Functional importance of ICAM-1 in the mechanism of neutrophil-induced liver injury in bile duct-ligated mice

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Gujral, Jaspreet S., Jie Liu, Anwar Farhood, Jack A. Hinson, and Hartmut Jaeschke. Functional importance of ICAM-1 in the mechanism of neutrophil-induced liver injury in bile duct-ligated mice. Am J Physiol Gastrointest Liver Physiol 286: G499–G507, 2004. First published October 16, 2003; 10.1152/ajpgi.00318.2003.—Cholestasis-induced liver injury during bile duct obstruction causes an acute inflammatory response. To further characterize the mechanisms underlying the neutrophil-induced cell damage in the bile duct ligation (BDL) model, we performed experiments using wild-type (WT) and ICAM-1-deficient mice. After BDL for 3 days, increased ICAM-1 expression was observed along sinusoids, along portal veins, and on hepatocytes in livers of WT animals. Neutrophils accumulated in sinusoids [35 ± 44 neutrophils/20 high-power fields (HPF)] and >50% extravasated into the parenchymal tissue. Plasma alanine transaminase (ALT) levels increased by 23-fold, and severe liver cell necrosis (47 ± 11% of total cells) was observed. Chlorotyrosine-protein adducts (a marker for neutrophil-derived hypochlorous acid) and 4-hydroxynonenal adducts (a lipid peroxidation product) were detected in these livers. Neutrophils also accumulated in the portal venules and extravasated into the portal tracts. However, no evidence for chlorotyrosine or 4-hydroxynonenal protein adducts was detected in portal tracts. ICAM-1-deficient mice showed 67% reduction in plasma ALT levels and 83% reduction in necrosis after BDL compared with WT animals. The total number of neutrophils in the liver was reduced (126 ± 25/20 HPF), and 85% of these leukocytes remained in sinusoids. Moreover, these livers showed minimal staining for chlorotyrosine and 4-hydroxynonenal adducts, indicating a substantially reduced oxidant stress and a diminished cytokine response. Thus neutrophils relevant for the aggravation of acute cholestatic liver injury in BDL mice accumulate in hepatic sinusoids, extravasate into the tissue dependent on ICAM-1, and cause cell damage involving reactive oxygen formation.

hepatotoxicity; chlorotyrosine-protein adducts; 4-hydroxynonenal adducts; oxidant stress

POLYMORPHONUCLEAR LEUKOCYTES or neutrophils (PMNs) are one of the primary defense mechanisms against invading microorganisms and are also involved in the removal of necrotic cells. However, in certain clinical conditions, recruitment of neutrophils can aggravate an existing injury. This has been seen to occur in the liver during ischemia–reperfusion (transplantation or Pringle maneuver) (23, 27), alcoholic hepatitis (2), sepsis and endotoxemia (28, 40), remote organ damage (20), and acute cholestatic liver injury (15). Neutrophil accumulation in the liver can be triggered by a variety of proinflammatory mediators such as TNF-α, IL-1, complement factors, platelet-activating factor, and chemokines (1, 3, 9, 25, 39, 46). Neutrophils accumulate in sinusoids, branches of the portal vein, and postsinusoidal venules (5, 13, 15, 51). In contrast to portal and postsinusoidal venules (10, 13, 35, 55), adhesion molecules do not seem to play a role in neutrophil sequestration in sinusoids (26). On the other hand, the subsequent step of neutrophil transmigration or extravasation into the tissue is dependent on the CD18 integrins, Mac-1 (CD11b/CD18) and lymphocyte function-associated antigen (LFA-1; CD11a/CD18). ICAM-1, and VCAM-1 (8, 9, 28). Extravasation also results in the full activation of neutrophils for reactive oxygen formation from their initial primed state (35). Moreover, ICAM-1 and CD18 integrins have also been implicated in the adherence of neutrophils to target cells, e.g., hepatocytes (41). This results in a long-lasting adherence-dependent oxidant stress (48), which is a major factor in neutrophil-mediated liver cell killing (29). A major oxidant generated by neutrophils is hypochlorous acid, which can react with proteins to form chlorotyrosine protein adducts (7, 19). In addition, reactive oxygen formation can lead to lipid peroxidation as indicated by 4-hydroxynonenal protein adducts (53).

