-2 familie en tot de zogenaamde IAP familie. De Bcl-2 familie reguleert onder andere de buitenmembraan van de mitochondriën. De IAP familieleden remmen apoptose door cruciale enzymen in dit proces, de zogenaamde caspases, te remmen. Bcl-2 familieleden die door NF-κB gereguleerd worden in hepatocyten zijn anti-apoptotisch Bcl-XL en A1/Bfl. Daarnaast reguleert NF-κB ook pro-apoptotische genen die behoren tot de Bcl-2 familie, namelijk Bak en Bid. Van de IAP familie wordt alleen het gen dat de genetische code bevat voor cIAP2 gereguleerd door NF-κB. Omdat remming van NF-κB de balans doet verschuiven naar apoptose in hepatocyten, zijn anti-apoptotisch cIAP2 en A1/Bfl dominant ten opzichte van pro-apoptotisch Bak en Bid.

Samengevat: tijdens ontstekingscondities functioneert NF-κB als een anti-apoptotische factor door de regulatie van o.a. de genen cIAP2 en A1/Bfl. Wanneer de humane homologe variant van het cIAP2 gen (HIAP1) in hepatocyten wordt gebracht met behulp van recombinant adenovirus, wordt door de hepatocyten een grote hoeveelheid van het HIAP1 eiwit aangemaakt dat celdood in deze cellen remt.

We hebben ook een ander gen onderzocht dat door NF-κB wordt gereguleerd, namelijk inducible nitric oxide synthase (iNOS) en zijn product stikstof oxide (NO·) Hoewel wij en andere onderzoekers sterke aanwijzingen hebben dat iNOS beschermend werkt tegen apoptose, is dit nog niet volledig opgehelderd. Wel hebben we aangetoond dat wanneer we een NO·-donor aan hepatocyten geven, apoptose geremd wordt in aan cytokine blootgestelde hepatocyten. We hebben echter niet verder onderzocht of NO· en iNOS potentieel therapeutisch toepasbaar zijn voor de behandeling van acute leverschade, omdat met name NO· ook voor veel nadelige effecten kan zorgen in de cel.

Omdat de humane homologe variant van het cIAP2 gen (HIAP1) beschermend werkt in hepatocyten, hebben we in **Hoofdstuk 4** onderzocht of het HIAP1 gen beschermt tegen acute leverschade in een diermodel. Alvorens acuut leverfalen op te wekken in muizen, zijn deze muizen eerst behandeld met recombinant adenovirus waarin het HIAP1 gen aanwezig is. Na 3 dagen zal het eiwitproduct, dat afkomstig is van het HIAP1 gen, met name in de hepatocyten aanwezig zijn. Op dat tijdstip is er een schadelijk stofje ingespoten dat afkomstig is van bacteriën (LPS), samen met een transcriptieremmer (Galactosamine). Dit veroorzaakt specifiek in hepatocyten massale celdood gevolgd door acute leverschade. Uit onze studie blijkt dat de acute leverschade niet minder wordt wanneer we het HIAP1 gen hebben toegediend. In tegendeel, de leverschade en celdood van hepatocyten worden juist erger.

Samenvattend: HIAP1 is dus niet therapeutisch voor de behandeling van acuut leverfalen. Dit gaat tegen alle verwachtingen in. Daarom wordt in hoofdstuk 4 uitgebreid besproken wat de oorzaak hiervan kan zijn. Uitgesloten kan worden dat het aan het ingebrachte adenovirus ligt, want studies in een speciale muizensoort die een soortgelijk gen heeft, genaamd XIAP, en dit eiwitproduct ook in grote hoeveelheden aanmaakt (transgene muizen) leveren dezelfde resultaten op. Onze studies onderstrepen de complexiteit van wat een simpele therapeutische benadering lijkt. In een gecontroleerde situatie zoals in cellen (hepatocyten) is

HIAP1 beschermend (hoofdstuk 3), maar in een complexe situatie als acuut leverfalen, waarin veel meer interacties zijn, is het moeilijker om signaalroutes, en dus het lot van de cel, te beïnvloeden (hoofdstuk 4). Hoofdstuk 4 is een relevante studie voor de ontwikkeling van gentherapie die als doel heeft in te grijpen in cel signaleringroutes tijdens leverziekten. Daarom gaat Hoofdstuk 8 (algemene discussie en perspectieven) nog wat dieper in op deze materie.

