Gene Expression The Journal of Liver Research, Vol. 19, pp. 215–228
Printed in the USA.

1052-2166/19 $90.00 + .00
DOI: https://doi.org/10.3727/105221619X15614873062730
E-ISSN 1555-3884
www.cognizantcommunication.com

Review

Inflammation and Cell Death During Cholestasis: The Evolving Role of Bile Acids

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Cholestasis results in blockage of bile flow whether the point of obstruction occurs extrahepatically or intrahepatically. Bile acids are a primary constituent of bile, and thus one of the primary outcomes is acute retention of bile acids in hepatocytes. Bile acids are normally secreted into the biliary tracts and then released into the small bowel before recirculating back to the liver. Retention of bile acids has long been hypothesized to be a primary cause of the associated liver injury that occurs during acute or chronic cholestasis. Despite this, a surge of papers in the last decade have reported a primary role for inflammation in the pathophysiology of cholestatic liver injury. Furthermore, it has increasingly been recognized that both the constituency of individual bile acids that make up the greater pool, as well as their conjugation status, is intimately involved in their toxicity, and this varies between species. Finally, the role of bile acids in drug-induced cholestatic liver injury remains an area of increasing interest. The purpose of this review is to critically evaluate current proposed mechanisms of cholestatic liver injury, with a focus on the evolving role of bile acids in cell death and inflammation.

Key words: Bile acid; Metabolism; Cholestasis; Liver; Neutrophil; Inflammation; Cytokine; Review; Cirrhosis; Drug-induced liver injury (DILI); Cholangiocytes

INTRODUCTION

Cholestatic liver injury is a common clinical occurrence wherein intrahepatic impairment of bile formation and excretion or extrahepatic blockade of the biliary tracts results in retention of bile acids or bile salts. Clinical obstructive cholestasis can be caused by events such as gallstones, impingement of pancreatic cancer onto the bile ducts, sclerosing cholangitis, or biliary stricture—all of which feature varying degrees of obstruction[1–3]. Acute cholestatic liver injury in patients, such as with gallstones, can commonly be resolved surgically. However, chronic diseases like biliary atresia, primary biliary cholangitis (PBC), progressive familial intrahepatic cholestasis (PFIC), and primary sclerosing cholangitis (PSC) are difficult to manage without liver transplantation. These diseases present with similar pathology including inflammation, major blockage of bile flow resulting in alterations in bile acid disposition, increases in serum transaminase levels indicative of liver injury, and substantial fibrosis eventually progressing to cirrhosis and liver failure. While the root cause is fundamentally different, these diseases converge upon obstruction of biliary flow as the primary instigator of liver damage. Unfortunately, therapeutic development in this area has lagged behind other areas for multiple reasons, and ursodeoxycholic acid (UDCA) remains one of few nonsurgical treatment options. Surgical treatments, including liver transplantation, are available, but the shortage of livers worldwide makes this option impossible for many patients. The lack of solid models for any of these diseases further makes development of therapeutics difficult[4]. Finally, many patients with these diseases present with significant obstruction, or even cirrhosis. At this point, it then becomes necessary to reverse the process rather than just limit damage to the liver, which complicates therapeutic

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development. New agents are in clinical trials, but limited success has been observed outside of recent success with bezafibrate in PBC[5]. Limiting hepatic injury, reducing fibrosis, and increasing choleresis to remove excessive bile acid levels remain likely mechanisms through which the disease states could be beneficially modulated; however, an increased understanding of how and why cholestasis causes liver injury is required to inform development of new therapeutics.

Normal enterohepatic circulation of bile acids results in the unobstructed flow from hepatocytes, the cellular point of synthesis, through the biliary tracts, into the intestines, and then back to the liver through the hepatic portal vein. Bile acid uptake and export through tissues is mediated by a series of uptake and export proteins selectively expressed on specific tissues to mediate this process (Table 1)[6,7]. Expression of these proteins is largely controlled by transcription factors such as the farnesoid X receptor (FXR), as well as circulating hormones like fibroblast growth factor 15[6–9]. Ultimately, the purpose of this flow is to aid in digestion, assist in excretion of endogenous and exogenous compounds, and regulate metabolism for nutrient uptake and usage. This is a highly efficient process, with minimal bile acid loss daily[6,7,9].

Obstruction of any point in the biliary tracts results in substantial pathology. Cholestasis occurs most commonly either intrahepatically in the small cholangioles of the liver, at the apical membrane of hepatocytes, or extrahepatically in the common bile duct. Similarly, a number of laboratory models have been developed that attempt to mimic this process through either direct physical obstruction or biochemical disruption of the biliary tracts[10,11]. Consistently, all of these models result in: 1) disruption of the bile acid pool in regard to its constituent members, the quantities in different compartments, and the molecular mechanisms that control bile acid uptake and export and 2) substantial liver inflammation, especially the recruitment and activation of cytotoxic neutrophils. Mechanisms of cholestatic liver injury described by our group and others have largely focused on these events as precipitating factors[2,6,8,12–20]. In spite of this, considerable debate still exists in the field as to what induces the characteristic hepatocyte cell death caused by cholestasis[14,21]. The purpose of this review is to critically evaluate mechanisms of cholestatic liver injury, with a focus on the interplay between bile acids, cell death, and inflammation. We will attempt to point out major differences between animal models and what is understood about the human condition, while focusing on gaps in knowledge that can be addressed to push the field forward.

