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GUIDELINES FOR BASIC SCIENCE

Novel insight into mechanisms of cholestatic liver injury

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Abstract

Cholestasis results in a buildup of bile acids in serum and in hepatocytes. Early studies into the mechanisms of cholestatic liver injury strongly implicated bile acid-induced apoptosis as the major cause of hepatocellular injury. Recent work has focused both on the role of bile acids in cell signaling as well as the role of sterile inflammation in the pathophysiology. Advances in modern analytical methodology have allowed for more accurate measuring of bile acid concentrations in serum, liver, and bile to very low levels of detection. Interestingly, toxic bile acid levels are seemingly far lower than previously hypothesized. The initial hypothesis has been based largely upon the exposure of μmol/L concentrations of toxic bile acids and bile salts to primary hepatocytes in cell culture, the possibility that *in vivo* bile acid concentrations may be far lower than the observed *in vitro* toxicity has far reaching implications in the mechanism of injury. This review will focus on both how different bile acids and different bile acid concentrations can affect hepatocytes during cholestasis, and additionally provide insight into how these data support recent hypotheses that cholestatic liver injury may not occur through direct bile acid-induced apoptosis, but may involve largely inflammatory cell-mediated liver cell necrosis.

Key words: Bile acids; Cholestasis; Apoptosis; Necrosis; Neutrophils; Innate immunity; Bile duct ligation

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CHOLESTATIC LIVER INJURY - CLINICAL RELEVANCE

A reduction in bile flow (cholestasis) can result from gall stones, impingement from a local tumor, intrahepatic cholestasis of pregnancy, genetic deficiency in bile export proteins or in autoimmune disorders amongst other etiologies[1,2]. Cholestasis results in a dramatic increase in liver and serum bile acid levels that eventually lead to acute liver toxicity, proliferation of bile ducts, and fibrosis progressing to cirrhosis[3]. Neonatal disorders such as progressive familial intrahepatic cholestasis and biliary atresia are particularly difficult to treat effectively[4]. While surgical means can prevent disease progression in the case of gall stones or biliary atresia, there are a limited number of treatment options pharmacologically for diseases such as primary sclerosing cholangitis or primary biliary cirrhosis[5]. Ursodiol, a pharmaceutical form of ursodeoxycholic acid, is the current mainline defense against cholestasis, although its efficacy is limited in

some circumstances[5,6].

While many of the etiologies of disease initiation are well described, the molecular mechanisms behind the early liver injury associated with cholestasis are extensively studied, but not well understood. A major hypothesis was based on the assumption that the buildup of bile acids in hepatocytes and blood results in hepatocellular apoptosis dependent upon bile acid concentrations[6,7]. While these studies on the direct toxicity of bile acids have been supported by many *in vitro* studies from multiple groups, recent methodology measuring the concentrations of individual bile acids after the onset of cholestasis *in vivo* has cast some doubt that bile acid levels may actually reach the levels necessary for cell toxicity[8-10]. A growing volume of work has instead focused on how sterile inflammation and innate immunity may result in the initial injury[11-15]. This review will discuss current and developing paradigms in the pathophysiology of early cholestatic liver injury, and provide insight into how recent advances in methodology may affect these theories.

DIRECT BILE ACID TOXICITY AS A MODEL FOR CHOLESTATIC LIVER INJURY

High concentrations of bile acids given to cultured hepatocytes *in vitro*[16] and bile acid-induced cholestasis[17], or bile duct ligation *in vivo*[18], have long been established as models of toxicity to hepatocytes. After the discovery that ursodeoxycholic acid could be used pharmacologically as a choleretic agent with protective effects against cholestasis[19], a renewed interest in the subject emerged. While previous papers had focused on the detergent properties of amphipathic bile acids[20,21] recent research resulted in a series of papers illustrating a detailed mechanism of how toxic bile acids or the respective bile salt, could induce apoptosis in primary human and rat hepatocytes[22-27]. Much of this work has spearheaded the current revival of direct bile acid toxicity as a model of cholestasis, or liver injury, *in vitro* and *in vivo*.

