Early Growth Response Factor-1 Limits Biliary Fibrosis in a Model of Xenobiotic-Induced Cholestasis in Mice

Bradley P. Sullivan,* Wei Cui,† Bryan L. Copple,*1 and James P. Luyendyk*2

*Department of Pharmacology, Toxicology and Therapeutics and †Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, Kansas, 66160

1Present address: Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824.
2To whom correspondence should be addressed at Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, 3901 Rainbow Boulevard, MS-1018, Kansas City, KS 66160. Fax: (913) 588-7501. E-mail: jluyendyk@kumc.edu.

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Hepatic expression of the transcription factor early growth response-1 (Egr-1) is increased in livers of patients with cholestatic liver disease. Bile acid induction of inflammatory genes in hepatocytes is Egr-1 dependent, and Egr-1 expression is increased in livers of mice after bile duct ligation. Of importance, Egr-1 deficiency reduces liver inflammation and injury in that model. However, it is not known whether Egr-1 promotes inflammation in other models of cholestasis. We tested the hypothesis that Egr-1 contributes to liver inflammation in mice exposed chronically to the bile duct epithelial cell (BDEC) toxicant alpha-naphthylisothiocyanate (ANIT). Egr-1-knockout (Egr-1−/−) mice and wild-type mice were fed a diet containing 0.025% ANIT for 2 weeks. Expression of Egr-1 mRNA and protein was significantly increased in livers of mice fed ANIT diet. Egr-1 deficiency did not significantly affect ANIT diet–induced hepatocellular injury, inflammatory gene induction, BDEC hyperplasia, or hepatic neutrophil accumulation. In contrast, the deposition of Type I collagen was significantly increased in livers of Egr-1−/− mice fed ANIT diet compared with wild-type mice fed ANIT diet. Interestingly, this increase in liver fibrosis occurred in association with elevated expression of the β6 integrin (Itgb6) gene, suggesting the potential for increased local activation of transforming growth factor beta. Taken together, the results indicate that Egr-1 does not contribute to liver injury or inflammation in mice fed a diet containing ANIT. Rather, these studies indicate that Egr-1 deficiency worsens liver fibrosis in conjunction with enhanced expression of the profibrogenic Itgb6 gene.

Key Words: liver; cholestasis; fibrosis; early growth response-1; alpha-naphthylisothiocyanate.
Of importance, bile acids directly stimulate the expression of Egr-1 in cultured mouse primary hepatocytes, and Egr-1 is required for bile acid induction of proinflammatory genes in these cells and in the livers of mice subjected to BDL (Allen et al., 2010, 2011). These studies strongly suggest that Egr-1 participates in the inflammatory response elicited by bile acids during cholestasis.

Chronic tissue injury during cholestasis initiates multiple repair processes, including the production of extracellular matrix (i.e., collagens), which in excess can lead to liver fibrosis. In addition to its role in inflammation, Egr-1 has been shown to play a role in regulating fibrosis in multiple mouse models. For example, Egr-1 deficiency reduced bleomycin-induced dermal and lung fibrosis in mice (Wu et al., 2009). In contrast, levels of hepatic collagen deposition were elevated in Egr-1-knockout mice treated with carbon tetrachloride (CCl4), suggesting that Egr-1 inhibits fibrosis during hepatic injury. However, hepatic collagen deposition was unaffected by Egr-1 deficiency in mice subjected to BDL, despite a marked reduction in liver injury (Kim et al., 2006). Taken together, these findings suggest that the role of Egr-1 in the development of fibrosis is likely tissue and model dependent. In this study, we utilized Egr-1-deficient mice to test the hypothesis that Egr-1 enhances inflammation and fibrosis in mice fed ANIT diet.

**MATERIALS AND METHODS**

**Animal care.** About 8- to 10-week-old female wild-type C57BL/6NTac (Taconic, Germantown, NY) and Egr-1-knockout mice (B6.129-Egr1<sup>m1Jmi</sup>N12; Taconic) backcrossed 11 generations on a C57BL/6NTac background were maintained in an AAALAC-accredited facility at the University of Kansas Medical Center. Mice were housed at an ambient temperature of approximately 22°C with alternating 14/10 h light/dark cycles and provided water and rodent chow *ad libitum* (Teklad 8604; Harlan, Indianapolis, IN) prior to feeding custom diets. All animal procedures were performed according to the guidelines of the American Association for Laboratory Animal Science and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

**ANIT diet and sample collection.** The custom diets were prepared by Dyets, Inc. (Bethlehem, PA). AIN-93M, a purified diet formulated for maintenance of mature rodents, was used as the control diet. The ANIT diet and sample collection.