BILE ACIDS IN CHOLESTATIC LIVER INJURY

Synthesis, Regulation, and Metabolism of Bile Acids

Bile acids are involved in a number of different diverse liver processes including metabolism, regeneration, and hormone signaling[6,8]. Bile acids are synthesized in the liver through well-described pathways involving oxidative metabolism by cytochrome P450s (Fig. 1)[6,7]. Cholesterol goes through a multienzymatic process ultimately controlled by CYP7A1 as the rate-limiting enzyme to produce one of two primary bile acids, cholic acid (CA) or chenodeoxycholic acid (CDCA)[22,23]. This process is acutely regulated by the transcription factor FXR, which is present in multiple tissues exposed to bile acids[24–26]. Activation of FXR by bile acids results in downregulation of CYP7A1 and other bile acid-metabolizing enzymes, downregulation of bile acid uptake proteins such as sodium taurocholate cotransporting polypeptide (NTCP), and simultaneous upregulation of proteins associated with bile acid export such as organic solute transporters (OSTs)[26–34]. These adaptive changes take place in multiple tissues and are designed to promote the excretion of bile acids to reduce systemic levels (Table 1). Primarily in hepatocytes, bile acids are exported by the bile salt export pump (BSEP) and multidrug resistance (mdr) family proteins[6,7]. BSEP is a major driving force for bile flow and, thus not surprisingly, BSEP inhibition by therapeutic compounds is a noted cause of cholestatic liver and a major source of toxicity in potential therapeutics[7,14,35]. Similarly, genetic loss of MDR3 in humans results in progressive familial intrahepatic choles-

Table 1. Expression Patterns of Multiple Transporters in Multiple Tissues

Tissue	Uptake Transporters	Export Transporters
Liver (cell type)		
Hepatocytes	From serum: OSTα/ß, NTCP, OATP	To bile: MRP2, MDR2, BSEP To serum: OSTα/ß, MRPs
Cholangiocytes	From bile: ASBT	To serum: OSTα/ß
Colon	From feces: ASBT	To serum: OSTα/ß
Kidneys	From serum: ASBT	To serum/urine: MRP2, OSTα/ß

NTCP, sodium taurocholate transporting polypeptide; MRP, multidrug resistance-associated protein; MDR, multidrug resistance protein; OST, organic solute transporters; ASBT, apical sodium-dependent bile acid transporters, organic anion-transporting polypeptide.

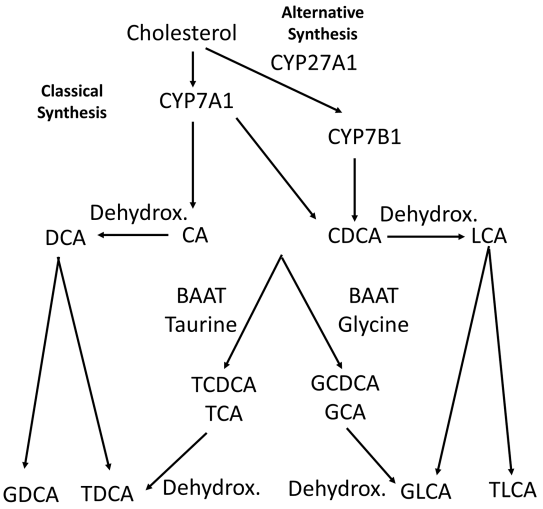

Figure 1. Bile acid synthesis. Bile acids are synthesized from cholesterol by cytochrome p450s (CYP) to either cholic acid (CA) or chenodeoxycholic acid (CDCA). Dehydroxylation by gut bacteria results in lithocholic acid (LCA) or deoxycholic acid (DCA). Conjugation of CA or CDCA to taurine/glycine results in taurochenodeoxycholic/taurocholic acid (TCDCA/TCA) or glycochenodeoxycholic/glycocholic acid (GCA/GCDCA). Dehydroxylated bile acids can also be conjugated to taurine/glycine. Dehydrox., dehydroxylated.

tasis, a genetic condition with prominent cholestatic liver injury, which is recapitulated in the mouse[10]. In summary, bile acid synthesis is tightly controlled by FXR and other bile acid synthetic genes, both physiologically and during cholestasis, to control bile acid synthesis and enhance excretion, with the goal of maintaining normal metabolism and nutrient uptake.

While they are commonly used experimentally as primary bile acids, the majority of circulating primary bile acids do not exist as CA or CDCA; rather, they are conjugated to either glycine or taurine by the enzyme bile acid-CoA:amino acid N-acyltransferase (BAAT) to promote hydrophilicity, presumably to help with solubility and reduce toxicity, or they are metabolized into other bile acids[36–40]. Taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), and glycochenodeoxycholic acid (GCDCA) are the most common circulating bile acids in humans in bile and serum, especially during cholestasis whether this is due to general obstruction or chronic disease such as with primary sclerosing cholangitis (Fig. 2)[2,39,40]. While rats also conjugate bile acids to glycine, multiple groups have now noted that glycine conjugation in the mouse is nearly absent[2,39–41]. While the mechanism is not well understood, murine BAAT strongly prefers taurine as a substrate, and thus bile acid conjugation is likely due to the preference of BAAT, but may be in part linked to either dietary taurine/glycine intake, glycine metabolism from serine, or taurine synthetic metabolism[36–38]. Notably, the percentage of conjugated to unconjugated bile acids increases dramatically during liver injury[2,39,40,42].