Cholestatic liver injury occurs in humans due to bile duct obstruction in a variety of clinical settings, such as gallstone impaction, cholangiocarcinoma, extrinsic compression by tumors or enlarged lymph nodes, and primary sclerosing cholangitis (21). Long-lasting cholestasis leads to liver fibrosis (33, 47). A well-established experimental animal model for cholestasis and liver fibrosis is bile duct ligation (BDL) in rodents (33). Cholestasis results in the accumulation of hydrophobic bile acids, which may be responsible for hepatocellular apoptosis and necrosis in the liver. As a consequence of this initial injury, hepatic inflammation develops, involving Kupffer cell activation, the release of cytokines and chemokines, and, subsequently, neutrophil recruitment to the liver (32, 37, 42, 45). There is increased expression of the neutrophil-attracting CXC chemokines and adhesion molecules, such as ICAM-1, in the cholestatic liver (18, 45). We have recently shown that neutrophils can aggravate cholestatic liver injury after BDL (15). Attenuation of the injury in partial CD18-deficient mice suggested that β2-integrins on neutrophils are involved in the injury process. Because β2-integrins can bind to a number of molecules, including ICAM-1 and -2, fibrinogen, and IC3b (22, 30), the importance of ICAM-1 in the neutrophil-induced injury mechanism remained unclear in the BDL model of cholestatic liver injury. In addition, there is a continuing

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controversy regarding the importance of neutrophils accumulating in and extravasating from sinusoids vs. those adhering in and transmigrating from venules (5, 31, 51, 52). Therefore, we addressed these issues by using the BDL model in wild-type (WT) and ICAM-1-deficient mice. We compared liver injury and neutrophil-induced oxidative stress, as detected by immunohistochemistry for the chlorotyrosine and 4-hydroxynonenal adducts, in the parenchymal tissue and in the portal tracts of WT and ICAM-1-deficient mice.

MATERIALS AND METHODS

Animals and experimental protocol. WT (C57BL/6J) and ICAM-1-deficient mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals received humane care according to the criteria outlined in Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIIH, Bethesda, MD 20205]. All experimental protocols were approved by the Animal Use Committees of the University of Arkansas for Medical Sciences and the University of Arizona. Before surgery, the mice were intramuscularly injected with a cocktail of anesthetics consisting of ketamine (225 mg/kg Ketaset; Fort Dodge Animal Health, Fort Dodge, IA), xylazine (11.4 mg/kg Rompun; Bayer, Shawnee Mission, KS), and acepromazine (2.3 mg/kg acepromazine maleate; VEDCO, St. Joseph, MO). After a midline laparotomy, the common bile duct was ligated twice with 4-0 silk sutures and then sectioned between the sutures. Sham-operated animals, in which the same procedures were performed except BDL and section, served as controls. After surgery, the animals were intraaperitoneally injected with a combination of the antibiotics ciprofloxacin, rehydrated, and then incubated sequentially with the Immunopure peroxidase suppressor (Pierce, Rockford, IL) for 1 h and a protein block (DAKO, Carpinteria, CA) for 2 h. This was followed by incubation with the primary antibody, anti-ICAM-1 (1:100), anti-chlorotyrosine (1:300), or anti-4-hydroxynonenal (1:100) overnight at room temperature. Color was developed by using a horseradish peroxidase-based labeled streptavidin biotin kit (DAKO) and diaminobenzidine chromogen (ICAM-1, chlorotyrosine; DAKO) or the AEC chromogen (4-hydroxynonenal; DAKO), according to the manufacturer’s instructions. Slides were mounted with Faramount aqueous mounting medium (DAKO).