Apoptosis and intracellular signaling mechanisms in bile acid-induced toxicity

The most commonly studied bile acid for its toxicity to hepatocytes is glycochenodeoxycholic acid (GCDCA), or its bile salt glycochenodeoxycholate, due to it being a major constituent of human serum and bile after cholestasis[28] and its relatively higher level of toxicity when compared to other bile acids[29]. Galle *et al*[22] first established GCDCA as toxic to primary human hepatocytes and in the same paper also established ursodeoxycholate (UDCA) as protective against the GCDCA-induced injury. Due to the limited availability of primary human hepatocytes, much of the subsequent research into mechanism of injury occurred in rodent, especially rat hepatocytes. GCDCA-induced hepatocellular death was first established as apoptotic using electron microscopy[23]. Incubation of primary rat hepatocytes with 50 μmol/L GCDCA induced DNA fragmentation, chromatin margination and condensation, cellular shrinkage and blebbing of the membrane with intact organelles enclosed, all of these classical hallmark characteristics of apoptosis[23]. While it has been extensively shown that DNA fragmentation is common to both necrosis and apoptosis[30-33], the morphological changes present after GCDCA exposure provided strong evidence of bile acid-induced apoptosis of primary hepatocytes. In addition, caspase activation is a feature of GCDCA-induced apoptosis[27,34-36] and caspase inhibitors were shown to protect[27,34,36].

Investigation into intracellular mechanisms of bile acid toxicity showed cellular apoptosis was associated with mitochondrial instability and mitochondrial membrane permeability transition pore (MPTP) formation that was preventable by either UDCA, ketohexoses such as fructose, or cyclosporine A and trifluoroperazine[36-39]. As mitochondria are essential for ATP production and GCDCA depleted ATP levels[29], the idea that UDCA, or other protective interventions, were sustaining ATP levels was tested; however, protection was not due to reduced ATP depletion, but by blockage of the MPTP[37]. This suggested that UDCA may prevent injury during cholestasis not only by enhancing bile flow, but by preventing initiation of the MPTP[37], a facet which has been confirmed in other models[40-42]. Mitochondrial reactive oxygen species (ROS) production and MPTP initiation was later also shown in human hepatocytes exposed to toxic bile acids such as GCDCA indicating the mitochondrial dysfunctions occur in more than just rodent species[43]. Adult rats fed a diet supplemented with deoxycholic acid (DCA) for 10 d also showed significant mitochondrial swelling, peroxynitrite formation and ROS production[44]. These data suggest that exposure of cultured hepatocytes to high levels of hydrophobic bile acids causes formation of ROS, reactive nitrogen species, and initiation of the MPTP, which is directly involved in the injury. In immortalized cell lines transfected with the basolateral transporter NTCP, bile acids like GCDCA could directly stimulate both Fas-dependent apoptosis *via* activation of Fas receptor[26] and Fas-independent apoptosis *via* previously established mitochondrial damaging mechanisms[41,42]. These experiments indicate that uptake of bile acids can cause both intrinsic and extrinsic apoptosis, and that cell death could be ameliorated by protecting against MPTP formation. These observations were critical in establishing the role of intracellular signaling and mitochondrial stress in the pathophysiology of bile acid-induced toxicity, as previous hypotheses revolved largely around the idea of inherent bile acid toxicity.

Although GCDCA and its bile salt have been extensively studied, other toxic bile acids have also effects on intracellular signaling mechanisms. Taurolithocholic acid (TLCA), taurochenodeoxycholic acid, and DCA increase intracellular ROS species in hepatocytes[45,46] and toxicity from these bile acids is responsive to antioxidants such as N-acetylcysteine and α-tocopherol[45]. DCA has also been shown to induce apoptosis and mitochondrial

instability in adult rats and primary hepatocytes at high concentrations[44,47,48]. Interestingly the taurine conjugate of UDCA, TUDCA, is protective against bile acid-induced apoptosis through activation of mitogen-activated protein kinase survival pathway signaling including p38 and extracellular signal-regulated kinase[55]. While GCDCA may be one of the most toxic and perhaps the most interesting bile acid scientifically and mechanistically, the total bile acid milieu probably contributes to the overall effect more than a single bile acid, with anti-apoptotic bile acids such as TUDCA and UDCA counteracting some of the effects of pro-apoptotic bile acids such as GCDCA.