Bile acids are also metabolized by gut bacteria, as CA is dehydroxylated to deoxycholic acid (DCA), while CDCA is dehydroxylated to lithocholic acid (LCA)[6,8]. These secondary bile acids can also be conjugated to glycine or taurine. While other metabolic processes including sulfation and other hydroxylation reactions have been established, levels of these bile acids are relatively minor in most species, and the predominant reactions are conjugation by BAAT and dehydroxylation by gut bacteria[43–46].

Bile Acid Disposition in Cholestatic Liver Injury

Cholestasis dramatically alters bile acid concentrations in different compartments. Acute cholestasis in laboratory models results in dramatic increases in serum bile acid levels, that taper off, but remain elevated for a significant period[47]. The presence of high bile acid levels in serum alone is insufficient to induce liver injury though[48–50]. In case studies, patients with constitutive loss of NTCP had extremely high serum bile acid levels and hyperbilirubinemia, but with minimal signs of overt liver toxicity[48,49]. Similarly, Myrcludex B, an NTCP-inhibiting polypeptide in use in patients for treating hepatitis B infection, is well tolerated in both rodents and human patients despite drastic increases in serum bile acid levels associated with loss of hepatic uptake[51,52]. Moreover, Myrcludex B is protective against the primary laboratory cholestasis models including bile duct ligation (BDL), wherein the bile duct is surgically ligated to induce extrahepatic cholestasis, and 3.5-diethoxycarbonyl-1.4-dihydrocollidine (DDC) administration, wherein DDC induces intrahepatic loss of bile flow[53]. Surprisingly this did not extend to the MDR2$^{-/-}$ mouse[53]. While the mechanism has not been established, serum bile acids may also increase during noncholestatic liver injury[42]. This may be due to necrosis of hepatocytes with impaired uptake and increased release of intracellular stores, and is likely not a contributing mechanism to injury in all cases[2,42].

Bile acids can be excreted into the urinary tracts and then removed from the body as an alternative means for excretion; however, long-term exposure results in kidney damage and indicates that the kidneys are not designed to continue this process chronically[54,55]. Cholemic nephropathy is largely understudied clinically, but a recent study indicates that this process likely occurs in patients as well, and may represent an area of underappreciated clinical concern[56]. Overall, it appears that shunting bile acids into the plasma is a protective measure used by the liver to prevent cholestatic liver injury by reducing overall bile acid levels via urinary excretion and preventing accumulation in hepatocytes.

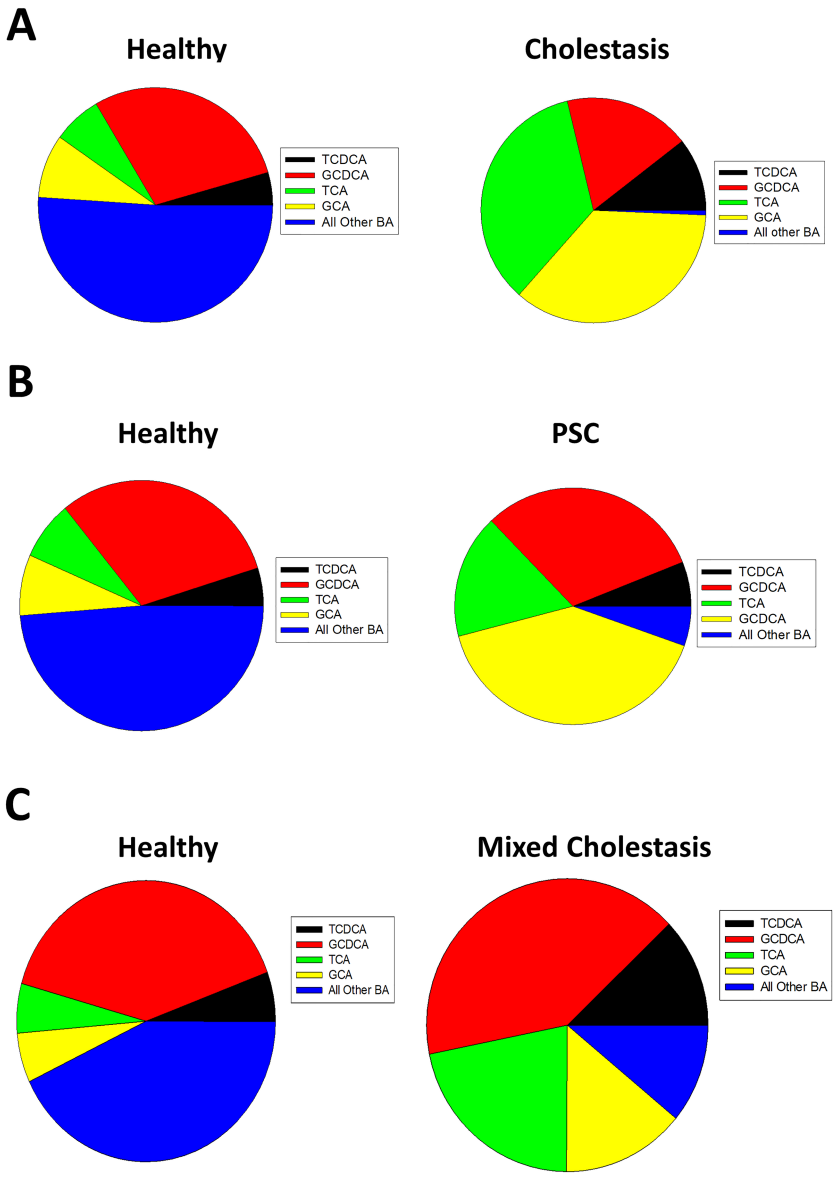

Figure 2. Serum bile acid levels in human patients with or without cholestasis. Serum bile acids were measured in healthy or cholestatic patients (A), or healthy or patients with primary sclerosing cholangitis (B), or healthy or cholestatic patients with mixed etiology of cholestasis (C). Data adapted from References 2, 39–40. BA, bile acid.

