Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Bile Acids Induce Inflammatory Genes in Hepatocytes

A Novel Mechanism of Inflammation during Obstructive Cholestasis

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Inflammation contributes to liver injury during cholestasis. The mechanism by which cholestasis initiates an inflammatory response in the liver, however, is not known. Two hypotheses were investigated in the present studies. First, activation of Toll-like receptor 4 (TLR4), either by bacterial lipopolysaccharide or by damage-associated molecular pattern molecules released from dead hepatocytes, triggers an inflammatory response. Second, bile acids act as inflammagens, and directly activate signaling pathways in hepatocytes that stimulate production of proinflammatory mediators. Liver inflammation was not affected in lipopolysaccharide-resistant C3H/HeJ mice after bile duct ligation, indicating that Toll-like receptor 4 is not required for initiation of inflammation. Treatment of hepatocytes with bile acids did not directly cause cell toxicity but increased the expression of numerous proinflammatory mediators, including cytokines, chemokines, adhesion molecules, and other proteins that influence immune cell levels and function. Upregulation of several of these genes in hepatocytes and in the liver after bile duct ligation required early growth response factor-1, but not farnesoid X receptor. In addition, early growth response factor-1 was up-regulated in the livers of patients with cholestasis and correlated with levels of inflammatory mediators. These data demonstrate that Toll-like receptor 4 is not required for the initiation of acute inflammation during cholestasis. In contrast, bile acids directly activate a signaling network in hepatocytes that promotes hepatic inflammation during cholestasis.   *(Am J Pathol 2011, 178: 175–186; DOI: 10.1016/j.ajpath.2010.11.026)*

Cholestatic liver disease develops when bile flow from the liver is interrupted.[1,2] This can occur during autoimmune reactions, congenital disorders, pregnancy, drug hepatotoxicities, and other forms of liver disease.[1] Cholestasis causes concentrations of bile acids to rapidly increase in liver and plasma.[3,4] As this disease progresses, hepatic inflammation and hepatocyte injury ultimately develop.[5] If cholestasis is not corrected and hepatocyte injury persists, portal myofibroblasts and hepatic stellate cells are stimulated to proliferate and produce extracellular matrix, a process that ultimately results in the development of biliary fibrosis and eventually cirrhosis.[6]

A major consequence of acute cholestasis, especially obstructive cholestasis, is the development of severe liver injury.[7] It is generally assumed that exposure of hepatocytes to high concentrations of potentially toxic bile acids is mainly responsible for cholestatic liver injury.[8] In support of this concept, several studies have demonstrated that high concentrations of certain bile acids produce hepatocyte cell death *in vitro* through activation of proapoptotic pathways.[9–11] In these studies, deoxycholic acid (DCA), lithocholic acid, and their metabolites are frequently used.[11–13] One caveat of these studies, however, is that plasma and liver concentrations of secondary bile acids (eg, lithocholic acid and DCA) do not dramatically increase in humans with cholestasis or in animal models of cholestasis,[14–16] because these bile acids are formed in the intestine from primary bile acids, and intestinal concentrations of primary bile acids decrease during cholestasis because of diminished biliary excretion.[14,15] On the other hand, serum levels of the most

abundant primary bile acids, such as cholic acid and muricholic acid, increase to 200–250 μmol/L in mice subjected to bile duct ligation (BDL) for 7 days.[14] In contrast to DCA and lithocholic acid, however, these bile acids are relatively nontoxic to hepatocytes in culture.[17] Accordingly, these data raise the question of whether direct bile acid toxicity can be entirely or even partially responsible for liver injury observed during obstructive cholestasis.[18]

Recent studies suggest that inflammatory cells such as neutrophils are activated and recruited into the liver during obstructive cholestasis and cause significant liver injury in a murine model of bile duct ligation.[5] This inflammatory response correlated with substantial formation of pro-inflammatory cytokines and expression of intercellular adhesion molecule-1 (ICAM-1).[19] Because liver injury in mice deficient in CD18 or ICAM-1 was almost eliminated, it was concluded that infiltrating neutrophils and not bile acids were responsible for hepatocellular injury.[5,19] In support of these findings from animal studies, serum levels of the neutrophil chemokine IL-8 are increased in patients with cholestatic liver disease.[20,21] Furthermore, ICAM-1 is up-regulated and neutrophils are present in the livers of these patients.[22] Thus, both animal and human studies support the hypothesis that an inflammatory response during obstructive cholestasis plays a critical role in the injury process.

What remains unclear is the molecular mechanism by which cholestasis initiates this inflammatory response in the liver. Two hypotheses were investigated in the present studies to explain this mechanism. First, activation of Toll-like receptor 4 (TLR4), either by bacterial lipopolysaccharide (LPS) released from the gut or by damage-associated molecular pattern molecules (DAMPs) released from dead hepatocytes exposed to pathological concentrations of bile acids, triggers an inflammatory response. Second, bile acids act as inflammagens and directly activate signaling pathways in hepatocytes that regulate production of pro-inflammatory mediators that stimulate recruitment of neutrophils into the liver. A better understanding of the initiating events of the inflammatory response may point to clinically relevant therapeutic strategies against cholestatic liver injury and even fibrosis, without affecting the vital host defense functions of neutrophils.

Materials and Methods

Animals

Mice used for these studies were male, ranging in age from 8 to 10 weeks: C57BL/6 (Harlan Laboratories, Indianapolis, IN), C57BL/6NTac (Taconic, Germantown, NY), early growth response factor-1 (Egr-1) knockout (B6.129-$Egr1^{tm1Jmi}$ N12; Taconic), C3H/HeJ (Jackson Laboratories, Bar Harbor, ME), and C3Heb/FeJ (Jackson Laboratories). Farnesoid X receptor (FXR) knockout mice, described previously,[23] were provided by Dr. Grace Guo, Ph.D., University of Kansas Medical Center. All animals were maintained on a 12-hour light/dark cycle under controlled temperature (18–21°C) and humidity.

Food (Teklad rodent chow; Harlan Laboratories, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

Hepatocyte Isolation

Hepatocytes were isolated from the livers of mice by collagenase perfusion, as we have described previously.[24] The hepatocytes were cultured in Williams' medium E containing 10% fetal bovine serum and penicillin-streptomycin. Hepatocytes were treated with bile acids within 16 hours of plating. For caspase inhibition studies, cells were incubated with the pan-caspase inhibitor quinoline-val-asp-difluorophenoxymethylketone (MP Biomedicals, Santa Ana, CA), for 30 minutes before bile acid treatment.

Bile Duct Ligation

Mice were subjected to BDL as described previously.[24]

Human Liver Samples

Research involving human liver was reviewed by the University of Kansas Medical Center Human Research Protection Program. The specimens were provided by the KU Liver Center Tissue Bank. Diseased liver tissue used for these studies was collected from patients with primary biliary cirrhosis (4 females, ages 59, 56, 60, and 61 years; 1 male, age 47 years) or primary sclerosing cholangitis (3 males, ages 44, 45, and 57 years). Liver samples without histological evidence of cholestasis, fibrosis, or severe inflammation were selected as control tissues (5 females, ages 41, 48, 56, 52, and 66 years; 5 males, age 57, 45, 57, 58, and 48 years).