**RNA isolation, complementary DNA synthesis, and real-time PCR.** Total RNA was isolated from 100 mg of snap-frozen liver using TRI Reagent (Molecular Research Center, Cincinnati, OH), from which 1 μg of complementary DNA (cDNA) was synthesized using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA) on a C100 thermal cycler (Bio-Rad, Hercules, CA). Subsequent real-time PCR analysis was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) and 2X iTaq Supermix (Bio-Rad) as per the manufacturer’s recommended protocol. The mRNA levels of KC growth-regulated protein (KC, Mm00433859_m1), intercellular adhesion molecule 1 (ICAM-1, Mm00516023_m1), monocyte chemotactic protein-1 (MCP-1, Mm00441818_m1), tissue inhibitor of metalloproteinase 1 (TIMP1, Mm00441818_m1), TGF-β1 (Mm00441724_m1), beta 6 integrin (Igb6, Mm00445326_m1), and 18S ribosomal RNA (18S, Hs99999901) were determined using TaqMan gene expression assays (Applied Biosystems), and early growth response factor 1 (Egr-1, NM_007913, forward primer: AGCGCCCTCATCACTCCAAG-3’; reverse primer: 5’-TTTGGCTGGGATACTCCTG-3’; and probe: 5’-56-FAM/CACCTCATT/ ZEN/GAGCACCTGACCCA3ABKQ-3’), collagen 1 (Col1a1, NM_007742, forward primer: 5’-CAT AAA GGG TCA TGG TGG CT-3’, reverse primer: 5’-TTG AGT CCG TCT TGT CCA G-3’, and probe: 5’-56-FAM/TGG TGA ACA/ ZEN/AGG CCC CTC TGG/3ABKQ-3’), and macrophage inflammatory protein 2 (MIP-2, NM_009140, forward primer: 5’-GAA GTC ATA GCC ACT CTC AAG G-3’, reverse primer: 5’-TGG TGA GAC AGC-3’, and probe: 5’-56-FAM/TCC TTT CCA/ ZEN/AGG CAG TTA GCC TTG C/3ABKQ-3’). DNA technologies, Coralville, IA) Levels of each gene were adjusted to the levels of 18S rRNA and the relative expression of each gene was evaluated using the comparative CT method.

**Statistics.** Comparison of two groups was performed using Student’s t-test. Comparison of three or more groups was performed using one- or two-way ANOVA, as appropriate, and the Student-Newman-Keuls post hoc test. Data not conforming to a normal distribution were Log<sub>10</sub> transformed prior to statistical evaluation. The criterion for statistical significance was p < 0.05.
RESULTS

Effect of Egr-1 Deficiency on ANIT Diet–Induced Liver Injury and BDEC Hyperplasia

Compared with wild-type mice fed control diet, hepatic Egr-1 mRNA levels were significantly increased in wild-type mice fed ANIT diet (Supplementary fig. 1A). Nuclear Egr-1 staining was also observed in livers of wild-type mice fed ANIT diet (Supplementary fig. 1). Similar to previous studies, aberrantly high Egr-1 mRNA expression was observed in livers of Egr-1/C0 mice (Supplementary fig. 1). Of importance, we did not detect Egr-1 protein expression in livers of Egr-1/C0 mice fed ANIT diet (Supplementary figs. 1C and 1E). WT and Egr-1/C0 mice fed control diet demonstrated unremarkable morphology (Supplementary figs. 2A and 2B). In contrast, WT and Egr-1/C0 mice fed ANIT diet showed a similar increase in portal inflammatory infiltration, composed of small lymphocytes, neutrophils, and eosinophils (Supplementary figs. 2C and 2D). Furthermore, biliary ductal hyperplasia was evident in both WT and Egr-1/C0 mice (Supplementary figs. 2E and 2F). Serum ALT activity increased to a similar extent in wild-type mice and in Egr-1/C0 mice fed ANIT diet (Fig. 1A). Moreover, biliary hyperplasia, as assessed by quantification of hepatic CK-19-positive staining, was unaffected by Egr-1 deficiency in mice fed the ANIT diet (Figs. 1B–F). The results suggest that Egr-1 does not contribute to liver injury or BDEC hyperplasia in mice fed the ANIT diet.