In contrast to serum levels, biliary levels of bile acids can change depending upon the point of cholestasis. After lithocholic acid feeding, biliary levels can rise acutely, but can alter over time due to the influx of water and other solutes that alter total volume[15,57]. This is partially accommodated through expansion and filling of the gallbladder, which is also a noted aspect of BDL and gall bladder physiology. In contrast, loss of BSEP or mdr2 results in loss of normal bile acid export and reduced biliary bile acid levels acutely and chronically[10,58,59]. This is consistent with the proposed function of BSEP, which shunts bile acids into the biliary ducts and promotes biliary flow[58,59]. Notably, both extrahepatic and intrahepatic cholestasis dramatically reduce the formation of secondary bile acids as they do not reach the intestine and thus are not metabolized by gut bacteria[2,47].

Cholestasis also results in alterations in intestinal bile acid levels (generally reducing them due to blockage in flow), which may be a therapeutic target[8]. Intestinal bile acids are typically reabsorbed by the apical sodium-dependent bile acid transporter (ABST)[60,61]. Recent efforts to reduce bile acid levels during cholestastic diseases using inhibition of ASBT have demonstrated that systemic reduction of bile acid levels can be highly

beneficial to liver disease, including cholestasis[62,63]. This is recapitulated by the use of cholestyramine, an agent that binds bile acids and forces their excretion through defecation[64,65]. More work is needed in this area to determine if bile acid pools can safely be restricted via this method, although this is a promising area for therapeutic development.

Finally, cholestasis results in dramatic increases in bile acid levels within hepatocytes[47]. This is potently counterregulated by FXR, and likely other mechanisms, such that the initial rise in bile acid levels tapers off over time; however, the spike in intrahepatic bile acid levels has widely been attributed to be the primary cause of hepatocyte cell death[18,46,66–68]. Furthermore, the presence of infarctions in the biliary tracts results in localized increases in bile acids, potentially exposing hepatocyte to mM levels of bile acids acutely that generate small foci of cell death. In spite of these data, species differences in susceptibility, metabolism, and physiology have made it difficult to translate in vitro findings to human disease, and bile acid-induced toxicity continues to be widely studied.

Bile Acid-Induced Hepatocyte Cell Death

Different bile acids induce cell death at different concentrations, and this is due to both relative hydrophobicity and the current conjugation status of the individual bile acid[2,18,69]. Because it induces well-defined apoptosis in rat hepatocytes, or human hepatocellular carcinoma cells that overexpress NTCP, one of the most commonly used bile acid to induce in vitro cell death is GCDCA; however, neither mouse nor human hepatocytes undergo appreciable levels of apoptosis when exposed to these concentrations of GCDCA, and both require dramatically higher concentrations to undergo any degree of cell death at all[2,70,71]. Importantly, mouse and human hepatocytes undergo necrosis, and not apoptosis, when exposed to other bile acids[2,57]. Similarly, human patients have limited levels of caspase-cleaved cytokeratin-18 release associated with apoptosis, but dramatic increases in full-length cytokeratin-18 release associated with necrosis[2]. Blockade of apoptosis was cited as the primary mechanism of protection in a number of studies with interventions against cholestatic liver injury; however, a majority of the commonly used laboratory models do not demonstrate gold standard markers such as caspase cleavage, caspase activity, or histological evidence for apoptosis[11,72–78]. Even in the BDL model in the rat, wherein higher concentrations of intrahepatic GCDCA would be expected, only limited apoptosis is found, and this is countered by activation of nuclear factor κ light chain enhancer of activated B cells (NF-κB) that prevents widespread apoptosis[79,80]. As such, while bile acid-induced apoptosis was a leading hypothesis in the field for many years, the likelihood that it extends broadly to human disease is very unlikely. Although this does not preclude bile acid-induced cell death, the mechanisms that control this in both human and mouse hepatocytes are poorly explained because they do not follow the established mechanisms present in rat hepatocytes.

Despite differences in interpretation, a number of studies support the ideas that excessive bile acid levels can kill hepatocytes. While rats undergo apoptosis at GCDCA concentrations above 50 μM, human cells are largely resistant until concentrations above 500 μM to 1 mM in vitro[2,70]. Whether this difference in susceptibility between rat and human hepatocytes is due to relatively higher exposure to glycine-conjugated bile acid under normal conditions or other mechanisms is not currently known. As the normal bile acid pool in the mouse consists primarily of taurine-conjugated bile acids, the relative toxicity of the pool on its own is minimal[2,39,40,81]. In contrast, toxification of the bile acid pool by feeding hydrophobic bile acids results in considerable toxicity[15,57,69]. Both cholic acid feeding and lithocholic acid feeding result in cholestasis and cell death when given at high concentrations in the feed[15,57,69]. Notably in the lithocholic acid model, a role for inflammation was ruled out, and administration of bile acid concentrations equivalent to what hepatocytes are predicted to be exposed to was toxic in vitro supporting the idea that hydrophobic bile acids, when present in sufficient concentrations in vivo, can be directly toxic[57]. Importantly in this model, direct cholestasis, that is a reduction in bile flow due to obstruction, was observed confirming the cholestatic phenotype[15]. Notably though, LCA levels either conjugated or unconjugated are usually very low in cholestasis, and the likely reason is that gut bacteria metabolism is necessary for their generation and cholestasis blocks flow to the gut, and thus this model is highly artificial relative to human disease[39,40].