Measurement of Hepatocyte Viability

Cells were plated and treated as described above. Cell death was evaluated by measuring the release of alanine aminotransferase (ALT) into the medium as an indicator of necrotic cell death, as described previously.[25] Cleavage of the Ac-DEVD-AMC substrate was measured to quantify caspase 3 activity. Briefly, cells were lysed with cell lysis buffer (50 mmol/L HEPES, pH 7.4; 100 mmol/L NaCl; 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 0.1 mmol/L ethylene-diamine-tetraacetic acid; 1 mmol/L dithiothreitol). The lysate was centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was collected and incubated with 200 μmol/L of Ac-DEVD-AMC substrate (BD Biosciences San Diego, CA) in assay buffer (50 mmol/L HEPES (pH 7.4); 100 mmol/L NaCl; 0.1% CHAPS; 0.1 mmol/L ethylene-diamine-tetraacetic acid; 10% glycerol; 10 mmol/L dithiothreitol). Cleavage of the Ac-DEVD-AMC substrate was measured kinetically by liberation of AMC using a Synergy 2 plate reader (BioTek, Winooski, VT) (380 nm excitation and 430–460 nm emission wavelengths).

Table 1.   Sequences of Real-Time PCR Primers

Gene	Forward primer	Reverse primer
Mouse *Egr-1*	5′-GGCAGAGGAAGACGATGAAG-3′	5′-GACGAGTTATCCCAGCCAAA-3′
MIP-2	5′-CTCAGACAGCGAGGCACATC-3′	5′-CCTCAACGGAAGAACCAAAGAG-3′
Mouse *ICAM-1*	5′-CGACGCCGCTCAGAAGAA-3′	5′-GTCTCGGAAGGGAGCCAAGTA-3′
Mouse *18S*	5′-TTGACGGAAGGGCACCACCAG-3′	5′-GCACCACCACCCACGGAATCG-3′
Mouse *PAI-1*	5′-AGTCTTTCCGACCAAGAGCA-3′	5′-ATCACTTGCCCCATGAAGAG-3′
VCAM-1	5′-TGGCTGTGACTCCCCTTCTTT-3′	5′-AGAGCTCAACACAAGCGTGGA-3′
Ccl7	5′-AAGATCCCCAAGAGGAATCTCA-3′	5′-CAGACTTCCATGCCCTTCTTT-3′
Human *18S*	5′-AACTTTCGATGGTCTCGCCG-3′	5′-CCTTGGATGTGGTAGCGTTT-3′
Human *Egr-1*	5′-TACTCCTCTGTTCCCCCTGCTT-3′	5′-GAAAAGGTTGCTGTCATGTCCG-3′
Human *ICAM-1*	5′-CGGCTGACGTGTGCAGTAATAC-3′	5′-GGCTTCGTCAGAATCACGTTG-3′
IL-8	5′-TCCTTGTTCCACTGTGCCTTG-3′	5′-TGCTTCCACATGTCCTCACAA-3′
Human *PAI-1*	5′-GTTCATTGCTGCCCCTTATGAA-3′	5′-AGCCTGGTCATGTTGGCCTTTC-3′
Snail	5′-TTTTGCTGACCGCTCCAAC-3′	5′-TGCTTGTGGAGCAAGGACAT-3′

Real-Time PCR

RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO), and was reverse-transcribed into cDNA, as we have described previously.[24] Real-time PCR was performed on an ABI 7900 real-time PCR instrument (Applied Biosystems, Foster City, CA) with the ABI SYBR Green DNA PCR kit. Sequences of primers are listed in Table 1. A customized TaqMan qPCR array (Applied Biosystems) was used to measure mRNA levels of the genes listed in Supplemental Table S1 (see http://www.ajp.amjpathol.org). PCR was performed according to the manufacturer's recommendations.

Immunohistochemistry

Sections of frozen liver were incubated with rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1) antibody diluted 1:50 (Millipore Bioscience Research Reagents, Temecula, CA). The sections were washed and incubated with goat anti-rat conjugated to Alexa 594 (Invitrogen, Carlsbad, CA).

Protein Quantification

Hepatocytes were lysed in RIPA (radio-immunoprecipitation assay) buffer containing complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor (both from Roche Applied Science, Indianapolis, IN). Equal amounts of protein were separated on a 7.5% or 4.5%–15% gradient Criterion polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was incubated with anti-ICAM-1 antibody (R&D Systems, Minneapolis, MN), anti-Egr-1 antibody (Cell Signaling Technology, Danvers, MA) or anti-β-actin antibody (Sigma-Aldrich), followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized using the Immun-Star HRP Substrate Kit (Bio-Rad). Macrophage inflammatory protein-2 (MIP-2) was measured in cell culture medium using the Bio-Plex assay (Bio-Rad), according to the manufacturer's instructions.

Quantification of Liver Injury in BDL Mice

ALT was measured in serum using a commercially available kit, as described previously.[24]

Quantification of Neutrophil Accumulation and Extravasation

Neutrophil accumulation and extravasation were quantified as described previously.[5]

Statistical Analysis

Results are presented as the mean $\pm$ SEM. Data were analyzed by analysis of variance or *t*-test. Analysis of variance was performed on $\log_x$-transformed data in instances in which variances were not homogeneous. Comparisons among group means were made using the Student-Newman-Keuls test. Spearman correlation was performed to identify correlations between Egr-1 mRNA levels and mRNA levels of other inflammatory mediators in human livers. The criterion for significance was $P < 0.05$ for all studies.

Results

Neutrophil Accumulation and Extravasation in the Livers of BDL Mice Occurs Independently of TLR4 Signaling

To determine whether TLR4 is required for stimulation of inflammation in the liver during cholestasis, C3H/HeJ mice, which have an inactivating mutation in the TLR4 gene,[26] and C3Heb/FeJ mice, TLR4 wild type, were subjected to BDL. Plasma ALT activity and % area of liver necrosis after BDL were unaffected in C3H/HeJ mice, consistent with previous studies (Figure 1).[27] Similarly, total numbers of neutrophils in the liver and numbers of extravasated neutrophils after BDL were unaffected in C3H/HeJ mice (Figure 1). These results suggest that LPS does not initiate inflammation through activation of TLR4 during cholestasis.
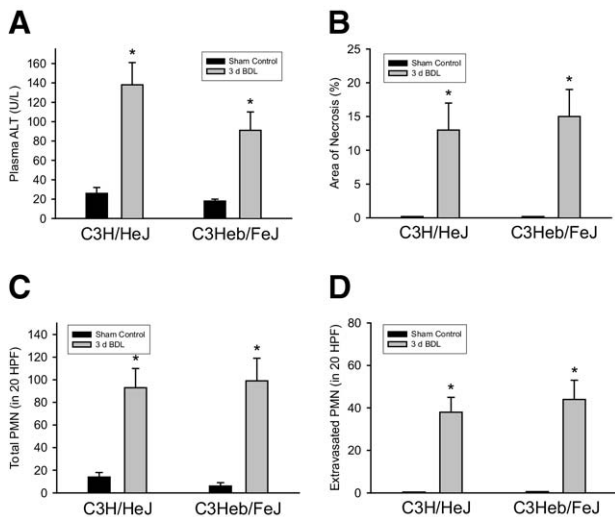
Figure 1. Role of Toll-like receptor 4 (TLR4) in inflammation in the liver during cholestasis. C3H/HeJ (TLR4 mutant) and C3Heb/FeJ (TLR4 wild-type) mice were subjected to bile duct ligation (BDL) or sham operation. Three days later, plasma alanine aminotransferase (ALT) (**A**), % area of necrosis (**B**), neutrophil (PMN) accumulation (**C**), and PMN extravasation (**D**) were measured. Data are expressed as mean ± SEM; $n = .6$. Values significantly different (*$P < 0.05$) from sham-operated mice. HPF, high-power field.