Effect of Egr-1 Deficiency on Proinflammatory Gene Induction and Neutrophil Accumulation in Livers of Mice Fed ANIT Diet

The levels of KC, ICAM-1, MIP-2, and MCP-1 mRNA were elevated in livers of wild-type mice fed an ANIT diet (Figs. 2A–D), and the induction of each gene was unaffected by Egr-1 deficiency. Increased neutrophil accumulation was evident in livers of wild-type mice fed ANIT diet (Figs. 3A and 3C), and mirroring changes in proinflammatory gene expression and hepatic neutrophil accumulation was unaffected by Egr-1 deficiency (Figs. 3B and 3D). The data suggest that Egr-1 does not contribute to hepatic inflammation in mice fed ANIT diet.

Effect of Egr-1 Deficiency on Hepatic Profibrogenic Gene Induction and Type 1 Collagen Deposition in Mice Fed ANIT Diet

Others and we have previously shown peribiliary Type 1 collagen deposition increases in livers of mice fed ANIT diet (Sullivan et al., 2010; Xu et al., 2004). Insofar as Egr-1 has been shown to play a differential role in multiple models of fibrosis, we determined the effect of Egr-1 deficiency on liver fibrosis in mice fed ANIT diet. Hepatic levels of mRNAs encoding the profibrogenic genes TGF-β1, TIMP1, Itgb6, and Col1a1 were significantly increased in wild-type mice fed an ANIT diet (Figs. 4A–D). Although Type 1 collagen mRNA levels tended to increase further in livers of Egr-1/C0 mice fed ANIT diet, this did not achieve statistical significance (Fig. 4D). Interestingly, the expression of Itgb6 mRNA was significantly increased in Egr-1/C0 mice fed ANIT diet compared with wild-type mice fed ANIT diet (Fig. 4C). Tracking with Col1a1 mRNA expression, increased levels of hepatic type 1 collagen protein were evident in livers of wild-type mice fed ANIT diet (Figs. 5A, 5C, and 5E). Interestingly, the ANIT diet–induced increase in hepatic collagen deposition was further enhanced by Egr-1 deficiency (Figs. 5C–E). The data indicate that Egr-1 deficiency increases collagen deposition in livers of mice fed ANIT diet.

DISCUSSION

Egr-1 has been shown to contribute to inflammation in several models of liver injury, and hepatic Egr-1 mRNA expression is increased in patients with PBC and primary sclerosing cholangitis (PSC) (Allen et al., 2011). Analogous to studies in mice subjected to BDL, hepatic Egr-1 expression was evident in hepatocytes and in cells adjacent to inflamed portal tracts in livers of mice fed ANIT diet (Kim et al., 2006). Previous studies have suggested that Egr-1 participates in multiple models of liver injury; however, the role of Egr-1 seems to be model dependent. For example, Egr-1/C0 mice have increased liver injury after treatment with carbon tetrachloride (Pritchard et al., 2010), whereas Egr-1 deficiency protects against lipopolysaccharide/galactosamine-induced liver injury and alcohol-dependent steatosis (McMullen et al., 2005; Pritchard et al., 2007). Interestingly, in a model of obstructive cholestasis, Egr-1 deficiency reduced liver injury as assessed by serum ALT in mice subjected to BDL (Kim et al., 2006). In contrast, we found that Egr-1 deficiency did not significantly affect the increase in serum ALT activity in the ANIT diet model of cholestasis. Similarly, Egr-1 did not alter BDEC hyperplasia in mice fed an ANIT diet. These contrasting results further highlight model-dependent differences in the role of Egr-1 in liver injury. Additional studies are required to identify critical Egr-1-expressing cell types contributing to liver fibrosis during cholestasis.

Injury elicited during cholestasis induces inflammation and initiates mechanisms that clear and repair the damaged tissue (Fioocchi and Lund, 2011; Smedsrod et al., 2009). Persistent liver injury can promote unremitting inflammation, which in turn contributes to disease progression during chronic cholestasis. Egr-1 has been shown to be a major regulator of the local inflammatory response by inducing the expression of target genes, such as MIP-2, ICAM-1, MCP-1, and others (Kim et al., 2006; Luyendyk et al., 2011; Pritchard and Nagy, 2005), which promote a local increase of inflammatory cells within the affected tissues. For instance, inflammation associated with bleomycin-induced pulmonary and dermal injury is reduced in Egr-1/C0 mice (Wu et al., 2009). Similarly, Egr-1/C0 mice...
subjected to BDL have reduced neutrophil accumulation, suggesting that Egr-1 also contributes to inflammation induced by obstructive cholestasis (Kim et al., 2006). In this study, we found that ANIT diet–induced hepatic expression of KC, MIP-2, ICAM-1, and MCP-1 mRNAs and hepatic neutrophil accumulation were independent of Egr-1. Taken together, these data suggest that injury to BDECs in the ANIT diet does not elicit an Egr-1-dependent inflammatory response.