Onderzoek naar chronische leverschade is beschreven in **Hoofdstuk 5**. Hieruit blijkt dat in levers van ratten met chronische leverschade weinig apoptotische celdood van hepatocyten optreedt. Dit gaat in tegen de gevestigde ideeën, maar wordt sinds kort ook gesteund door andere onderzoekers, die net als wij alleen necrotische celdood vinden. In ons diermodel, waarin de galgang wordt afgebonden (het zogenaamde galgang-ligatie model) hopen zich veel galzouten op in de hepatocyten. In hepatocyten die we geïsoleerd hebben uit een normale ratlever en kweken in het laboratorium, zorgt blootstelling aan galzouten voor apoptotische celdood. Omdat we geen apoptose van hepatocyten zien in een dier met chronische leverschade, zijn hepatocyten in deze situatie dus blijkbaar goed beschermd tegen apoptotische celdood. Wij hebben verder onderzocht hoe dat kan. We hebben uitgesloten dat het beschermende eiwit Bcl-2 (onderdeel van de Bcl-2 eiwitfamilie) hierbij betrokken is. Dit eiwit komt namelijk niet voor in hepatocyten, ook niet tijdens chronische leverschade.

Omdat NF-κB een belangrijke beschermde factor is in hepatocyten (Figuur 3), hebben we de rol van NF-κB onderzocht. Inderdaad wordt in hoofstuk 5 aangetoond dat NF-κB geactiveerd wordt tijdens chronische leverschade. Bovendien blijkt dat de genen cIAP2 en A1/Bfl-1 worden ‘geactiveerd’ door NF-κB onder invloed van cytokinen die tijdens chronische leverschade aanwezig zijn, zoals TNF-α.

In rat hepatocyten die in kweekschaaltjes worden gehouden, kan apoptotische celdood geactiveerd worden door het galzout glycochenodeoxycholaat (GCDCA). Wanneer we echter van tevoren deze cellen blootstellen aan cytokinen die NF-κB

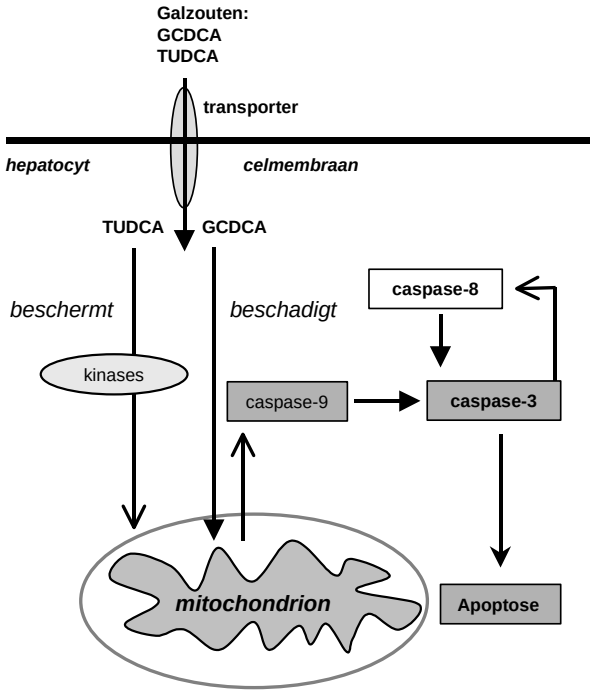
activeren, wordt apoptose geremd. Nog een aanwijzing dat NF-κB beschermend is. Bovendien hebben we laten zien dat adenovirale gentherapie met de humane homologe variant van het cIAP2 gen (HIAP1) beschermt tegen GCDCA geactiveerde apoptose.

Samenvattend: hepatocyten worden beschermd tegen galzout geactiveerde apoptose tijdens chronische leverschade, doordat ze in staat zijn om NF-κB te activeren onder invloed van een verhoogde concentratie van cytokinen. Deze resultaten hebben consequenties voor behandelmethoden die gericht zijn op het remmen van cytokinen van het afweersysteem tijdens chronische leverschade.