Bile Acids Increase Expression of ICAM-1 and MIP-2 in Hepatocytes

Bile acids activate several signaling pathways in hepatocytes.[28–30] Accordingly, we next determined whether bile acids increase expression of proinflammatory mediators in hepatocytes. For this study, hepatocytes were exposed to DCA, chenodeoxycholic acid (CDCA), and taurocholic acid (TCA). DCA was chosen because several studies have shown that DCA activates signal transduction pathways in hepatocytes.[28–30] CDCA was chosen because serum levels of CDCA increase in BDL mice.[14] TCA was chosen because serum levels of cholic acid increase in BDL mice,[14] and because we recently determined that >99% of cholic acid is conjugated with taurine (Copple, Jaeschke, and Klaassen, unpublished data). Exposure of primary mouse hepatocytes to DCA, CDCA, or TCA increased mRNA levels of ICAM-1 and macrophage inflammatory protein-2 (MIP-2), a neutrophil chemokine involved in cholestatic liver injury (Figure 2, A and B).[31]

Bile Acids Increase Expression of ICAM-1 and MIP-2 in Hepatocytes Independent of Effects on Viability

Next we investigated the mechanism by which bile acids increase expression of ICAM-1 and MIP-2 in hepatocytes. Studies have demonstrated that bile acids kill hepatocytes by apoptosis and necrosis *in vitro*.[28] Apoptotic bodies and proteins released from necrotic cells have been shown to increase expression of proinflammatory mediators in some cells types.[32] Accordingly, we next determined whether bile acids caused apoptosis or

necrosis in our studies and whether apoptosis contributed to the increase in ICAM-1 and MIP-2 mRNAs in bile acid-treated hepatocytes. Exposure of hepatocytes to DCA, CDCA, and TCA did not increase caspase 3 activity (Figure 3A), stimulate ALT release (Figure 3B), or cause morphological changes consistent with apoptosis or necrosis (Figure 3, C–F). Lastly, treatment of hepatocytes with quinoline-val-asp-difluorophenoxymethylketone, a pan-caspase inhibitor, did not prevent up-regulation of MIP-2 or ICAM-1 by DCA (Figure 3, G and H).

Farnesoid X Receptor Is Not Required for Up-Regulation of ICAM-1 and MIP-2 in BDL Mice

Previous studies demonstrated that FXR regulates ICAM-1 in HepG2 cells.[33] To determine whether this occurs *in vivo* during cholestasis, wild-type and FXR knockout mice were subjected to BDL. Bile duct ligation increased ICAM-1 mRNA levels to the same extent in wild-type and FXR knockout mice (Figure 4A). MIP-2 mRNA levels were increased to a greater extent in FXR knockout mice subjected to BDL, compared with BDL wild-type mice (Figure 4B). Hepatic neutrophil numbers were not significantly different between BDL wild-type and FXR knockout mice (Figure 4C).
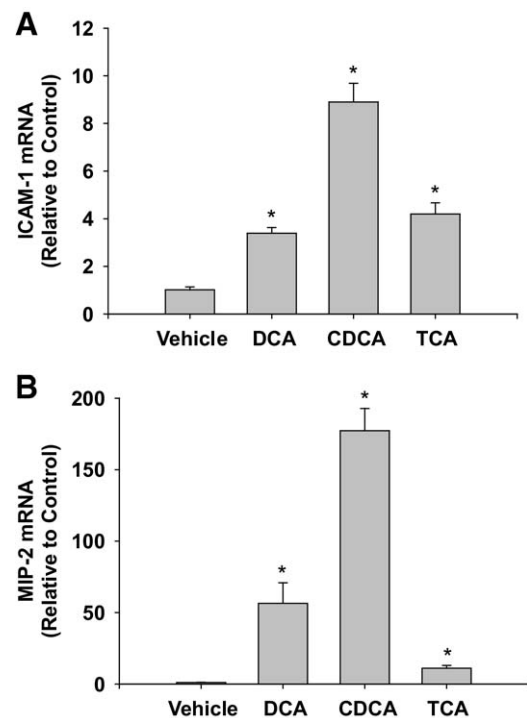
Figure 2. Up-regulation of intercellular adhesion molecule-1 (ICAM-1) and macrophage inflammatory protein-2 (MIP-2) in bile acid-treated hepatocytes. Hepatocytes were isolated from mice and treated with 200 μmol/L deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), or taurocholic acid (TCA). Six hours later, mRNA levels of ICAM-1 (**A**) and MIP-2 (**B**) were quantified by real-time PCR. Data are expressed as mean ± SEM; $n = 3$. Values significantly different (*$P < 0.05$) from vehicle-treated hepatocytes.
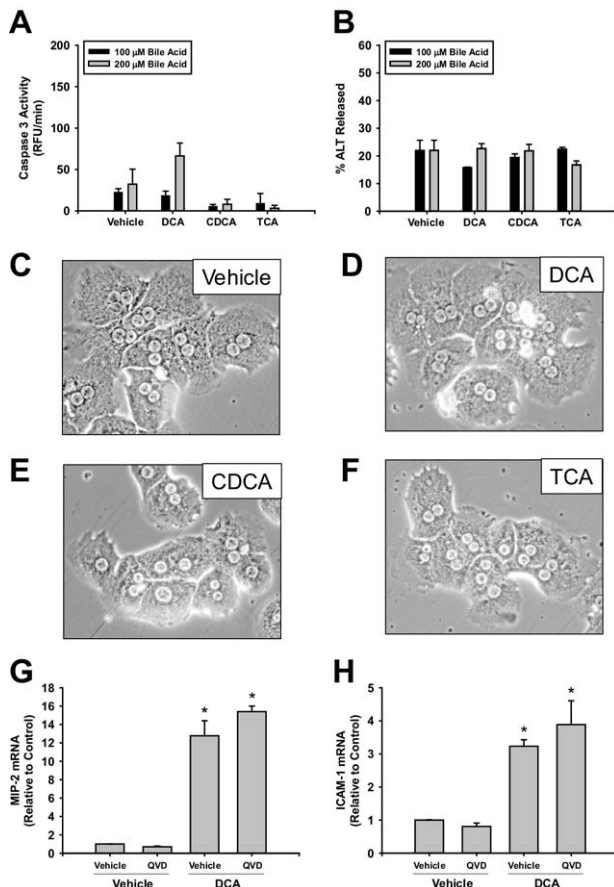
Figure 3. Bile acids do not affect the viability of hepatocytes. Hepatocytes were isolated from mice and treated with 200 μmol/L DCA, CDCA, or TCA. Six hours later, activities of caspase 3 (**A**) and ALT (**B**) were measured. Data are expressed as mean ± SEM; *n* = 3. Photomicrographs (200X) of cells treated with vehicle (**A**), DCA (**B**), CDCA (**C**), or TCA (**D**) were taken 6 hours after treatment. Primary hepatocytes were isolated and pretreated with quinoline-val-asp-difluorophe-noxymethylketone (QVD) or dimethyl sulfoxide vehicle followed by treatment with DCA. mRNA levels of ICAM-1 (**G**) and MIP-2 (**H**) were measured 6 hours later. Data are expressed as mean ± SEM; *n* = 3. Values significantly different (*P < 0.05) from vehicle-treated hepatocytes.

Up-Regulation of ICAM-1 and MIP-2 in Hepatocytes by Bile Acids Requires Early Growth Response Factor-1

Next, we investigated whether up-regulation of ICAM-1 and MIP-2 requires the transcription factor Egr-1. Exposure of primary mouse hepatocytes to DCA, CDCA, or TCA increased Egr-1 mRNA and protein (Figure 5, A and B). Exposure of hepatocytes isolated from wild-type mice to DCA, CDCA, or TCA increased mRNA and protein levels of ICAM-1 and MIP-2 (Figure 5, C–F). Up-regulation of ICAM-1 and MIP-2 by bile acids was attenuated in hepatocytes isolated from Egr-1 knockout mice (Figure 5, C–F).