Just as toxification of the bile acid pool increases cell death, detoxification of the bile acid pool reduces cell death. NorUrsodeoxycholic acid (nUDCA), a UDCA derivative, has been used to reduce liver injury caused by bile acids in a number of different models as it is a relatively mild bile acid in toxicity that also promotes biliary flow, which has been proposed to help with excretion of bile acids and consequently prevent mitochondrial damage induced by bile acid retention[82]. A recent experiment noted that mdr2$^{-/-}$ animals had higher levels of cholic acid, a more hydrophobic bile acid; however, when this mouse was fed hydrophilic bile acids, or when this mouse was crossed to the BSEP$^{-/-}$ mouse that produces largely hydrophilic bile acids, liver injury was reduced[83]. Experiments directly feeding a dose response of different bile acids, even up to 3% UDCA, did not produce a significant increase in serum ALT values, despite the fact that it increased liver bile acid concentrations[69]. In this same study though, 1% cholic acid feeding produced

significant increases in serum ALT, although this occurred without a change in overall liver bile acid levels[28]. Serum bile acid levels were dramatically increased though, and as such, the point measured may have occurred after FXR counterregulation and shunting of bile acid to serum[40]. These experiments largely combine to indicate alterations in bile acid pool size, and constituency dramatically affects the capacity of the liver to detoxify and prevent bile acid-induced injury.

Intrahepatic cholestasis has varying results with bile acid toxicity. Since its initial understanding, BSEP inhibition has routinely been proposed as a major cause of drug withdrawal due to drug-induced cholestatic liver injury[84]. The proposed mechanism was increased intrahepatic bile acid accumulation; however, BSEP knockout alone does not result in liver injury in mice or in cell lines[59,85,86]. In fact, many drugs that are BSEP inhibitors in vitro demonstrate alteration in the bile acid pool and bile acid concentrations in media at concentrations significantly below their toxicity level[87]. Furthermore, when troglitazone, a known BSEP inhibitor, was given to BSEP KO HepaRG cells, the toxicity was enhanced[88]. Troglitazone can reduce conjugation of bile acid to taurine and further polarizes the bile acid pool toward glycine-conjugated bile acid acids[89]. This may be a mechanism through which troglitazone induces cholestasis and subsequent liver injury[89]. As such, while excessive intrahepatic bile acid stores are likely a potential cause of cholestatic liver injury, simply blocking export is insufficient to induce toxicity in vitro, and thus other mechanisms may also be at play.

One of the characteristic histological findings with many types of cholestatic liver injury, including in laboratory models, is the presence of foci of liver necrosis (Fig. 3). These foci are thought to be due to infarction of the surrounding biliary tracts and leakage of bile into the hepatic parenchyma and are commonly referred to as bile infarcts or Charcot–Gombault necrosis[90]. Biliary infarcts are noted in the BDL model, the mdr2$^{-/-}$ model, the LCA administration model, and in human patients[2,16]. It has been hypothesized that mechanical stress weakens the small cholangioles in the liver, which increases susceptibility to infarction of the biliary tree resulting in leakage of bile[91]. A recent study has confirmed this using intravital two-photon imaging[90]. Biliary infarcts are initiated at the apical membrane of hepatocytes and expand from there along with hepatocyte cell death[68]. These infarcts are especially notable in the LCA model wherein electron microscopy pictures have detailed the formation of LCA precipitates that aggregate and irritate the cholangioles leading to rupture[15]. As these infarcts are commonly associated with major obstruction, it was surprisingly noted that UDCA levels actually worsened injury levels in these animals[16]. As UDCA is thought to be far less toxic and potentially even helpful to biliary injury, these data indicate that biliary rupture is likely highly damaging to hepatocytes, even when the bile is relatively detoxified[71]. In contrast, the use of nUDCA benefited mice with partial obstruction, although not complete obstruction[92]. Supporting these data, a recent study noted that knockout of sortilin, a trafficking protein that can affect bile acid metabolism, reduced bile acid pool size, which led to less infarction of the biliary tracts and a reduction in injury consistent with the idea that reduction in the bile acid pool size leads to reduced injury, likely due to reduced intrahepatic biliary pressure and reduced bile acid leakage into the parenchyma[93].

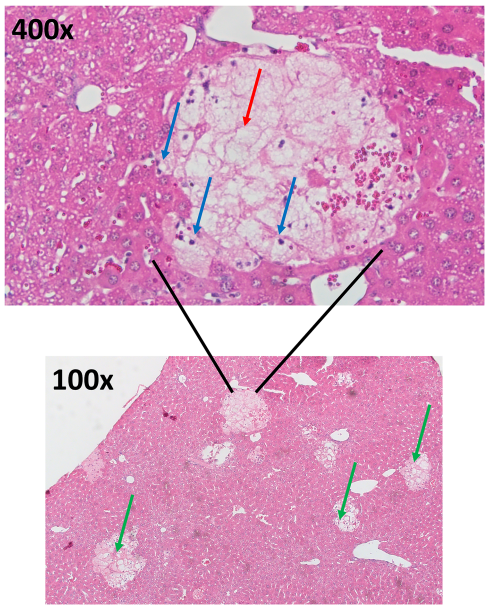

Figure 3. Bile duct ligation histology. H&E stain of a mouse liver 24 h post-bile duct ligation. Blue arrows represent inflammatory cells. Red arrows represent areas of feathery necrosis inside the infarct. Green arrows on the 100× images represent obvious areas of infarction and cell death.