Bile acids are also known to target intracellular nuclear receptors, especially the liver X receptor (LXR) family such as farnesoid X receptor (FXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR) and more[49]. The first LXRs identified as bile acid receptors were FXR[50,51], LXR[52], and later PXR[53]. FXR has been established as a key regulator in bile acid synthesis and transport[54]. Agonism of FXR with the synthetic drug GW4064 is hepatoprotective against both intra- and extrahepatic cholestasis[55] and agonism of PXR is protective against certain bile acid-induced cholestatic syndromes[56]. Nevertheless, the FXR knockout mouse were also protected against bile duct ligation-induced injury[57,58], which was found to be *via* increases in phase I detoxifying reactions and renal excretion of bile acids[59]. This suggests that both agonism and antagonism may be beneficial, depending on the type of cholestasis and the individual response to alterations in master regulators such as FXR. There is evidence for reduced bile acid load by either a reduction in synthesis in the case of agonism or enhanced excretion in antagonism of FXR. In cases of agonism a reduced bile acid load also correlated with a reduction in the hepatic inflammatory response in both steatotic and non-steatotic models[55,60] and loss of FXR has also protected against inflammatory injury in other organs[61]. These data indicate that increasing bile acid levels may have a strong signaling effect through FXR, PXR and more in hepatocytes that results in decreased bile acid synthesis, increased bile acid excretion and recruitment of inflammatory cells to the area. Early growth response factor-1 (Egr-1) has been clearly established as a master regulator in the sterile inflammatory response that hepatocytes express after exposure to high levels of bile acids[62]. Future research investigating interplay between FXR and Egr-1 in this signaling axis may be fruitful.

Bile acid concentrations: The in vivo to in vitro shift and back again

While it seems undeniable that bile acids have the capacity to elicit multiple different signaling cascades ranging from mitochondrial dysfunction and cell death to bile acid synthesis and glucose homeostasis[49,63], there remains some debate over the models used to dissect these actions and their physiological relevancy. In particular, there appears to be a discrepancy between the concentration of bile acids used *in vitro* to cause toxicity, and the levels of bile acids occurring during cholestasis both in rodents and in man[8,9].

Table 1 Human plasma bile acid concentrations in healthy volunteers and patients with extrahepatic cholestasis

BA	Healthy volunteers (nmol/L)	Cholestatic patients (nmol/L)
CDCA	257 ± 56	84 ± 60
TCDCA	120 ± 22	16 578 ± 4370
GCDCA	776 ± 112	28 535 ± 5734
CA	182 ± 83	173 ± 137
TCA	180 ± 47	54 487 ± 13 419
GCA	233 ± 56	56 222 ± 15 714
UDCA	138 ± 25	20 ± 11
TUDCA	5 ± 1	75 ± 22
LCA	13 ± 2	2 ± 1
TLCA	23 ± 4	12 ± 2
GLCA	16 ± 4	11 ± 2
LCA-S	7 ± 1	4 ± 2
DCA	387 ± 66	21 ± 16
TDCA	45 ± 12	213 ± 71
GDCA	246 ± 43	418 ± 91

BA: Bile acid; CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GDCA: Glycodeoxycholic acid; LCA: Lithocholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid; LCA-S: Lithocholic acid sulfate; GLCA: Glycolithocholic acid; TDCA: Taurodeoxycholic acid. Data adapted from Trottier *et al*[9].

Table 1 illustrates the increases in serum bile acids found in sex and age matched patients after multiple forms of cholestasis in man[9]. Full details on the patients are provided by Trottier *et al*[9] and Lai *et al*[64]. In brief, bile acid concentrations were measured in human serum from healthy volunteers and from patients with extrahepatic cholestasis[9]. Seventeen patients (8 men and 9 women) were recruited to the study. Clinical diagnoses of the patients included 8 common bile duct stones, 5 pancreatic tumors, 1 bile duct tumor, 1 benign common bile duct stenosis and 1 chronic pancreatitis. Age and sex matched (20 male, 20 female) non-cholestatic volunteers were used as controls[9], selected from the Genetics of Lipid Lowering Drugs and Diet Network study[64].