In **Hoofdstuk 5 en 6** hebben we onderzocht op wat voor manier galzouten apoptotische celdood in cultures van rat hepatocyten veroorzaken. Niet alle galzouten zijn direct schadelijk voor hepatocyten. De taurine geconjugeerde galzouten, tauro-chenodeoxycholaat (TCDCA) en tauro-ursodeoxycholaat (TUDCA), en het glycine geconjugeerde galzout, glyco-ursodeoxycholaat (GUDCA), veroorzaken geen apoptose. Daarentegen is glyco-chenodeoxycholaat wel schadelijk. In hoofdstuk 6 hebben we laten zien dat galzouten eerst getransporteerd moeten worden door de celmembraan om hun invloed te kunnen uitoefenen op de hepatocyt. We hebben gevonden dat GCDCA schade toebrengt aan de mitochondriën. Op deze manier initieert GCDCA apoptotische celdood (Figuur 4). Ook is gebleken dat de activering van caspases in eerste instantie niet via een receptor gaat, maar via de mitochondriën. Daarmee volgt de caspase-activering niet de klassieke volgorde, die vaak start bij caspase-8, maar via caspase-9, caspase-3 en dan caspase-8. Dit resulteert uiteindelijk in apoptotische celdood.

Patiënten die leiden aan chronische lever schade worden vaak behandeld met een galzout, namelijk ursodeoxycholaat (UDCA). Het mechanisme achter de beschermende rol van UDCA is nog niet volledig opgehelderd. Daarom hebben wij in **hoofdstuk 6** onderzocht hoe TUDCA beschermt tegen GCDCA geactiveerde celschade. Gebleken is in hoofdstuk 6 dat TUDCA beschermend werkt door de activering van beschermingsroutes. Hierbij is NF-κB niet betrokken, maar worden

er onder invloed van TUDCA direct enzymen (zogenaamde kinases) geactiveerd die op hun beurt weer andere beschermende eiwitten activeren. We hebben in hoofdstuk 6 laten zien dat deze kinases behoren tot de MAP kinase familie, namelijk p38 en ERK, en dat ze samen met PI3 kinase betrokken zijn bij de bescherming tegen GCDCA (Figuur 4). TUDCA werkt zeer snel. Het kan zelfs na het toevoegen van GCDCA worden toegediend aan hepatocyten om beschermend te werken. Ook laten andere onderzoekers zien dat de bescherming van TUDCA via directe effecten op de membraan van de mitochondriën kan plaatsvinden (Figuur 4).



Figuur 4. Overzicht van beschermende effecten van het galzout TUDCA tegen het apoptotische galzout GCDCA in rat hepatocyten. Opname van deze galzouten gebeurt via transport door de celmembraan. GCDCA zorgt er voor dat pro-apoptotische factoren vanuit de mitochondriën in de cel terechtkomen. Deze factoren initiëren apoptotische celdood via de activering van caspases. TUDCA beschermt de cel hiertegen via directe remming op de membraan van de mitochondriën of door activering van beschermingsroutes. In deze routes worden zogenaamde kinases geactiveerd die beschermende eiwitten activeren.

Samenvattend, GCDCA is een schadelijk galzout dat apoptotische celdood activeert in hepatocyten via de mitochondriën. Apoptose kan worden voorkomen

en geremd door het beschermende galzout TUDCA, dat werkt via kinase afhankelijke signaalroutes en via directe effecten op de mitochondriën.

In **Hoofdstuk 7** wordt beschreven hoe selectieve gentherapie kan worden uitgevoerd in de geactiveerde stellaatcel tijdens leverfibrose. Om leverfibrose terug te dringen is de geactiveerde stellaatcel een doelcel. Een mogelijke therapie is het activeren van apoptotische celdood in deze cellen. Daarom hebben wij een transportmiddel ontwikkeld dat selectief een gen in de geactiveerde stellaat cellen kan brengen dat bijvoorbeeld een beschermingsmechanisme van de cel doorbreekt.

Wij hebben gebruik gemaakt van een receptor die verhoogd aanwezig is op de celmembraan van de geactiveerde stellaatcel tijdens leverfibrose. Deze receptor is de PDGF-receptor waaraan normaal gesproken de stof PDGF bindt. Door nu het bindende gedeelte van PDGF te koppelen aan een stofje dat bindt aan de buitenkant van een recombinant adenovirus, ontstaat een nieuw fusie-eiwit. In Figuur 1b van hoofdstuk 7 wordt het concept weergegeven. Dit fusie-eiwit zorgt ervoor dat het recombinante adenovirus selectief bindt aan de PDGF-receptor. Op deze manier wordt het adenovirus, met een eventueel geassocieerd therapeutisch gen, specifiek in de geactiveerde stellaatcel geloodst door binding aan de PDGF-receptor. Het fusie-eiwit is gemaakt door de genetische informatie van het fusie-eiwit aan elkaar te zetten en dit DNA in speciale bacteriën te brengen. Deze bacteriën maken dan het fusie-eiwit, waarna wij het kunnen zuiveren en testen.

