Toxicology Letters 224 (2014) 186–195

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Osteopontin is an initial mediator of inflammation and liver injury during obstructive cholestasis after bile duct ligation in mice

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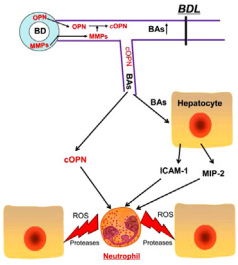
HIGHLIGHTS

- Cholestatic liver injury occurs partially by neutrophil mediated hepatic necrosis.
- Osteopontin-deficient mice are protected against early cholestatic liver injury.
- Metalloproteinases cleave osteopontin to a pro-inflammatory form in bile.
- MMP inhibitor prevents neutrophil recruitment and early cholestatic liver injury.
- Osteopontin mediates neutrophil recruitment leading to cholestatic liver injury.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 August 2013
Received in revised form 24 October 2013
Accepted 25 October 2013
Available online 2 November 2013

Keywords:
Inflammatory liver injury
Cholestasis
Bile duct ligation
Osteopontin
Neutrophils

ABSTRACT

Osteopontin (OPN) is a chemotactic factor which can be cleaved to the pro-inflammatory form by matrix metalloproteinases (MMPs). To test the hypothesis that OPN can modulate inflammatory liver injury during cholestasis, wild-type (WT) C57BL/6 and OPN knockout (OPN-KO) mice underwent bile duct ligation (BDL). OPN-KO mice showed significant reduction in liver injury (plasma ALT and necrosis) and neutrophil recruitment compared with WT animals at 24 h but not 72 h after BDL. In WT mice, a 4-fold increase in hepatic MMP-3 mRNA and elevated MMP activities and cleaved OPN levels were observed in bile. WT mice subjected to BDL in the presence of the MMP inhibitor BB-94 showed reduced liver injury, less neutrophil extravasation and diminished levels of cleaved OPN in bile. Thus, during obstructive cholestasis, OPN released from biliary epithelial cells could be cleaved by MMPs in bile. When the biliary system leaks, cleaved OPN enters the parenchyma and attracts neutrophils. In the absence of OPN, other chemoattractants, e.g. chemokines, mediate a delayed inflammatory response and injury. Taken together, our data suggest that OPN is the pro-inflammatory mediator that initiates the early neutrophil-mediated injury phase during obstructive cholestasis in mice.

Abbreviations: ALT, alanine aminotransferase; BDECs, bile duct epithelial cells; BDL, bile duct ligation; H&E, hematoxylin and eosin; HPF, high-power fields; ICAM-1, intercellular adhesion molecule-1; KO mice, gene knock-out mice; MMP, matrix metalloproteinase; OPN, osteopontin; WT, wild type.

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http://dx.doi.org/10.1016/j.toxlet.2013.10.030

1. Introduction

Cholestatic liver disease can be caused acutely by drug toxicities or obstruction of the bile duct by gallstones, and chronically by genetic defects or malignancies (Hirschfield et al., 2010). Acute obstructive cholestasis is characterized by hepatocellular injury, which is followed by bile duct proliferation, fibrosis and cirrhosis (Sellinger and Boyer, 1990). However, the molecular mechanisms of liver injury induced by obstructive cholestasis remain not well defined (Woolbright and Jaeschke, 2012). One hypothesis suggests that liver injury during BDL is caused by an inflammatory response involving neutrophil-mediated liver injury (Gujral et al., 2003, 2004b). However, for neutrophils to be a relevant factor in the pathophysiology, a prerequisite would be the formation of chemotactic agents that can activate and recruit neutrophils into the liver (Jaeschke, 2006). It was demonstrated that bile acids can induce the transcription factor early growth response factor-1 (egr-1), which regulates a number of pro-inflammatory genes including macrophage inflammatory protein-2 (MIP-2), keratinocyte chemoattractant (KC) and intercellular adhesion molecule-1 (ICAM-1) in hepatocytes (Allen et al., 2010, 2011; Kim et al., 2006). The fact that mice deficient in ICAM-1 or egr-1 showed reduced neutrophilic inflammation and less injury after BDL supported the importance of bile acid-induced inflammation in the pathophysiology (Gujral et al., 2004b; Kim et al., 2006). Most importantly, the pro-inflammatory effect was caused by bile acids at concentrations that are achieved in vivo after BDL (Zhang et al., 2012). However, an important, unanswered question remains which mediator(s) actually initiate this neutrophilic inflammatory response after BDL.

Osteopontin is a multifunctional glycophosphoprotein that can function as a neutrophil chemoattractant by binding to integrin receptors (Banerjee et al., 2008; Denhardt et al., 2001; Ramaiah and Rittling, 2008). In the liver, osteopontin is expressed in biliary epithelial cells and has been shown to be substantially induced in different models of biliary fibrosis including BDL (Banerjee et al., 2006b; De Minicis et al., 2007; Fickert et al., 2007, 2010). Osteopontin expression was also upregulated in biliary epithelium in human biliary atresia (Whitington et al., 2005). Because of the induction during BDL and its potent chemotactic properties, we tested the hypothesis that osteopontin may initiate the neutrophilic inflammatory response during BDL.

2. Material and methods

2.1. Animals

Eight to twelve week old male WT (C57BL/6) and OPN knockout (OPN-KO) mice, which are on a C57BL/6 background, were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were maintained in an environmentally controlled room with a 12 h light/dark cycle and allowed free access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals in research.