Bile Acids Increase Expression of Several Proinflammatory Genes in Hepatocytes by Egr-1-Dependent and -Independent Mechanisms

We next determined whether inflammatory genes other than ICAM-1 and MIP-2 are up-regulated in bile acid-

treated hepatocytes in an Egr-1-dependent manner. The genes that were measured are listed in Supplemental Table S1 (see *http://www.ajp.amjpathol.org*). The results of these studies are summarized in Tables 2, 3, and 4 (only those genes that were increased by bile acid treatment are included in the tables). DCA treatment of hepatocytes isolated from wild-type mice increased mRNA levels of several chemokines, including CXCL1, CXCL10, CXCL11, Ccl2, Ccl5, Ccl7, and Ccl20 (Table 2). In addition, DCA treatment increased mRNA levels of VCAM-1, plasminogen activator inhibitor-1 (PAI-1), uroki-
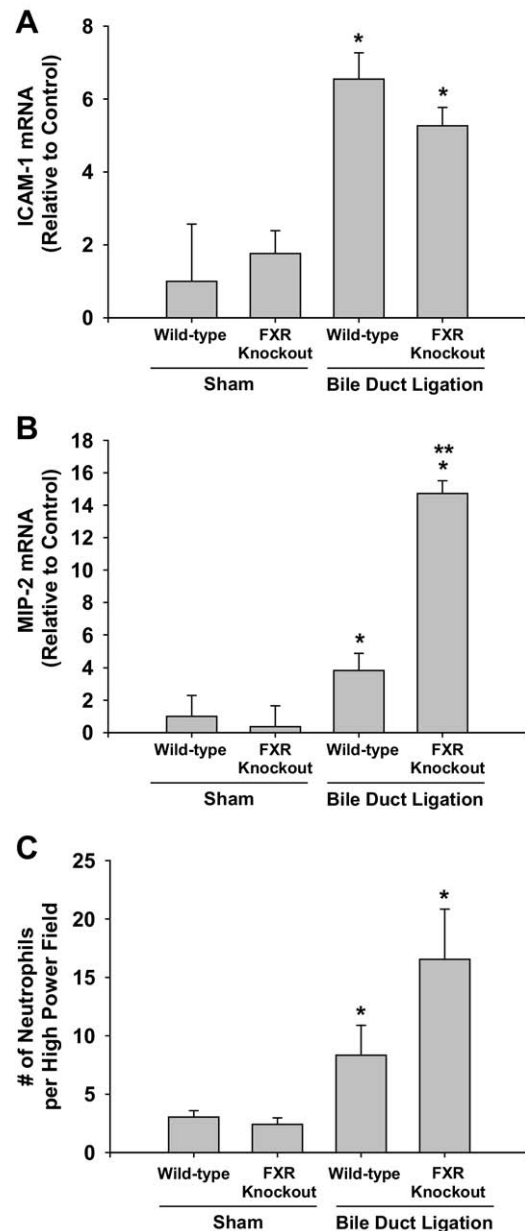
Figure 4. Role of FXR in inflammation in the liver during cholestasis. Wild-type and farnesoid X receptor (FXR) knockout mice were subjected to BDL or sham operation. Three days later, mRNA levels of ICAM-1 (**A**) and MIP-2 (**B**) were quantified and hepatic neutrophil accumulation (**C**) was measured. Values significantly different (*P < 0.05) from sham-operated mice; and significantly different (**P < 0.05) from wild-type mice subjected to BDL.
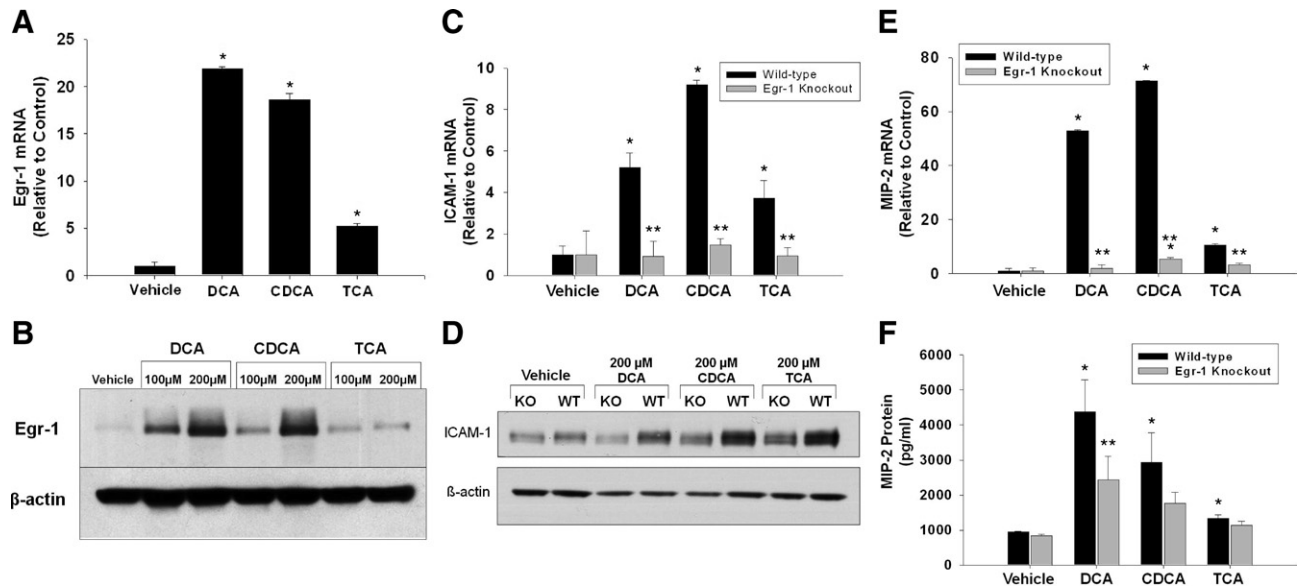
Figure 5. Role of early growth response factor-1 (Egr-1) in up-regulation of ICAM-1 and MIP-2 in hepatocytes. Hepatocytes were isolated from mice and treated with 200 μmol/L DCA, CDCA, or TCA. Two hours later, Egr-1 mRNA (**A**) and protein (**B**) were measured. Data are expressed as mean ± SEM; *n* = 3. Values significantly different (*P* < 0.05) from vehicle-treated hepatocytes are marked **a**. Hepatocytes were isolated from wild-type or Egr-1 knockout mice. Six hours later, (**C**) ICAM-1 and (**E**) MIP-2 mRNAs were measured. Eight hours later, ICAM-1 (**D**) and MIP-2 (**F**) protein levels were measured. Data are expressed as mean ± SEM; *n* = 3. Values significantly different (**P* < 0.05) from vehicle-treated hepatocytes; and significantly different (***P* < 0.05) from wild-type hepatocytes treated with bile acids.

nase plasminogen activator receptor, and cyclooxygenase-2 (COX-2). Lastly, DCA treatment increased levels of two hematopoietic growth factors, granulocyte macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF). Of the genes up-regulated by DCA, those encoding CXCL1, Ccl7, PAI-1, and GM-CSF were Egr-1-dependent (Table 2).

Exposure of wild-type hepatocytes to CDCA up-regulated all of the same proinflammatory genes as DCA, and in addition increased levels of CXCL16 and Ccl3 (Table 3). CDCA up-regulation of CXCL1, Ccl7, PAI-1, and GM-

CSF was partially Egr-1-dependent, similar to DCA (Table 3).