Uit onze studie blijkt dat modelcellen voor de geactiveerde stellaat cel, de zogenaamde 3T3 fibroblasten, het normale virus nauwelijks opnemen, maar het door ons aangepaste virus wel. Dit wordt gemeten door de activiteit van het eiwitproduct te meten dat afkomstig is van het gen dat in het virus aanwezig is. Na opname van het virus door de cel wordt dit gen ‘afgeschreven’ en het corresponderende eiwit aangemaakt door de cel. Verder is gebleken in hoofdstuk 7 dat de geactiveerde stellaatcellen ook een verhoogde opname laten zien van het door ons aangepaste virus. Daarentegen nemen de hepatocyten het door ons aangepaste virus niet op.

Samenvattend: het door ons aangepaste adenovirus wordt selectief via de PDGF-receptor door de geactiveerde stellaatcellen opgenomen en omzeilt opname door de hepatocyten. Nu moet worden onderzocht hoe het virus zich gedraagt in een diermodel met leverfibrose.

9.4 Perspectieven

Het begrijpen van de mechanismen die celdood van levercellen reguleren is van wetenschappelijk, maar ook van klinisch belang. Met deze kennis kunnen nieuwe therapieën worden ontwikkeld. Dit proefschrift beschrijft de beschermende mechanismen van hepatocyten tegen celdood, tijdens acute en chronische leverschade. De studies beschreven in dit proefschrift hebben geleid tot het identificeren van nieuwe interventie-strategieën om apoptotische celdood van hepatocyten te voorkomen. Daarnaast hebben wij een transportmiddel voor genen ontwikkeld dat selectief door de geactiveerde stellaatcellen wordt opgenomen. Hiermee kan apoptotische celdood worden geactiveerd in deze cellen, wat een relevante therapeutische strategie kan zijn tijdens chronische leverziekten.

Dankwoord

(Acknowledgements)

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Januari 2004,

Curriculum Vitae

List of publications

Marieke (Henriëtte) Schoemaker werd op 2 maart 1976 geboren te Groningen, waarna ze tot haar 18e jaar in Bedum woonde. In 1994 behaalde zij het VWO diploma aan het Wessel Gansfort College te Groningen. In datzelfde jaar startte zij met de studie Biologie aan de Rijksuniversiteit Groningen. Nadat zij het propedeutisch examen in 1995 met goed gevolg had afgelegd, begon zij met de specialisatie Medische Biologie. Hiervoor verrichte ze haar eerste afstudeeronderzoek bij de vakgroep Moleculaire Virologie van Prof. Dr. J.C. Wilschut. Onder leiding van Dr. C.A.H.H. Daemen onderzocht zij de rol van dendritische cellen en de toepassing hiervan in een immuuntherapie. Vervolgens deed zij een tweede onderzoeksproject bij de groep Medische Biologie (Prof. Dr. L.F.M.H. de Leij) op het Academisch Ziekenhuis in Groningen. Hier optimaliseerde zij in het kader van de ontwikkeling van een kankertherapie, de productie van MAGE-3-positieve retrovirussen. Dit onderzoek werd verricht onder begeleiding van Dr. J. van Zanten. In augustus 1999 behaalde zij het doctoraal diploma. Diezelfde maand startte zij als assistent in opleiding (AIO) bij het Centrum voor Lever- Darm en Stofwisselingsziekten van het onderzoeksinstituut GUIDE, verbonden aan de Rijksuniversiteit Groningen. In het project werd een aantal studies gefinancierd door de Maag Lever Darm stichting (projectnummer WS99-28). Als AIO deed zij onderzoek naar mechanismen van celdood en celoverleving tijdens acute en chronische leverschade. In dit kader zocht zij naar aangrijpingspunten voor nieuwe mogelijke therapieën. Het onderzoek werd uitgevoerd in het laboratorium van de afdeling Maag-, Darm en Leverziekten (MDL) onder begeleiding van Prof. Dr. P.L.M. Jansen en Dr. H. Moshage. Daarnaast werkte zij aan dit project op de laboratoria van de afdelingen Therapeutische Gen Modulatie (TGM) en Farmakokinetiek & Drug Delivery onder begeleiding van respectievelijk Prof. Dr. H.J. Haisma en Prof. Dr. K. Poelstra. De resultaten van het onderzoek staan beschreven in dit proefschrift getiteld “Apoptotic cell death as a target for the treatment of acute and chronic liver injury”.