Bile acid-induced toxicity is clearly dependent on a number of factors relating to individual bile acid levels, bile acid disposition, and relative degree of obstruction. The majority of the data points toward the same idea: reducing pool size, promoting conjugation to taurine, pushing bile acids into serum or alternate excretion routes, and preventing complete obstruction all minimize liver injury.

CHOLANGIOCYTES: CRITICAL MEDIATORS OF THE EPITHELIAL BARRIER TO BILE ACID TOXICITY

Cholangiocytes are also critical mediators of cholestatic liver injury, and their relationship with cholestatic liver injury cannot be overlooked. The liver is interlaced internally with small biliary vessels termed bile canaliculi that are lined with cholangiocytes. Canaliculi dump

bile acids generated by hepatocytes into larger cholangioles and then into the greater biliary tracts. Export of bile acids generated by hepatocytes is mediated by transporters such as BSEP and mdr2 as aforementioned. Bile acids such as TCA stimulate proliferation of the cholangioles in order to handle increased bile load and prevent infarction[94]. One of the most important effects of cholangiocytes is maintenance of bile acid-independent bile flow via the hormone secretin and the protein anion exchanger 2 in addition to glutathione[95,96]. These proteins regulate secretion of bicarbonate and chloride anion, which then regulate water flux and drive biliary flow[95]. Cholangiocytes also express a number of bile acid receptors including the apical sodium bile acid transporter (ASBT), sphingosine 1 phosphate receptor 2 (S1PR2), and TGR5[97]. These receptors can mediate cholestatic liver injury. Loss of S1PR2 reduces BDL-induced cholestasis and fibrosis, but had minimal effect on hepatic injury as measured by ALT/AST[97]. Activation of TGR5 stimulates proliferation of cholangiocytes and protects against death receptor-induced cell death, which may be protective through maintenance of normal epithelial barrier against biliary infarction via stabilization of junctional adhesion molecule-A, and thus TGR5 agonism may represent a therapeutic target in cholestatic liver injury[98,99]. ASBT shunts bile acids from bile back into the liver through as a cholehepatic shunt, which may yield alternate excretory mechanisms[100]. Secretin upregulates ASBT and prolongs bile acid transit time by enhancing the shunt of bile acids back into hepatocytes providing a potential feedback loop[101]. Overall, cholangiocytes express a number of bile acid transporters that react to alterations in biliary flow by altering bile acid uptake and bile acid-independent flow. Moreover, cholangiocytes provide the critical epithelial barrier against biliary rupture necessary for safely removing excess bile acid levels.

INFLAMMATION IN CHOLESTATIC LIVER INJURY: CAUSE OR CONSEQUENCE

Neutrophils and Cholestatic Liver Injury

Although cholestatic liver injury was hypothesized to be due to bile acid toxicity, other hypotheses also exist that explain why biliary rupture would be damaging to hepatocytes. Primarily, a number of studies have begun to elucidate intricate signaling networks mediated in part by the presence of high levels of bile acids that initiate a potent neutrophil-mediated inflammatory response. While a consensus has formed that inflammation occurs after biliary rupture or hepatic exposure to high levels of bile acids, the mechanisms that dictate inflammatory progression and its precise role in the injury process remain areas of intense study.

The most commonly cited inflammatory process after either BDL, bile acid feeding, or in the mdr2$^{-/-}$ model is the recruitment of neutrophils to areas of injury[10,102–104]. Initial results indicated a potential CXCL-mediated neutrophil recruitment pathway after BDL, which has largely been confirmed in the mouse[105,106]. Knockout of CCL2 resulted in sustained inhibition of BDL-induced liver injury and injury after cholic acid feeding[107,108]. Neutrophil recruitment happens as early as 6 h after BDL, consistent with the initial points of tissue damage, and continues throughout the disease[11]. Studies have shown that prevention of neutrophil adhesion through either knockout of CD18 or knockout of intercellular adhesion molecule-1 (ICAM-1) was protective against BDL-induced liver injury, as was knockout of P-selectin glycoprotein ligand-1 necessary for neutrophil adherence[103,104,109]. Neutrophils are hypothesized to kill hepatocytes through release of potent ROS forms such as hypochlorous acid, which induces cell death through increasing oxidative stress[102,110,111]. Furthermore, the *lpr* mutant mouse, which has a deficient immune response and autoimmune dysfunction due to mutation in *Fas* receptor, is also protected, independent of fas-induced apoptosis[76]. Similar results were obtained in the plasminogen activator inhibitor (PAI1$^{-/-}$) knockout mouse, or Egr-1$^{-/-}$ mouse, both of which have knockouts for proteins involved in initiating inflammation[112,113]. Knockout of osteopontin yielded an early decrease in liver injury after BDL that was not sustained, indicating it may have an acute role in the injury process[114]. The biliary release of osteopontin by cholangiocytes and cleavage by matrix metalloproteases generates a potent chemotactic factor, which is responsible for the initial recruitment of neutrophils and the early inflammatory injury[114]. In addition, biliary levels of bile acids induce chemokine formation in hepatocytes[81]. Notably, interleukin (IL)-17$^{-/-}$ animals had reduced injury, but this did not affect levels of bilirubin, serum bile acids, or alkaline phosphatase, indicating that the reduction in injury was purely associated with a change in inflammation, and not with the relative level of cholestasis in the animal[115]. As such, several lines of research have converged on the idea that inflammation can mediate a portion of the injury, especially after the initial biliary rupture[1,14,16,90]. Even still, this may be dependent on the model, and no data to the authors' knowledge has fundamentally demonstrated a role for inflammation in hepatocyte death in human patients with any specific form of cholestatic liver injury. Moreover, in human patients, significant quantities of glycine-conjugated bile acids are present that could justifiably induce cell death during cholestasis. Figure 4 depicts mechanisms of neutrophil-induced liver injury in the mouse BDL model.