Treatment of wild-type hepatocytes with TCA increased mRNA levels of numerous proinflammatory genes. The profile of mRNA changes was different from the changes that occurred after treatment with the unconjugated bile acids DCA and CDCA. Treatment of hepatocytes with TCA increased mRNA levels of several chemokine genes, including CXCL1, CXCL 5, CXCL10, CXCL11, CXCL13, CXCL16, Ccl2, Ccl3, Ccl4, Ccl5, Ccl7, Ccl20, and also several cytokine genes, including IL-1β

Table 2. Effect of DCA on Proinflammatory Gene Expression in Hepatocytes Isolated from WT Mice and Egr-1 Knockout Mice

Gene	Other names	Function	WT vehicle	Egr-1 knockout vehicle	WT DCA	Egr-1 knockout DCA
Cxcl1	KC	Neutrophil chemokine	1.1 ± 0.2	1.1 ± 0.3	3.5 ± 0.5*	1.6 ± 0.4[†]
Cxcl10	IP-10	T-cell chemokine	1.0 ± 0.2	1.0 ± 0.2	47.0 ± 11.6*	33.9 ± 14.1*
Cxcl11	I-TAC	T-cell chemokine	1.2 ± 0.2	1.1 ± 0.4	16.7 ± 7.7*	8.1 ± 3.6
Ccl2	MCP-1	Monocyte and T-cell chemokine	1.1 ± 0.2	1.0 ± 0.1	12.5 ± 1.9*	8.6 ± 3.7*
Ccl5	RANTES	T-cell, eosinophil, and mast-cell chemokine	1.0 ± 0.2	1.0 ± 0.1	3.5 ± 1.1*	4.4 ± 1.9*
Ccl7	MCP-3	Monocyte, T-cell, neutrophil, and eosinophil chemokine	1.1 ± 0.3	1.0 ± 0.1	9.7 ± 1.1*	2.7 ± 0.9[†]
Ccl20	MIP-3α (*LARC*)[‡]	T-cell and B-cell chemokine	1.2 ± 0.4	2.7 ± 2.3	6.5 ± 3.5	13.1 ± 2.0*
Vcam1	CD106	Adhesion molecule	1.0 ± 0.2	1.0 ± 0.1	7.3 ± 1.36*	4.6 ± 1.9*
Serpine1	PAI-I	Plasminogen activator inhibitor	1.0 ± 0.02	1.0 ± 0.1	4.3 ± 1.0*	1.0 ± 0.5[†]
Plaur	uPAR	uPA receptor	1.2 ± 0.1	1.0 ± 0.03	2.3 ± 0.2*	2.7 ± 0.2*
Ptgs2	COX-2	Arachidonic acid metabolism	1.0 ± 0.2	1.0 ± 0.1	2.9 ± 0.5*	2.6 ± 0.9*
Csf2	GM-CSF	Hematopoietic growth factor	1.2 ± 0.4	1.0 ± 0.2	6.6 ± 1.0*	2.4 ± 0.3[†]
Csf3	G-CSF	Hematopoietic growth factor	2.1 ± 1.6	0.2 ± 0.1	2.6 ± 1.0*	16.6 ± 2.9*[†]

Primary mouse hepatocytes were isolated from WT or Egr-1 knockout mice and treated with 200 μmol/L DCA. Six hours later, total mRNA was isolated and proinflammatory gene expression measured by quantitative real-time PCR. COX-2, cyclo-oxygenase-2; DCA, deoxycholic acid; uPAR, urokinase plasminogen activator receptor; WT, wild type.
*Significantly different (*P* < 0.05) from vehicle-treated hepatocytes.
[†]Significantly different (*P* < 0.05) from WT hepatocytes treated with DCA.
[‡]Human synonym given in parenthesis.

Table 3. Effect of CDCA on Proinflammatory Gene Expression in Hepatocytes Isolated from WT Mice and Egr-1 Knockout Mice

Gene	Other names	Function	WT vehicle	Egr-1 knockout vehicle	WT CDCA	Egr-1 knockout CDCA
Cxcl1	KC	Neutrophil chemokine	1.1 ± 0.2	1.1 ± 0.3	7.1 ± 1.4*	4.1 ± 1.6*
Cxcl10	IP-10	T-cell chemokine	1.0 ± 0.2	1.0 ± 0.2	92.7 ± 21.4*	102.1 ± 33.8*
Cxcl11	I-TAC	T-cell chemokine	1.2 ± 0.2	1.1 ± 0.4	49.4 ± 14.2*	44.7 ± 15.0*
Cxcl16	SR-PSOX	T-cell chemokine	1.0 ± 0.04	1.0 ± 0.2	3.6 ± 0.2*	3.7 ± 1.6*
Ccl2	MCP-1	Monocyte and T-cell chemokine	1.1 ± 0.2	1.0 ± 0.1	26.5 ± 1.3*	20.1 ± 2.8*†
Ccl3	MIP-1α	Monocyte and T-cell chemokine	1.0 ± 0.2	1.0 ± 0.2	2.1 ± 0.3*	2.8 ± 0.3*
Ccl5	RANTES	T-cell, eosinophil, and mast cell chemokine	1.0 ± 0.2	1.0 ± 0.1	6.5 ± 1.7*	8.2 ± 3.1*
Ccl7	MCP-3	Monocyte, T-cell, neutrophil, and eosinophil chemokine	1.1 ± 0.3	1.0 ± 0.1	26.1 ± 5.0*	10.3 ± 4.8*†
Ccl20	MIP-3α (*LARC*)‡	T-cell and B-cell chemokine	1.2 ± 0.4	2.7 ± 2.3	27.2 ± 11.4*	34.2 ± 8.8*
Vcam1	CD106	Adhesion molecule	1.0 ± 0.2	1.0 ± 0.1	21.9 ± 6.3*	10.7 ± 4.9*
Serpine1	PAI-1	Plasminogen activator inhibitor	1.0 ± 0.02	1.0 ± 0.1	6.6 ± 1.6*	3.4 ± 0.1*†
Plaur	uPAR	uPA receptor	1.2 ± 0.1	1.0 ± 0.03	3.9 ± 0.1*	3.87 ± 0.4*
Ptgs2	COX-2	Arachidonic acid metabolism	1.0 ± 0.2	1.0 ± 0.1	5.1 ± 1.3*	3.5 ± 1.8*
Csf2	GM-CSF	Hematopoietic growth factor	1.2 ± 0.4	1.0 ± 0.2	20.2 ± 4.1*	4.7 ± 1.5*†
Csf3	G-CSF	Hematopoietic growth factor	2.1 ± 1.6	0.2 ± 0.1	3.6 ± 1.5*	6.5 ± 3.5*

Primary mouse hepatocytes were isolated from WT or Egr-1 knockout mice and treated with 200 μmol/L CDCA. Six hours later, total mRNA was isolated and pro-inflammatory gene expression measured by quantitative real-time PCR. CDCA, chenodeoxycholic acid; COX-2, cyclo-oxygenase-2; uPAR, urokinase plasminogen activator receptor; WT, wild type.
*Significantly different ($P < 0.05$) from vehicle-treated hepatocytes.
†Significantly different ($P < 0.05$) from WT hepatocytes treated with CDCA.
‡Human synonym given in parenthesis.

and IL-10 (Table 4). In addition, mRNA levels of VCAM-1, COX-2, GM-CSF, and G-CSF genes were increased in hepatocytes after TCA treatment. The increase in Ccl2, Ccl7, Ccl20, IL-1β, VCAM-1, and GM-CSF mRNAs were partially Egr-1-dependent, whereas changes in CXCL1 and CXCL13 were completely Egr-1-dependent (Table 4).