Publications

Schoemaker MH, Ros JE, Homan M, Klok P, Trautwein C, Liston P, Poelstra K, van Goor H, Jansen PLM and Moshage H. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-κB-regulated inhibitor of apoptosis protein2 (cIAP2) prevents apoptosis. *(2002) Journal of Hepatology 36: 742-750*

Schoemaker MH and Moshage H. Die another Day. Beyond the journal. *(2003) Journal of Hepatology 38: 873-875*

Schoemaker MH, Gommans WM, Conde de la Rosa L, Homan M, Klok P, Trautwein C, van Goor H, Poelstra K, Haisma HJ, Jansen PLM and Moshage H. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation. *(2003) Journal of Hepatology 39: 153-161*

Schoemaker MH, Homan M, Liston P, Klok P, van Goor H, Poelstra K, Jansen PLM, Haisma HJ, and Moshage H. Adenoviral overexpression of caspase inhibitor HIAP1 is not protective in a mouse model of severe acute liver failure. *In preparation*

Schoemaker MH, Conde de la Rosa L, Homan M, Vrenken TA, Havinga R, Poelstra K, Haisma HJ, Jansen PLM and Moshage H. TUDCA protects hepatocytes from bile acid-induced apoptosis via activation of survival pathways. *Hepatology, in press*

Schoemaker MH, Rots MG, Beljaars L, Ypma AY, Jansen PLM, Poelstra K, Moshage H and Haisma HJ. Specific and enhanced gene transfer: redirection of PDGFβ-receptor retargeted adenovirus from hepatocytes to activated stellate cells. *Submitted*

Schoemaker MH and Moshage H. Defying death: The Hepatocyte's Survival Kit. Review *submitted*

Abstracts

Svegliati-Baroni G, Ridolfi F, Schoemaker MH, Saccomanno S, Buist-Homan M, Jansen PLM, Benedetti A, Moshage H. Bile acids induce survival signals in hepatic stellate cells through a PKC- and ERK-dependent mechanism. *(2002) Hepatology 36(4) pt 2; pp 316A; abstract 611*

Rots MG, Elferink MGL, Gommans WM, Schoemaker MH, Olinga P, Haisma HJ and Groothuis GMM. Screening of novel adenoviral vectors for specificity of gene transfer using the precision cut slice technology. *(2003) Molecular Therapy 7:14 abstract*

Conde de la Rosa L, Schoemaker MH, Buist-Homan M, Jansen PLM, and Moshage H. Reactive oxygen species induce hepatocyte cell death via different mechanisms; a balance between apoptosis and necrosis. *(2003) Hepatology 38(4) pt 2; pp 577A; abstract 866*

International presentations

Survival of hepatocytes in acute hepatitis: a balance between protective and apoptotic pathways. American Association for the Study of Liver Diseases (AASLD) 51st Annual meeting (2000) Dallas, USA. *Hepatology 32 (4) pt 2; pp 336A; abstract 705*

Changes in the expression of apoptosis related genes in rat liver fibrosis. AASLD Single Topic Conference: Mechanisms and therapy of hepatic fibrosis (2000). Warrenton, Virginia, USA.

Survival of hepatocytes in acute hepatitis: a balance between protective and apoptotic pathways. Falk symposium No 125; Cytokines in liver injury and repair (2001) Hannover, Germany.

Hepatocytes are protected against apoptosis in biliary fibrosis by increased expression of Bcl-2 family members. EASL Single Topic Conference; Liver fibrosis: from cell biology to clinical targets. (2001) Florence, Italy.

Increased resistance of rat hepatocytes against apoptosis in biliary fibrosis is due to cytokine- and bile acid-induced expression of anti-apoptotic genes. AASLD 52nd Annual meeting (2001) Dallas, USA. *Hepatology 34(4) pt 2; pp 274A; abstract 407*

Anti-apoptotic mechanisms in chronic liver diseases. Cancer Research Institute, Children's Hospital Ottawa (2002) Ottawa, Canada. *Oral presentation*

The protective mechanism of tauroursodeoxycholic acid against bile acid-induced apoptosis in hepatocytes. AASLD 53rd Annual meeting (2002) Boston, USA. *Hepatology 36(4) pt 2; pp 321A; abstract 631.*

Protective mechanisms of TUDCA against bile acid-induced apoptosis in primary rat hepatocytes. Apoptosis meeting; From signalling pathways to therapeutic tools (2003) Luxembourg, Luxembourg.