Chronic administration of α-naphthyl isothiocyanate (ANIT) shares many aspects of BDL-induced liver injury. Inflammation is also prominent in the model, and

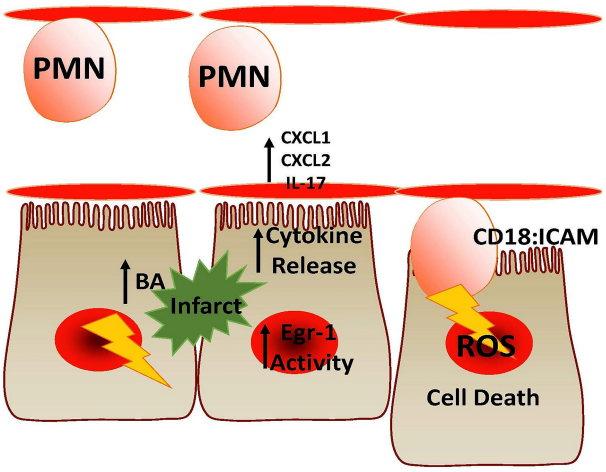

Figure 4. Proposed model of neutrophil-induced injury. Infarction of the biliary tract results in hepatocyte damage and release of bile acids (BA) and damage-associated molecular patterns (DAMPs). This releases cytokines like CXC-ligands 1 and 2 or IL-17 and increased early growth factor response-1 activity. Neutrophils (PMN) recognize these signals and adhere firmly to hepatocytes via CD18/intercellular adhesion molecule-1 (ICAM-1) and induce cell death through ROS production.

blockade of inflammation reduces liver injury. This is a neutrophil- and Egr-1-dependent injury process similar to BDL[116–118]. These data support the idea that inflammation, especially neutrophils, can promote cholestatic liver injury.

Kupffer Cells and Inflammatory Mediators in Cholestatic Liver Injury

A number of other inflammatory cells might be involved in inflammation during cholestasis. Kupffer cells are resident tissue macrophages in the liver that also have implications in BDL-induced liver injury. Kupffer cell inactivation with gadolinium chloride protects against BDL-induced liver injury and reduces neutrophil recruitment leading to reduced liver injury[73,119]. In contrast, Kupffer cell depletion with clodronate liposomes enhances injury[120]. Notably, IL-6 depletion also worsens injury and is thought to be mediated by Kupffer cells in the model, indicating that IL-6 might have an anti-inflammatory role in the model, which is recapitulated by the fact that recombinant IL-6 administration is also protective[120]. Both dendritic cells and T cells have also been observed to cause differences in BDL-exposed animals; however, the role of the adaptive immune system generally is less well understood[121–123].

Inflammation is also a likely consequence of most types of liver injury. Necrosis of hepatocyte results in release of sterile mediators referred to as damage-associated molecular patterns (DAMPs) that can initiate inflammation including mitochondrial DNA, nuclear DNA fragments, ATP, and more[124,125]. Receptors for many of these products are present on Kupffer cells and even hepatocytes and can initiate an inflammatory response[124]. Notably, bile acid levels are also increased dramatically in noncholestatic forms of liver injury such as with acetaminophen overdose[42]. Bile acids also have signaling pathways mediated by receptors such as G-protein-coupled bile acid receptor (TGR5) on Kupffer cells that mediate inflammation, indicating that bile acids themselves may be an underappreciated DAMP[65]. Moreover, bile acids also directly induce inflammation in hepatocyte in murine hepatocytes in an Egr-1-dependent manner[81,126]. Regardless, a causative role for neutrophil-mediated liver injury is implied through experiments with knockout of mediators of neutrophil adherence and recruitment that implicate inflammation directly[103,104,113,127]. Inflammation is directly tied to biliary pressure and degree of cholestasis, and thus, completely separating bile acid accumulation and inflammation is nearly impossible in determining a concrete mechanism.

Cholangiocytes and the Senescence-Associated Secretory Phenotype

Cholangiocytes are known to undergo senescence-like changes during cholestasis, resulting in the senescence-associated secretory phenotype that promotes inflammation. Biliary cells are especially prone to cellular senescence, and their presence is noted in chronic cholestatic diseases such as PBC[128]. Cellular senescence in the liver initiates a paracrine signaling pathway that exacerbates DDC-induced liver injury through enhanced secretion of proinflammatory and profibrotic mediators[128–130]. Senescence is known to promote inflammation, and thus biliary senescence may be a major mediator of inflammation, especially in the later stages of advanced cholestasis.