Previous work suggests serum bile acid levels are highly similar to total liver levels when comparing bile acid concentrations *in vivo*[8]. The bile acids that show the greatest increase in concentration in the serum are not toxic bile acids, but largely non-toxic bile acids such as TCA and GCA in man[9] or TCA, α- and β-muricholic acid (MCA) and TMCA in mice[8]. Levels of the primary toxic bile acid in humans, GCDCA, rise substantially, to almost 30 μmol/L under severe cholestatic conditions as measured by serum levels in man[9]. However, studies with primary human hepatocytes have shown little to no increase in toxicity until levels greater than 100 μmol/L for 24 h with significant toxicity beginning around 500 μmol/L[22,65]. The only paper to demonstrate toxicity at a level close to 30 μmol/L reported that GCDCA showed morphological characteristics in primary human hepatocytes that resembled

Table 2 Murine plasma bile acid concentrations after bile duct ligation

BA	Sham control (μmol/L)	BDL 6 h (μmol/L)
TMCA	0.229 ± 0.029	1.003 ± 133
TCA	0.122 ± 0.027	1.072 ± 137
TCDCA	0.029 ± 0.003	7 ± 0.557
GCA	0.007 ± 0.0002	1 ± 0.118
GCDCA	0.003 ± 0.003	0 ± 0
αMCA	0.697 ± 0.273	87 ± 41
βMCA	0.175 ± 0.23	253 ± 100
CA	0.219 ± 0.059	17 ± 10
CDCA	0.0001 ± 0.0001	0.144 ± 0.085
TUDCA	0.031 ± 0.002	16 ± 14
TDCA	0.016 ± 0.004	0.330 ± 0.107
TLCA	0.007 ± 0.002	0.027 ± 0.002
UDCA	0.131 ± 0.002	0.492 ± 0.215
DCA	0.042 ± 0.018	0.109 ± 0.032
LCA	0.054 ± 0.013	0.033 ± 0.022

BA: Bile acid; CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GDCA: Glycodeoxycholic acid; LCA: Lithocholic acid; TCA: Taurocholic acid; TCDCA: Taurochenodeoxycholic acid; TLCA: Taurolithocholic acid; TUDCA: Tauroursodeoxycholic acid; UDCA: Ursodeoxycholic acid; LCA-S: Lithocholic acid sulfate; GLCA: Glycolithocholic acid; TDCA: Taurodeoxycholic acid; BDL: Bile duct ligation. Data adapted from Zhang *et al*[8].

apoptosis and a 60% increase in DNA fragmentation at 50 μmol/L[66]. However, a significant increase in serum aspartate aminotransferase activities over control levels was only observed at 100 μmol/L GCDCA[66]. These data suggest that direct bile acid toxicity may not be the main mechanism of cholestatic liver injury in humans and seem to be supported by findings in mouse models of obstructive cholestasis[8,15]. Table 2 shows plasma bile acid concentrations in sham-operated controls or after 6 h of bile duct ligation (BDL)[8]. This is a time point corresponding with either maximal or near maximal increases in bile acid concentrations. Data are expressed as mean ± SE of $n = 6$ per group. For toxic bile acids such as GCDCA or LCA, the levels required to initiate toxicity in murine hepatocyte cultures are up to 3000-fold higher than the corresponding bile acid levels *in vivo*[8]. In the case of GCDCA, the most commonly used bile acid for *in vitro* toxicity, *in vivo* levels never exceed about 24 nmol/L throughout the first 3 d of injury[8]. This compares to 50 μmol/L GCDCA that is required to cause toxicity in cultured rodent hepatocytes *in vitro*[23,26]. In addition, during cholestasis *in vivo*, hepatocytes are exposed to a mixture of bile acids, some of which have actually an anti-apoptotic effect by causing the upregulation of inhibitor of apoptosis proteins, which may serve to counterbalance the apoptotic effect of specific bile acids[67,68].