Specific and enhanced gene transfer: redirection of PDGF-receptor retargeted adenovirus from hepatocytes to activated stellate cells. American Society for Gene Therapy (ASGT) (2003) Washington, USA. *Molecular Therapy 7:132*

Adenoviral gene targeting for the treatment of chronic liver injury. AASLD 54th Annual meeting (2003) Boston, USA. *Hepatology 38 (4) pt 2; pp 224A; abstract 143. Oral presentation*

List of abbreviations

ActD	actinomycin-D
AdHIAP1	adenovirus encoding for HIAP1
Ad5IkBAA	adenovirus encoding for dominant-negative IκB-α
Ad5LacZ	adenovirus encoding for β-galactosidase
ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
Bcl-2 family	pro-apoptotic members, like: Bax, Bak, Bid; anti-apoptotic members, like:Bcl-2,Bcl-XL, A1/Bfl-1
caspase	cysteine aspartyl protease, like: caspase-3
CAR	coxsackie virus and adenovirus receptor
CM	cytokine mixture
EMSA	electrophoretic mobility shift assay
FADD	Fas-associated protein with death domain
GalN	D-galactosamine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCDCA	bile acid: glyco-chenodeoxycholic acid
GUDCA	bile acid: glyco-ursodeoxycholic acid
HIAP1	human homologue of cIAP2
IAP family	Inhibitor of Apoptosis Protein family
IFNγ	cytokine: interferon-γ
IκB	Inhibitory protein of NF-κB, like: IκB-α
IL-1β	cytokine: interleukin-1β
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase, like: ERK1/2, p38
NF-κB	transcription factor: nuclear factor-kappa B
$NO^{\bullet}$	nitric oxide
PDGF	platelet derived growth factor
PDGF-Rβ	platelet derived growth factor-receptor
PI3-kinase	phosphatidylinositol-3 kinase
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gelectrophoresis
TCDCA	bile acid: tauro-chenodeoxycholic acid
TNF-α	cytokine: tumor necrosis factor α
TNFR-1	TNF-α type 1 receptor
TRADD	TNFR-associated death domain protein
TRAF2	TNFR-associated factor 2
TUDCA	bile acid: tauro-ursodeoxycholic acid
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling
UDCA	bile acid: ursodeoxycholic acid
XIAP	X-chromosome-linked IAP

Appendix

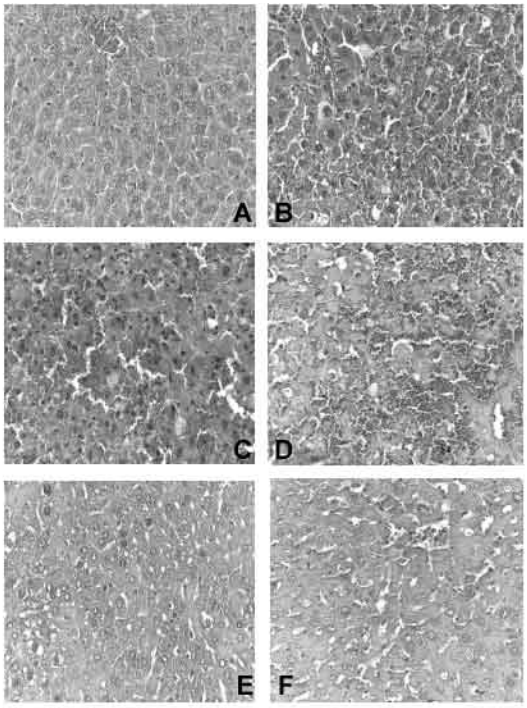
Chapter 4

Figure 3. Liver histology and morphology displayed by hematoxylin-eosin staining. (A) Normal mouse liver. (B) Liver exposed to LPS/GalN. (C) Mouse liver pre-treated with 5 x 10^9 pfu of Ad5LacZ followed by the onset of acute liver injury using LPS/GalN. (D) Mouse liver pre-treated with 5 x 10^9 pfu of AdHIAP1 followed by induction of acute liver injury using LPS/GalN. (E) Mouse liver pre-treated with 5 x 10^9 pfu of AdHIAP1. (F) Mouse liver exposed to 10 mg/kg of LPS (original magnification 400x). Representative data of 5 to 10 animals per group are shown.

Chapter 5

Figure 2 B. Limited apoptosis in rat livers beyond 1 week of bile duct ligation (BDL). (B) Active caspase-3 staining. SHAM animals served as control. D-Gal/LPS treated livers served as positive control.

Chapter 4:
figure 3



Chapter 5:
figure 2b