BILE ACID-INDUCED PROINFLAMMATORY SIGNALING: A NEW HYPOTHESIS?

In contrast to experimental evidence in favor of a neutrophil-mediated injury response, defining the signaling pathway is polluted and made more difficult by activation of a number of proinflammatory signaling cascades through generalized inflammation and cell death. As such, detailed investigations into the inflammatory process have proven difficult. One area where a consensus is building is in proinflammatory signaling induced by bile acids in isolated hepatocytes. Initial studies indicated that a number of different conjugated and unconjugated bile acids can induce proinflammatory signaling changes in isolated murine hepatocytes[81]. This was most prevalent with TCA, which dramatically enhances expression and secretion of cytokines such as

CXCL1 and CXCL2 as well as expression of ICAM-1[81]. This pathway is dependent on Egr-1[126]. These same cytokines have been directly implicated in BDL-induced injury, and this process was noted to occur independently of FXR, meaning an entirely separate bile acid signaling pathway is present in hepatocytes that mediates this interaction independently, which does not occur with isolated nonparenchymal cells[81,107]. TCA-mediated increases in CCL2 may also mediate other forms of injury such as carbon tetrachloride, wherein the process is dependent on c-jun N-terminal kinase signaling[131]. Subsequent studies showed increases in IL-17A and IL-23A expression after TCA exposure in hepatocytes, indicating the hepatocyte-mediated proinflammatory pathway likely has a direct linkage to the subsequent inflammation found in the BDL model[115]. The receptor or mechanism responsible for the initiation of this signaling pathway is not currently well understood, although multiple receptors have been established as bile acid receptors including S1PR2, TGR5, and likely more[65,132]. Figure 5 depicts a simplified version of this pathway.

Bile acid-induced proinflammatory signaling changes as observed in mouse hepatocytes were not repeatable in primary human hepatocytes or in HepaRG cells, a hepatocyte-like cell line that expresses some bile acid transporters, exposed to TCA[2,133]. However, later studies indicated that GCDCA did induce expression of human cytokines at concentrations of 50 μM[107]. A diverse array of diseases present with increased inflammation and increased serum bile acid levels, and subsequent enhancement of inflammation may be involved broadly in liver inflammation in addition to the role of bile acids in metabolism.

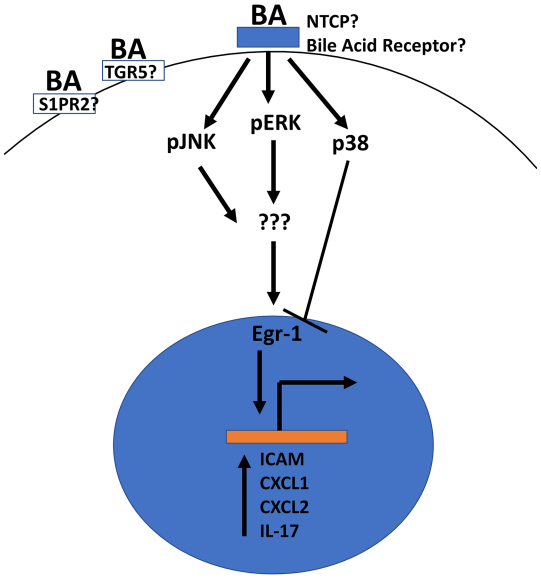

Figure 5. Proposed model of bile acid inflammatory signaling. Bile acids activate an unknown receptor, potentially sphingosine 1 phosphate receptor 2 (S1PR2) or G-protein-coupled bile acid receptor (TGR5), to initiate mitogen-activated protein kinase and c-Jun N-terminal kinase pathways. These increase early growth factor response-1 (Egr1) activity and induce proinflammatory gene induction.

FUTURE PERSPECTIVES

Cholestasis definitively results in considerable hepatocyte cell death. Recent studies have indicated separate roles for bile acids and inflammatory cells, but both likely contribute to the disease. Moreover, bile acids themselves are likely a highly proinflammatory DAMP-like molecule, and their removal may benefit other disease states. Depending on the model, the degree of inflammation may be sharply tied to the degree of cholestasis, and thus, the degree to which inflammation contributes is likely dependent on the location of obstruction and pathological sequelae. As such, therapeutics that enhance excretion of bile acids in these patients are likely to be of benefit for both the reduction in inflammation and the reduction in intrahepatic bile acid levels. Reducing levels of toxic bile acids may also benefit patients. Critical questions remain in the field though, primarily: 1) How do we safely alter conjugation status of bile acid pools to promote conjugation to taurine and reduce direct bile acid toxicity? 2) What is the role of inflammation in human diseases with prominent cholestasis and can reduction in inflammation acutely or chronically benefit patients and/or stave off liver transplantation? 3) Can alterations in bile acid-induced inflammation reduce injury in other disease states with increased bile acid levels? 4) What is the most effective way to reduce systemic bile acid levels without inducing toxic effects? Novel studies answering these questions could potentially reshape patient treatment in this disease space in the near future.

ACKNOWLEDGMENTS: This work was supported by the National Institutes of Health (grant R01 DK102142) and the National Institute of General Medical Sciences (P20 GM103549 and P30 GM118247) from the National Institutes of Health to H.J. The authors declare no conflicts of interest.

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