While it is known that cultured hepatocytes lose expression of bile acid transporters such as NTCP and the OATPs over time in culture, cells freshly taken from the liver express nearly the same amount of transporter as whole liver tissue[69], and nearly the same level at 3 h after plating[70], when many of these experiments begin[23,71,72]. Even given a 30% decrease in mRNA levels of transporters such as NTCP 3 h after plating, cells maintain over 50% of their NTCP function up to 24 h after plating as measured by kinetic parameters[70]. Thus, it stands to reason that these hepatocytes are concentrating bile acids *in vitro* at levels within a 2 fold difference of the *in vivo* physiology, yet it takes multiple orders of magnitude, up to a 3000 fold difference in the case of GCDCA, to account for the difference between bile acid levels used on rodent hepatocytes *in vitro* and levels observed in murine samples *in vivo*. Still, even given that serum or liver levels may underestimate the true concentrations of bile acids hepatocytes are exposed to after BDL when bile leaks back into the parenchyma[73,74], the principle bile acid composition in bile is similar to serum, i.e. the non-toxic bile acids predominate, thus it is unlikely that cells are exposed to micromolar concentrations of the *toxic* bile acids *in vivo*[75].

INFLAMMATORY LIVER INJURY AFTER BDL - A NOVEL HYPOTHESIS

If bile acids are not directly responsible for cholestatic liver injury *in vivo,* then what is the mechanism of injury, and what role do bile acids play in the pathophysiology and progression of the injury? Accumulation of neutrophils, largely around sites of cell injury, within 24 h after bile duct ligation has been reported numerous times[11,12,76,77]. In addition, an increase in macrophage accumulation is observed approximately one week post ligation[77,78]. Figure 1 shows typical histology and neutrophil extravasation after bile duct ligation in mice. H&E staining shows multiple areas of hepatic focal necrosis throughout the liver. Neutrophils are present around the site of injury as early as six hours post BDL, and peak around 48-72 h after BDL, which is also the peak of liver injury[8,11,77].

Neutrophil accumulation has been directly associated with the injury, as deficiency of adhesion molecules such as ICAM-1 or CD18 reduced the number of neutrophils that extravasate into liver parenchyma, and drastically reduced liver injury[11,12]. A neutrophil is a particularly toxic leukocyte due to its capacity to produce superoxide *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the potent oxidant hypochlorous acid *via* myeloperoxidase[79]. Neutrophil accumulation after bile duct ligation has been associated with an increase in myeloperoxidase activity[80] and intracellular chlorotyrosine adduct formation in hepatocytes[11,12,81]. This indicates that neutrophil-derived hypochlorous acid can cause an intracellular oxidant stress in hepatocytes, which is responsible for cell injury[82,83]. Inhibition of NADPH oxidase reduces neutrophil-mediated oxidant stress and protects against neutrophil cytotoxicity[84,85]. In addition, hepatic NADPH oxidases located in hepatic stellate cells (Nox1) and Kupffer cells (Nox2) contribute to BDL-induced fibrosis[86,87] but their contribution to the initial liver injury (bile infarcts) has not been determined.