Up-Regulation of PAI-1, VCAM-1, Ccl7, and Snail in the Livers of BDL Mice Requires Egr-1

Next, we determined whether the inflammatory genes that were up-regulated in bile acid-treated hepatocytes in an Egr-1-dependent manner (Tables 2–4), were also in-

Table 4. Effect of TCA on Proinflammatory Gene Expression in Hepatocytes Isolated from WT Mice and Egr-1 Knockout Mice

Gene	Other names	Function	WT vehicle	Egr-1 knockout vehicle	WT TCA	Egr-1 knockout TCA
Cxcl1	KC	Neutrophil chemokine	1.1 ± 0.2	1.1 ± 0.3	3.1 ± 0.2*	1.6 ± 0.2†
Cxcl5	LIX, GCP-2	Neutrophil chemokine	1.1 ± 0.4	3.5 ± 1.9	2.7 ± 0.2	10.2 ± 2.6*†
Cxcl10	IP-10	T-cell chemokine	1.0 ± 0.2	1.0 ± 0.2	5.18 ± 0.8*	5.9 ± 0.7*
Cxcl11	I-TAC	T-cell chemokine	1.2 ± 0.2	1.1 ± 0.4	5.9 ± 0.9*	4.9 ± 1.9*
Cxcl13	BLC	B-cell arrest chemokine	1.2 ± 0.4	1.2 ± 0.3	4.8 ± 1.1 *	1.3 ± 0.2*†
Cxcl16	SR-PSOX	T-cell chemokine	1.0 ± 0.04	1.0 ± 0.2	1.8 ± 0.2	2.0 ± 0.5*
Ccl2	MCP-1	Monocyte and T-cell chemokine	1.1 ± 0.2	1.0 ± 0.1	5.2 ± 0.1*	3.6 ± 0.5*†
Ccl3	MIP-1α	Monocyte and T-cell chemokine	1.0 ± 0.2	1.0 ± 0.2	7.3 ± 0.7*	9.5 ± 1.6*
Ccl4	Mip-1β	T-cell chemokine	1.1 ± 0.3	1.0 ± 0.0	7.6 ± 0.6*	8.9 ± 1.0*
Ccl5	RANTES	T-cell, eosinophil, and mast-cell chemokine	1.0 ± 0.2	1.0 ± 0.1	1.6 ± 0.2	2.1 ± 0.2*
Ccl7	MCP-3	Monocyte, T-cell, neutrophil, and eosinophil chemokine	1.1 ± 0.3	1.0 ± 0.1	6.5 ± 0.4*	2.0 ± 0.1*†
Ccl20	MIP-3α (*LARC*)‡	T-cell and B-cell chemokine	1.2 ± 0.4	2.7 ± 2.3	26.8 ± 4.1*	13.0 ± 0.5*†
Il1b	IL-1β	Lymphocyte activator, acute phase response	1.2 ± 0.4	1.0 ± 0.1	17.6 ± 1.4*	4.9 ± 1.9*†
Il10	IL-10 (*CSIF*)‡	Antiinflammatory cytokine	1.2 ± 0.4	1.3 ± 0.5	8.2 ± 1.3*	6.3 ± 0.9*
Vcam1	CD106	Adhesion molecule	1.0 ± 0.2	1.0 ± 0.1	12.0 ± 1.2*	3.8 ± 1.9*†
Ptgs2	COX-2	Arachidonic acid metabolism	1.0 ± 0.2	1.0 ± 0.1	6.9 ± 0.3*	8.7 ± 1.5*
Csf2	GM-CSF	Hematopoietic growth factor	1.2 ± 0.4	1.0 ± 0.2	7.2 ± 1.6*	2.0 ± 0.7*†
Csf3	G-CSF	hematopoietic growth factor	2.1 ± 1.6	0.2 ± 0.1	6.0 ± 1.8*	33.6 ± 5.8*

Primary mouse hepatocytes were isolated from WT or Egr-1 knockout mice and treated with 200 μmol/L TCA. Six hours later, total mRNA was isolated and pro-inflammatory gene expression measured by quantitative real-time PCR. COX-2, cyclo-oxygenase-2; TCA, taurocholic acid; WT, wild type.
*Significantly different ($P < 0.05$) from vehicle-treated hepatocytes.
†Significantly different ($P < 0.05$) from WT hepatocytes treated with TCA.
‡Human synonym given in parenthesis.
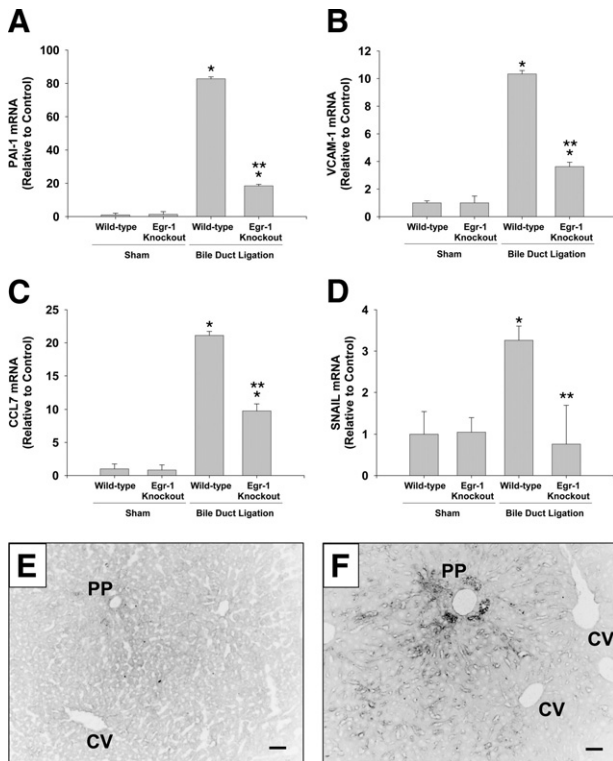
Figure 6. Role of Egr-1 in up-regulation of PAI-1, VCAM-1, CCL7, and SNAIL in the liver during cholestasis. Wild-type and Egr-1 knockout mice were subjected to BDL or sham operation. Seven days later, mRNA levels of PAI-1 (**A**), VCAM-1 (**B**), CCL7 (**C**), and SNAIL (**D**) were quantified by real-time PCR. Values significantly different (*$P < 0.05$) from sham-operated mice; and significantly different (**$P < 0.05$) from wild-type mice subjected to BDL. VCAM-1 protein was detected in sections of liver from sham-operated (**E**) and BDL (**F**) mice. Scale bar = 50 μm. PP, periportal zone; CV central vein.

creased in BDL mice in an Egr-1-dependent manner. BDL in Egr-1 knockout mice for 10 days resulted in decreased areas of bile infarcts and reduced ALT activity, confirming our previous studies at 14 days after BDL (data not shown).[24] BDL increased expression of PAI-1, VCAM-1, and Ccl7 mRNAs to a greater extent in the livers of wild-type mice than in Egr-1 knockout mice (Figure 6, A–C).

Levels of Snail are increased in bile acid-treated hepatocellular carcinoma cells[34] and in the liver after BDL.[35] Studies have demonstrated that Egr-1 regulates Snail in some cell types.[36] Accordingly, we determined whether up-regulation of Snail in the liver after BDL requires Egr-1. mRNA levels of Snail increased after BDL (Figure 6D). Up-regulation of Snail was completely prevented in Egr-1 knockout mice (Figure 6D).

Minimal immunostaining for VCAM-1 was observed in sham-operated livers (Figure 6E). In liver sections from BDL mice, VCAM-1 immunostaining was observed on bile duct epithelial cells and within the sinusoids, primarily in periportal regions of liver (Figure 6F).