2.2. Experimental design

WT and OPN-KO mice were subjected to BDL for 1 or 3 days as described previously (Gujral et al., 2003). Sham-operated animals served as controls ($n = 5$ for each time point). For experiments with inhibition of MMPs, the MMP inhibitor Batimastat (BB-94) at a dose of 20 mg/kg was used (Wielockx et al., 2001). The compound was sonicated into suspension with PBS/0.01% Tween 20. WT mice subjected to BDL for 1-day were intraperitoneally injected with either BB-94 or vehicle, just after BDL and then 6 h after BDL. The animals were sacrificed at various time points by cervical dislocation and exsanguination. Blood, bile and liver samples were collected at the time of sacrifice from the animals. Bile was obtained via rupture of the gall bladder into a plastic tube. Gall bladder was excluded from protein used in western blot assays. Bile was flash frozen in liquid nitrogen and stored at −80 °C. Plasma was used to determine alanine aminotransferase (ALT) activities. Liver samples were either snap-frozen in liquid nitrogen or fixed in phosphate-buffered formalin. Formalin-fixed livers were embedded in paraffin, and 5 μm sections were cut and used for histology.

2.3. Histology

Liver sections were stained with hematoxylin and eosin (H&E) for evaluation of liver injury. The tissue for histological analysis was always derived from similar mid-sections from the same liver lobes, i.e. the tissue sections used for H&E staining and immunohistochemical analysis were of similar size for all animals in all groups. For counting of the infarcts per section, the tissue sections were de-identified and evaluated blindly. To assess neutrophil accumulation in the liver, sections were stained for chloroacetate esterase, a marker for neutrophils (Jaeschke et al., 1990), using a Naphthol-ASD Chloroacetate Esterase Kit (Sigma, St. Louis, MO). Neutrophils present in the sinusoids and extravasated into the parenchyma were counted in 20 randomly selected high-power fields (HPF). The sum of sinusoidal and extravasated neutrophils were expressed as the total neutrophil sequestration in the liver (Gujral et al., 2003, 2004b).

2.4. Real-time PCR

mRNA expression of the selected genes (OPN, MMP-2, -3, -7, and -13) was quantified using real-time RT-PCR analysis as previously described (Bajt et al., 2008). Briefly, RNA was isolated using TRI reagent (Sigma–Aldrich, St. Louis, MO), and was reverse-transcribed into cDNA. After normalizing cDNA concentration, the SYBR green DNA PCR kit (Applied Biosystems) was used for real-time PCR analysis. Primer pairs used included as follows: 5′-GTATGACTCCACTCACGGCAAA-3′ and 3′-GGTCTCGCTCCTGGAAGATG-5′ for β-actin; 5′-ACACTTTCACTCCAATCGTCC-3′ and 3′-TGCCCTTTCCGTTGTTGTCC-5′ for OPN; 5′-CACCTGGTTTCACCCTTTCTG-3′ and 3′-AACGAGCGAAGGGCATACAA-5′ for MMP-2; 5′-CCCACCAAGTCTAACTCTCTGGAA-3′ and 3′-GGGTGCTGACTGCATCAAAGA-5′ for MMP-3; 5′-GGTCACCTACAGGATCGTATCATAT-3′ and 3′-CATCACTGCATTAGGATCAGAGGAA-5′ for MMP-7; and 5′-GACCTTGTGTTTGCAGAGCACTAC-3′ and 3′-TTCTCGGAGCCTGTCAACTGT-5′ for MMP-13. The relative differences in expression between groups were expressed using cycle time (Ct) values generated by the ABI 7900 instrument (Applied Biosystems). All genes evaluated were first normalized to β-actin, a housekeeping gene, as an internal control, and then expressed as a fold increase relative to control arbitrarily set as 1.0. Calculations are made by the $2\hat{(}-\Delta\Delta Ct)$ formula.

2.5. Immunohistochemistry

Sequential liver sections were deparaffinized in xylene and rehydrated in serial ethanol: water dilutions. Endogenous hydrogen peroxide was quenched and the tissue was probed for OPN using an anti-OPN antibody (anti-mouse OPN. Cat. # AF808, R&D systems, Minneapolis, MN). Stain was visualized using a donkey anti-goat HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and DAB peroxidase (Vector Labs, Burlingame, CA) and counterstained with hematoxylin.

2.6. Western blotting

OPN in bile and liver tissue was evaluated by Western Blot analysis, as described (Bajt et al., 2000). The following antibodies were used: a rabbit anti-OPN antibody (Abcam, Cambridge, MA), which recognizes both the full-length OPN (66 kDa) and MMP-cleaved OPN (32 kDa); a donkey anti-rabbit IgG (Santa Cruz) was used as secondary antibody. Each lane was loaded with 30 μg protein for bile or liver samples. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

2.7. Gelatin zymography

Zymography of protease activity was performed as described (Ramachandran and Balasubramanian, 2000). Briefly, bile samples (100 μg protein) were separated on 12% polyacrylamide gels containing 0.2% gelatin. After electrophoresis, the gels were soaked in 2% Triton X-100, followed by incubation in reaction buffer (0.1 M Tris–HCl, pH 7.5 with 10 mM $CaCl_2$) overnight at 37 °C and then stained with Coomassie brilliant blue.

2.8. Statistical analysis

Data are given as means ± SE. Comparison between two groups were performed with Student t test or 1-wayANOVA followed by Bonferroni t test for multiple groups. If the data were not normally distributed, the Mann–Whitney test was applied for comparison of 2 groups. $P < 0.05$ was considered significant.