BDECs form a protective conduit (i.e., bile ducts) separating high concentrations of bile acids from the surrounding liver parenchyma. Damage to bile ducts can disrupt bile flow and can result in the release of bile acids into the liver, which can be directly toxic to hepatocytes (Palmeira and Rolo, 2004). Interestingly, treatment of cultured hepatocytes with subtoxic doses of bile acids can elicit a signaling response and induce expression of Egr-1 (Allen et al., 2010). In line with the severity of cholestasis in mice subjected to BDL, circulating bile acid concentrations are much higher in these mice compared with mice fed ANIT diet. To this end, the concentration of pericellular bile acids achieved in mice after BDL may achieve a threshold required to provoke Egr-1-dependent inflammatory gene induction (Kim et al., 2006). Consistent with this hypothesis, we have previously shown that mice lacking circulating fibrinogen, a component of blood clots, had significantly elevated levels of serum bile acids compared with control mice after ANIT diet feeding (Luyendyk et al., 2011). Of importance, this was associated with an increased induction of hepatic Egr-1 mRNA and enhanced induction of its target genes KC, MIP-2, and MCP-1 mRNAs compared with wild-type mice (Luyendyk et al., 2011). Taken together, this suggests that BDEC injury itself is not sufficient to illicit Egr-1-dependent inflammation during cholestasis; however, in more severe cholestatic models such as in BDL and potentially late-stage PBC and PSC, Egr-1 may act as a secondary sensor to initiate additional inflammation in response to toxic concentrations of bile acids.
Chronic injury and repair during cholestasis can develop into fibrosis and cirrhosis if not adequately controlled. Egr-1 has also been implicated in the progression of fibrosis, and its contribution seems to be dependent on the model of injury. Egr-1 deficiency reduced fibrosis in models of bleomycin-induced pulmonary and dermal injury, suggesting that it can play a role in fibrosis.

**FIG. 2.** Effect of Egr-1 deficiency on proinflammatory gene induction in livers of mice fed ANIT diet. Egr-1-deficient (Egr-1<sup>−/−</sup>) mice or age-matched wild-type mice were fed AIN-93M diet containing 0.025% alpha-naphthylisothiocyanate (ANIT diet) or a purified AIN-93M control diet for 14 days. Hepatic levels of (A) KC, (B) ICAM-1, (C) MIP-2, and (D) MCP-1 mRNA were determined by quantitative PCR. Data are expressed as fold change versus wild-type mice fed control diet and as mean ± SEM. *Statistically different from mice of the same genotype fed control diet. p < 0.05. n = 4–9 mice per group.

**FIG. 3.** Effect of Egr-1 deficiency on hepatic neutrophil accumulation in livers of mice fed ANIT diet. Egr-1-deficient (Egr-1<sup>−/−</sup>) mice or age-matched wild-type mice were fed AIN-93M diet containing 0.025% alpha-naphthylisothiocyanate (ANIT diet) or a purified AIN-93M control diet for 14 days. (A–D) Representative photomicrographs of hepatic neutrophil staining (neutrophils appear dark) performed as described in "Materials and Methods" section from (A and C) wild-type and (B and D) Egr-1<sup>−/−</sup> mice fed a (A and B) control or (C and D) ANIT diet.
a profibrogenic role (Wu et al., 2009). In contrast, another study found that Egr-1-deficient mice treated with CCl₄ have increased hepatic deposition of collagen, suggesting that Egr-1 inhibits fibrosis (Pritchard et al., 2010). Interestingly, Egr-1 did not affect liver fibrosis induced by BDL, despite decreasing both liver inflammation and injury (Kim et al., 2006). In this study, we found that Egr-1-deficient mice had increased hepatic Type I collagen protein levels compared with wild-type mice. Of importance, Egr-1−/− mice subject to BDL may actually have enhanced liver fibrosis when adjusted for the total liver injury load. Because wound healing and fibrosis are generally considered to be related to the extent of tissue injury, Egr-1−/− mice may have more collagen fibrosis than when adjusted for the total liver injury load.