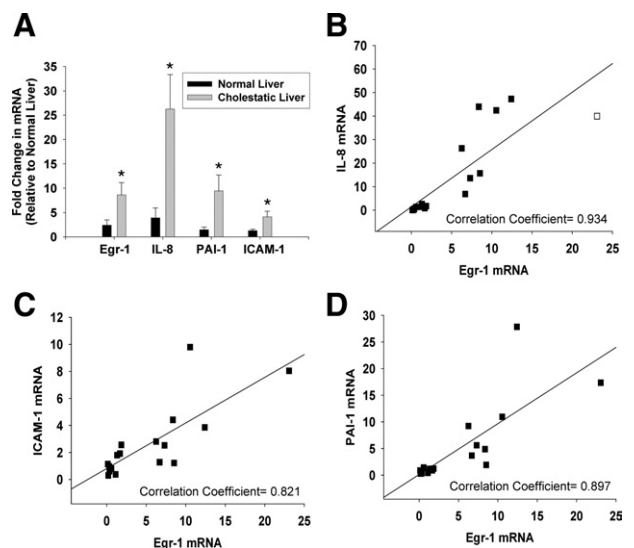## Up-Regulation of Egr-1, PAI-1, ICAM-1, and IL-8 in Livers of Humans with Cholestatic Liver Disease

Human liver was obtained from control donors and from patients with primary biliary cirrhosis or primary scleros-

ing cholangitis. Levels of Egr-1, PAI-1, ICAM-1, and IL-8 mRNAs were increased in livers of patients with cholestasis from these diseases (Figure 7A). In addition, Spearman correlation indicated a significant correlation between Egr-1 mRNA levels in these patients and mRNA levels of IL-8, PAI-1, and ICAM-1 (Figure 7, B–D).

Discussion

LPS Does Not Promote Inflammation in the Liver during Acute Phases of Cholestasis

It has been proposed that bacterial LPS may be an important stimulus for inflammation in the liver during cholestasis.[37,38] Studies have demonstrated that reduced bile flow increases translocation of bacteria and LPS from the gut, which may stimulate proinflammatory gene expression in the liver.[38] In BDL animals, however, up-regulation of inflammatory mediators that affect neutrophil accumulation (eg, MIP-2 and ICAM-1) and hepatic neutrophil accumulation occur within the first 24 hours, whereas plasma LPS levels are not increased at this time.[31,37–40] Furthermore, Seki et al[27] demonstrated recently that mice deficient in TLR4 (the receptor for LPS) and mice deficient in TLR4 on immune cells are not protected from liver injury after BDL, suggesting that gut-derived LPS is not necessary for neutrophil-dependent injury during cholestasis. Consistent with this report, our present data demonstrate the novel finding that neutrophil accumulation and extravasation in the liver 3 days after BDL is not affected in LPS-resistant C3H/HeJ mice (Figure 1). Collectively, these data suggest that activation

Figure 7. Up-regulation of inflammatory mediators in the livers of humans with cholestatic liver disease. **A:** Real-time PCR was used to measure mRNA levels of Egr-1, ICAM-1, IL-8, and PAI-1 in normal human livers and in cholestatic livers from patients with primary biliary cirrhosis and primary sclerosing cholangitis. Values significantly different (*$P < 0.05$) from normal liver. The mRNA levels of IL-8 (**B**), ICAM-1 (**C**), and PAI-1 (**D**) were plotted against mRNA levels of Egr-1. Spearman correlation indicated a significant correlation (specified in each panel) between Egr-1 mRNA levels and mRNA levels of ICAM-1, IL-8, and PAI-1 in normal human livers and liver from patients with cholestatic liver disease at $P < 0.05$.

of macrophages by LPS, and subsequent release of proinflammatory cytokines such as tumor necrosis factor-α, is not important for the acute phase of inflammation that occurs during cholestasis. Consistent with this finding, tumor necrosis factor-α mRNA levels are not increased in hepatic macrophages at 24 hours after BDL, and depletion of macrophages does not protect from liver injury after BDL.[41,42] All together, the present data and these studies suggest that during early stages of cholestasis the activation of hepatic macrophages by LPS from the gut is not important for inflammatory liver injury.

Bile Acid-Induced Hepatocyte Cell Death Is Not a Stimulus for Inflammation in the Liver during Cholestasis

Studies have demonstrated that DAMPs released from necrotic cells, such as high-mobility group box 1 protein, activate macrophages by a TLR4-dependent mechanism that stimulates a sterile inflammatory response.[43] This mechanism is important for inflammation in the liver after hepatic ischemia-reperfusion.[44] Our results suggest, however, that this mechanism is not responsible for initiation of inflammation in the liver during cholestasis. In BDL mice, serum concentrations of cholic acid and muricholic acid reach approximately 200–250 μmol/L by 7 days after BDL.[14] We recently determined that >99% of cholic acid in the serum of BDL mice is conjugated with taurine (Copple, Jaeschke, and Klaassen, unpublished data). In the present studies, exposure of hepatocytes to 200 μmol/L TCA, a concentration that occurs in BDL mice,[14] did not produce hepatocyte cell death (Figure 3). Serum concentrations of CDCA and its conjugates increase to approximately 4 μmol/L in BDL mice,[14] and in our studies concentrations as high as 200 μmol/L did not produce toxicity in cultured hepatocytes (Figure 3). Similarly, exposure of hepatocytes to 200 μmol/L DCA did not produce toxicity in our studies (Figure 3). Because concentrations of DCA typically decrease during cholestasis,[14,15] however, it is unlikely that it contributes to liver toxicity and initiation of inflammation during cholestasis.

We also demonstrated that bile acid-dependent up-regulation of ICAM-1 and MIP-2 was independent of caspase activation (Figure 3). These results are consistent with previous studies in which the pan-caspase inhibitor z-Val-Ala-Asp-fluoromethylketone did not prevent inflammation or attenuate liver injury in BDL mice.[45] Collectively, these studies demonstrate that exposure of hepatocytes to bile acids, which increase in serum during cholestasis, do not directly cause cell death and therefore are unlikely to initiate a sterile inflammatory response. Consistent with this finding, inflammation was not affected in TLR4 mutant mice (Figure 1), further supporting the idea that DAMPs released from dead cells are not responsible for initiating inflammation during cholestasis.

Bile Acids Stimulate Production of Inflammatory Mediators by Hepatocytes

Our results indicate that LPS from the gut and DAMPs released from necrotic cells do not stimulate production of proinflammatory cytokines during early stages of cholestasis, suggesting that another mediator triggers inflammation during cholestasis. Furthermore, this other stimulus elicits inflammatory mediator production from cell types other than macrophages, because depletion of macrophages does not attenuate injury after BDL.[41,42] Hepatocytes are exposed to high concentrations of bile acids during cholestasis,[14,15] and bile acids activate numerous signaling pathways in hepatocytes.[29,30,46] We therefore next investigated whether bile acids stimulate production of proinflammatory mediators by hepatocytes. Exposure of hepatocytes to bile acids increased levels of numerous mediators, including cytokines (IL-1β, IL-10), chemokines (KC, IP-10, I-TAC, MCP-1, RANTES, MCP-3, MIP-1α, MIP-1α, MIP-2, MIP-3α, LIX, SR-PSOX, MCP-3, BLC, SR-PSOX), adhesion molecules (ICAM-1, VCAM-1), enzymes in arachidonic acid metabolism (COX-2), and other proteins that influence immune cell levels and function (PAI-1, uPAR, G-CSF, GM-CSF) (Tables 2–4). The concentration of TCA that increased expression of these proinflammatory mediators was similar to concentrations that occur in the serum of BDL mice.