Together these data strongly implicate neutrophils

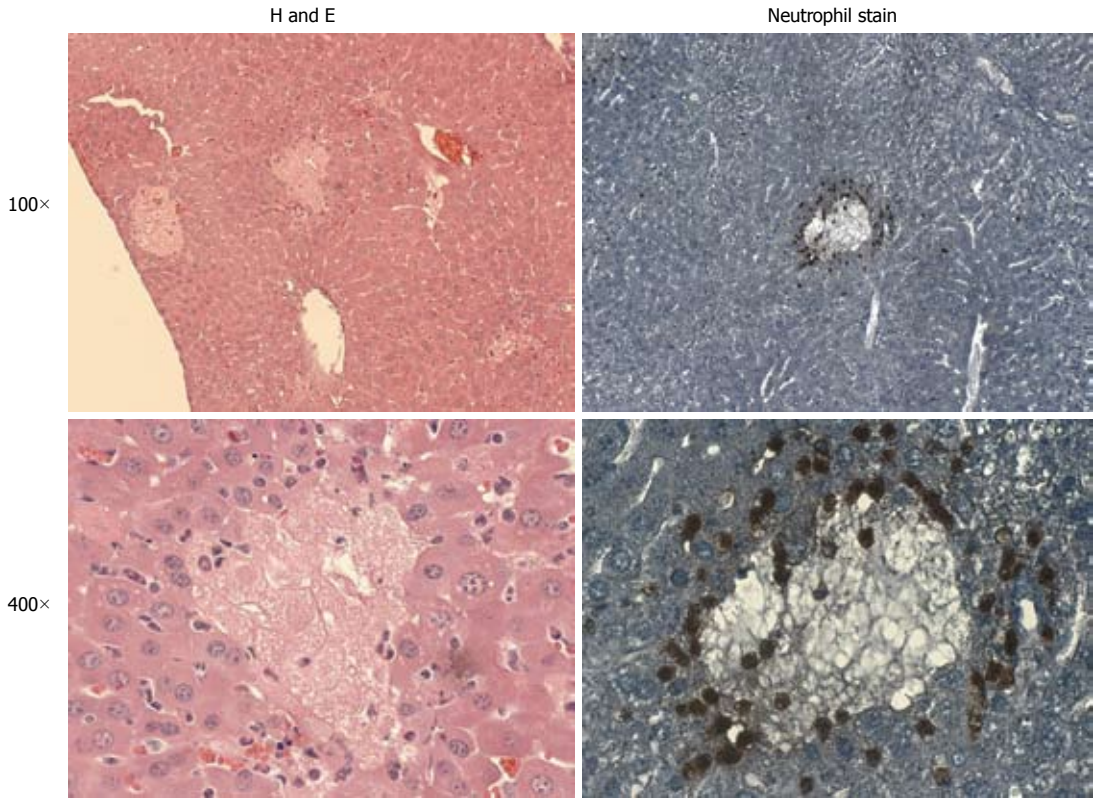

Figure 1 Sample liver sections from C57Bl/6 mice illustrating neutrophil accumulation after bile duct ligation. C57BL/6 mice were subjected to bile duct ligation and sacrificed 24 h later. Hematoxylin and eosin (H and E) stained sections showing focal necrosis at 100× and 400× magnification. Sections stained with an anti-neutrophil antibody. Neutrophils are present largely around areas of focal necrosis in this model.

in the pathogenesis of early cholestatic liver injury. In support of this hypothesis is recent data that exposure of cultured hepatocytes to both toxic and non-toxic bile acids at levels that recapitulate the *in vivo* concentrations result in dramatic increases in chemotactic factors such as MIP-2 and mKC, and adhesion molecules such as ICAM-1, all factors that can be involved in hepatic neutrophil recruitment[15]. The inflammatory mediator induction is dependent on the activation of the transcription factor Egr-1 in bile duct-ligated mice and in bile acid-exposed hepatocytes[15,62]. Egr-1 expression is also increased in human patients with cholestatic liver disease and the hepatic Egr-1 levels correlate with interleukin-8 and ICAM-1 induction[15]. Therefore, we initially hypothesized that after BDL, bile leaks back into the parenchyma after rupture of bile ducts and hepatocytes are locally exposed to high concentrations of pro-inflammatory bile acids such as TCA, β-MC and TMC that initiate an inflammatory response[8,13,15]. Additionally these hepatocytes are exposed to low concentrations of cytotoxic bile acids such as DCA, TLCA, and GCDCA that may initiate a low level of mitochondrial dysfunction that stresses the hepatocytes, but is insufficient to directly kill the cells[8,29,37,38,41-43]. This bile acid-induced stress triggers the expression of ICAM-1, MIP-2, and mKC in hepatocytes[8,15]. The subsequent release of these CXC chemokines can activate neutrophils in circulation[88] and provide a chemotactic gradient for the extravasation of neutrophils, which then attack the stressed hepatocytes and cause cell death through reactive oxygen formation[11,12,81,82]. Given a dramatic enough increase in toxic bile acid concentrations by feeding e.g. a lithocholic acid-containing diet[89], it is possible to directly cause cell death *via* bile acid toxicity but independent of neutrophils (Woolbright and Jaeschke unpublished observation). However the *in vivo* bile acid concentrations in models such as BDL are insufficient to meet this threshold, and thus the neutrophil-mediated cell death is dominant.