One mechanism whereby Egr-1 could inhibit fibrosis in the ANIT diet is by limiting expression of profibrogenic Itgb6 mRNA. This gene encodes a subunit of the αVβ6 integrin, a cell surface receptor that can bind and activate latent-TGF-β1 and TGF-β3, the rate-limiting step of TGF-β activation. Local TGF-β signaling has been shown to be an important regulator of fibrosis in models of biliary fibrosis including the ANIT diet (Sullivan et al., 2010). Of importance, TGF-β signaling is required for differentiation of portal fibroblasts into myofibroblasts, the main cell type responsible for collagen deposition during biliary fibrosis (Dranoff and Wells, 2010). Of interest, previous studies have shown that inhibition of the αVβ6 integrin can reduce fibrosis in rodent models of cholestasis, including the ANIT diet (Patsenker et al., 2008; Sullivan et al., 2010; Wang et al., 2007). In this study, we found that Egr-1 deficiency was associated with increased Itgb6 mRNA expression. This suggests a potential mechanism whereby Egr-1 reduces fibrosis after chronic BDEC injury by limiting the expression of the αVβ6 integrin and subsequently reducing local concentrations of active TGF-β.

Additional studies are needed to clarify the interplay between Egr-1 deficiency and Itgb6 expression and to determine whether the αVβ6 integrin contributes to the enhanced fibrosis in Egr-1−/− mice during ANIT diet.

The substantial increase in Type I collagen protein in livers of Egr-1−/− mice fed ANIT diet in the absence of a large increase in collagen mRNA expression suggests an alternative mechanism whereby Egr-1 could limit liver fibrosis. Egr-1 has been shown previously to regulate the expression of MMPs, enzymes responsible for degradation of extracellular matrix (Rockel et al., 2009). Dysregulated MMP expression or altered expression of TIMPs could underlie the increased collagen protein levels in Egr-1−/− mice. Increased liver injury in Egr-1−/− mice was associated with increased TIMP-1 mRNA expression in a model of CCl₄-induced liver fibrosis (Pritchard and Nagy, 2010). In contrast, hepatic TIMP-1 mRNA levels were not reduced in Egr-1−/− mice fed an ANIT diet (Fig. 4B). We were also unable to detect a significant increase in gelatinase (MMP-2/9) activity in livers of mice fed ANIT diet. In addition, utilizing a fluorescence resonance energy transfer-based MMP

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**FIG. 4.** Effect of Egr-1 deficiency on hepatic profibrogenic gene induction in mice fed ANIT diet. Egr-1-deficient (Egr-1−/−) mice or age-matched wild-type mice were fed AIN-93M diet containing 0.025% alpha-naphthylisothiocyanate (ANIT diet) or a purified AIN-93M control diet for 14 days. Hepatic levels of (A) TGF-β1, (B) TIMP-1, (C) Itgb6, and (D) Col1a1 mRNAs were determined by quantitative PCR. Data are expressed as fold change versus wild-type mice fed control diet and as mean ± SEM. *Statistically different from mice of the same genotype fed control diet. #Statistically different from wild-type mice fed ANIT diet. p < 0.05. n = 4–9 mice per group.
activity assay did not reveal a significant change in total liver MMP activity in mice fed ANIT diet (data not shown). Although these studies suggest that enhanced MMP activity is not a predominant mechanism whereby Egr-1 deficiency increases liver fibrosis in mice fed the ANIT diet, additional studies examining the activity of individual MMPs could be revealing.

In summary, Egr-1 is induced during chronic cholestasis elicited by ANIT diet. Although Egr-1 deficiency did not affect ANIT diet–induced liver injury or inflammation, Egr-1–deficient mice had significantly increased levels of hepatic collagen deposition. Of interest, the increase in fibrosis in Egr-1–deficient mice was accompanied by induction of the gene encoding the β6 integrin subunit, suggesting a potential role for Egr-1 in restricting local activation of TGF-β and subsequent biliary fibrosis. This finding is fundamentally different from the lack of effect of Egr-1 deficiency on liver fibrosis in mice subjected to BDL. A better understanding of the differential contribution of Egr-1 to liver disease between these two models of cholestasis is essential for the development of therapies targeting Egr-1 in cholestatic liver disease.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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