Several of the mediators that were up-regulated by bile acids, including ICAM-1, MIP-2, and KC, are known to be involved in neutrophil trafficking in liver and have been shown to contribute to liver injury during cholestasis.[19,31] In addition to neutrophils, studies have shown that T cells and B cells infiltrate the liver during cholestasis.[39] Our studies demonstrated that hepatocytes exposed to bile acids may contribute to the chemokine milieu that is responsible for lymphocyte and neutrophil recruitment into the liver. Although systemic chemokine formation may not always contribute to inflammatory liver injury,[47,48] a more selective chemokine formation by hepatocytes establishing a chemotactic gradient toward the parenchyma has been shown to trigger neutrophil extravasation and injury.[49] Collectively, these results suggest that bile acids at pathological concentrations are inflammatory mediators, and that hepatocytes may be an important source of cytokines and chemokines during cholestasis after exposure to high concentrations of bile acids. In further support of proinflammatory signaling in hepatocytes during cholestasis, recent studies demonstrated that hepatocytes stain positive for the chemokines IL-8, Gro-α, and ENA-78 by immunohistochemistry in the livers of patients with cholestasis.[50] In addition, hepatocyte expression of chemokines has been described in animal models of cholestasis.[51]

Bile Acids Increase Levels of Proinflammatory Mediators in Hepatocytes by Egr-1-Dependent and -Independent Mechanisms

Egr-1-dependent signaling has been shown to enhance inflammation in the vasculature, lungs, and liver.[24,52–55]

We demonstrated previously that Egr-1 expression increases after BDL, and up-regulation of Egr-1 was dependent on activation of Erk1/2 signaling and occurred independently of FXR activation.[24,30] We also demonstrated that Egr-1 knockout mice subjected to BDL exhibited reduced inflammation and liver injury, suggesting a potential role for Egr-1 in regulation of proinflammatory mediator production during cholestasis.[24] These studies did not, however, identify the stimulus for up-regulation of Egr-1, nor did they determine the influence of Egr-1 signaling on expression of proinflammatory mediators in hepatocytes. Accordingly, we determined whether bile acids increase cytokine and chemokine expression in hepatocytes by Egr-1-dependent mechanisms. Of the genes that increased in wild-type hepatocytes after exposure to bile acids, those for ICAM-1, MIP-2, CXCL1, CXCL13, Ccl2, Ccl7, IL-1β, PAI-1, G-CSF, GM-CSF, and VCAM-1 (Tables 2–4) were increased in an Egr-1-dependent manner. In addition, we demonstrated that Ccl7, PAI-1, VCAM-1, ICAM-1, and MIP-2 were increased in BDL mice in an Egr-1-dependent manner, consistent with the *in vitro* studies (Figure 6).[24] VCAM-1 protein levels were increased primarily in periportal regions, where intrahepatic bile acid concentrations would expected to be highest (Figure 6F). This is consistent with results in humans with cholestatic liver disease, where VCAM-1 levels were similarly increased in periportal regions of liver.[56,57]

In addition to findings in mice, our results also demonstrated the novel finding that Egr-1 expression is increased in livers of humans with cholestasis, and that levels of Egr-1 directly correlate with expression of ICAM-1, IL-8 (ie, human homolog of MIP-2 and KC), and PAI-1 (Figure 7). These results suggest that Egr-1 may directly regulate expression of these genes in livers of humans with cholestasis similar to BDL mice. Furthermore, these studies suggest that bile acids may be the stimulus for up-regulation of Egr-1 in the liver during cholestasis.

Recent studies indicated that the bile acid nuclear receptor FXR regulates ICAM-1 in HepG2 cells[33] Our results indicate, however, that FXR is not important for up-regulation of ICAM-1 in the liver during cholestasis, nor is it important for hepatic neutrophil accumulation (Figure 4). In contrast, Egr-1 is required for up-regulation of ICAM-1 in the liver during cholestasis, consistent with our *in vitro* studies (Figure 5).[24] Although these results indicate that FXR is not required for inflammation, results from FXR knockout mice need to be interpreted cautiously. FXR knockouts have higher bile acid concentrations and reduced bile duct proliferation after BDL.[15,23] In addition, hepatocyte necrosis in FXR knockout mice after BDL is pathologically distinct (ie, less bile infarcts) from wild-type mice.[15,23]

For many of the genes, increased expression in bile acid-treated hepatocytes occurred independently of Egr-1, suggesting that other signaling pathways are involved. Bile acids have been shown to activate a multitude of signaling pathways in hepatocytes. For example, mitogen-activated protein kinases are activated in bile acid-treated hepatocytes and in livers of BDL mice.[30] In addition, bile acids activate various isoforms of the pro-

tein kinase C family, p38, c-Jun N-terminal kinase, and pregnane X receptor.[46,58–61] It is possible that bile acids stimulate up-regulation of the proinflammatory genes by activation of one or more of these pathways. In support of this concept, studies have shown that activation of c-Jun N-terminal kinase signaling can up-regulate CXCL10 and RANTES in some cell types, and p38 signaling can up-regulate MIP-1α and COX-2 in some cell types.[62–64] It seems possible, therefore, that the collective up-regulation of these proinflammatory genes by bile acids may require activation of several signaling pathways; however, further studies are needed to specifically identify which signaling pathways are responsible for up-regulation of each mediator. Although nuclear factor-κB is an important regulator of many of these inflammatory mediators, it is unlikely that nuclear factor-κB contributes to up-regulation of inflammatory mediators in hepatocytes by bile acids, because studies have shown that bile acids do not activate nuclear factor-κB in primary hepatocytes.[65]

In summary, the present studies have identified a novel mechanism of inflammation in the liver that occurs independently of LPS and TLR4. Furthermore, these studies have identified bile acids as inflammagens that stimulate production of proinflammatory mediators by hepatocytes through Egr-1-dependent and -independent mecha-
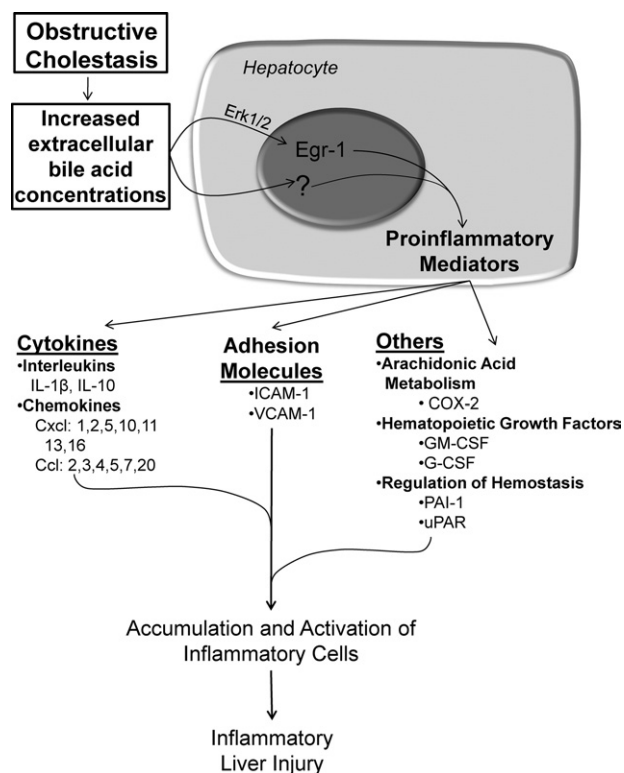
Figure 8. Proposed mechanism of inflammation in the liver during obstructive cholestasis. During obstructive cholestasis, extracellular concentrations of bile acids are increased. Bile acids activate Erk1/2 in hepatocytes which stimulates up-regulation of Egr-1. Egr-1 then regulates production of inflammatory mediators that promote accumulation and activation of inflammatory cells which cause liver injury. Bile acids also up-regulate inflammatory mediators in hepatocytes by an Egr-1-independent mechanism. In addition to hepatocytes, other cell types (not shown) may produce inflammatory mediators that promote inflammatory liver injury during cholestasis.

nisms that promote neutrophil accumulation, extravasation, and activation (Figure 8). In addition, bile acids stimulate production of cytokines that may promote accumulation of other immune cells, such as T cells that could potentially influence liver pathology during cholestasis. Whereas this signaling network has likely evolved to facilitate repair of the liver during acute episodes of cholestasis, during chronic cholestasis the persistent activation of this inflammatory pathway by bile acids promotes injury. Further identification of the signaling pathways that contribute to activation of this novel inflammatory signaling network in hepatocytes could be useful for the development of drugs that limit inflammation in the liver during cholestasis without affecting vital host defense function of the innate immune response.

Acknowledgment

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