APOPTOTIC AND NECROTIC CELL DEATH AFTER BDL

A central but controversial issue in the debate on the mechanisms of cell death in cholestatic liver disease is whether the injury occurs through apoptosis or necrosis[90]. This is a key point as the therapeutic options to prevent injury will progress in two very different directions depending on the answer. Necrosis is characterized by cellular swelling, membrane blebbing, DNA fragmentation and release of cellular components, whereas apoptosis is characterized by cellular shrinking, caspase activation, DNA fragmentation, chromatin condensation with initially no release of cellular contents[32]. These processes lead to dramatically different outcomes after cell death in regards to progression of disease with ne-

crosis leading to increased inflammation and further aggravation by inflammatory cells[81,90,91].

Although there is no question that high concentrations of certain hydrophobic bile acids can cause apoptotic cell death in cultured rodent hepatocytes[22-27, 34-42,67,68] as discussed earlier, the pathophysiological relevance of these observations for *in vivo* models, e.g., BDL, is questioned due to the fact that the pro-apoptotic bile acids do not achieve sufficiently high levels *in vivo* to directly cause cell death[8,9]. Thus, the evaluation of the mechanism of cell injury has to be based on the *in vivo* evidence. Early studies concluded that BDL-induced liver injury was caused by apoptosis mainly due to the positive terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay[92-94] which is clearly not specific for apoptotic cell death[30-32]. In addition, immunofluorescence was used to detect cells with active caspases 3/7[95] but this was not confirmed by others[96]. Additional arguments for apoptosis were the reduced BDL-induced liver injury in Fas receptor-deficient *lpr* mice[94] and after anti-sense treatment to reduce Bid expression[97]. However, *lpr* mice are protected due to the reduced inflammatory response[98] and genetically Bid-deficient mice did not show reduced liver injury[96]. In contrast, numerous studies did not find any morphological evidence of apoptosis[11,12,68,98-101] and no evidence of caspase activation (enzyme activity and/or active fragments) or immunohistochemical staining for cytokeratin 18 cleavage products or active caspase-3/7[96,98,99]. In addition, potent caspase inhibitors, which effectively eliminated caspase activation and cell death in Fas- or TNF-mediated apoptosis models[98,102], did not protect against BDL-induced liver injury[98]. The one study reporting reduced injury after a caspase inhibitor in the absence of relevant caspase activation[95] may be due to off-target effects on other proteases[103]. Likewise, the anti-sense knock-down of Bid gene expression may have caused compensatory protective gene activation, which may have attenuated BDL-induced liver injury[95]. Clearly, Bid-deficient mice were not protected against BDL-induced liver injury[96]. The cathepsin B knockout mouse also showed protection against apoptotic injury[92], although apoptosis was assessed entirely based on the TUNEL assay. While the reduction in injury was attributed to lack of cathepsin B, and thus a lack of apoptotic processing proteins, the paper also showed a significant reduction in the neutrophilic inflammatory response[92]. Thus, the reduced injury in cathepsin B-deficient mice may have been due to reduced inflammation rather than apoptotic cell death. Taken together, the preponderance of experimental evidence supports the conclusion that liver injury after BDL involves mainly necrotic cell death.

In conclusion, when using morphological criteria of apoptosis and caspase activation in combination with positive controls of apoptotic cell death *in vivo*, the overwhelming experimental evidence supports the conclusion that there is no relevant apoptotic cell death but almost exclusively necrosis after BDL. The main paradigm shift comes from the recognition that the well-known hepatotoxic bile acids never reach concentrations in the liver after BDL that would directly cause cell death. In contrast, the most common bile acids that reach close to mmol/L levels trigger inflammatory mediator formation, which initiates an inflammatory response and cell death caused by neutrophils through oxidant stress. Initial data from human patients with cholestatic liver disease appear to confirm several aspects of the mouse model. Thus, the focus of future research should be on evaluating the cell stress mechanisms in hepatocytes considering pathophysiologically relevant bile acid mixtures and on further characterizing the initiation of the inflammatory response after BDL. Understanding these early injury mechanisms may not only reveal therapeutic approaches to reduce liver injury but also the subsequent fibrosis.

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