Liver injury and plasma bilirubin levels. ALT activities were determined in the plasma by using the DG 159-UV kit (Sigma), according to the manufacturer’s instructions. Sections of formalin-fixed paraffin-embedded liver samples were stained with hematoxylin and eosin for evaluation of liver cell injury. Cell necrosis was quantitated in these sections. The percentage of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared with the entire histological section (14-16). Total bilirubin levels were measured in plasma as a marker for cholestasis by using a total bilirubin kit (Sigma).
Real-time RT-PCR analysis. Expression of the selected genes was quantified by using real-time RT-PCR analysis as described (36, 54). Briefly, total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers. The forward and reverse primers for selected genes were designed by using Primer Express software (Applied Biosystems, Foster City, CA). The SYBRgreen DNA PCR kit (Applied Biosystems) was used for real-time PCR analysis. Primer pairs used for PCR included 5'-GACCCCTCACACTCAGATCATTTCT-3' and 3'-CCTCAATGTTGGTTTGCT-5' for TNF-α; 5'-GTATGACTCCACTCACGGAAGATG-3' and 3'-CCTCCACTTGGTGGTTTGCT-5' for β-actin; 5'-CCAAGCCTTATCGGAAATGATC-3' and 3'-GATTTCGAGCCATGCT-5' for IL-10; and 5'-GCCACCAAGAACGATAGTCA-3' and 3'-GAAGGCACTGGAAGTCTCT-5' for IL-6. The relative differences in expression among groups were expressed by using cycle time (Ct) values. Ct values for the genes we were interested in were first normalized with that of β-actin in the same sample, and then relative differences among groups were expressed by using the 2^(-ΔΔCt) formula. Standard curve analysis was also performed according to the method of Liu and Saint (38), and the results were largely comparable with the above method, except for TNF-α, whose expression efficacy was ~90%, and the standard curve was used for the calculation.

Statistics. Data are expressed as means ± SE. Comparisons among multiple groups were performed with one-way ANOVA followed by Bonferroni t-test. If the data were not normally distributed, the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparisons test was performed. P < 0.05 was considered significant.

RESULTS

Previously described pilot experiments (15) demonstrated that BDL caused significant neutrophilic inflammation and liver injury in C57BL/6J mice without any mortality 3 days...
after BDL. Therefore, we used this time point to evaluate the role of ICAM-1 in the pathophysiology. Liver sections were immunostained to determine ICAM-1 expression in sham-operated WT and ICAM-1-deficient mice as well as after BDL for 3 days. Slightly positive staining was observed in the livers from sham-operated WT mice, mainly around portal venules and sometimes along sinusoids (Fig. 1A). Three days after BDL, ICAM-1 was upregulated in the WT livers and could be seen along sinusoids, portal venules, and hepatocytes, especially around necrotic foci (Fig. 1B). Moreover, this positive staining colocalized with the extravasated neutrophils seen in and around the foci of necrotic hepatocytes. Neither sham-operated nor BDL ICAM-1-deficient mice had any positive staining for ICAM-1 (data not shown).

To evaluate hepatic neutrophil sequestration, liver sections were stained for chloroacetate esterase, a specific marker for neutrophils. Livers from sham-operated mice had very few neutrophils in sinusoids and portal venules with virtually no extravasation into the parenchyma or the portal tracts (data not shown). On the other hand, 3 days after BDL, a number of neutrophils (arrows in Fig. 2) accumulated in sinusoids and portal venules of WT mice and then extravasated into the parenchymal tissue (Fig. 2A) and the portal tracts (Fig. 2B), respectively. Only a small number of neutrophils accumulated in livers of ICAM-1-deficient animals 3 days after BDL (Fig. 2C and D). Neutrophil accumulation in these livers was quantified separately for sinusoids, parenchymal extravasation, portal venules, and portal tract extravasation (Fig. 3). The sum of the neutrophils in sinusoids and those extravasated in the parenchyma was expressed as the total number of neutrophils in 20 HPF. In sham-operated animals, only a few neutrophils accumulated in the liver (10 ± 2 per 20 HPF) with minimal extravasation (Fig. 3A). However, 3 days after BDL, WT animals had over a 35-fold increase in the total number of hepatic neutrophils. More than 50% of these neutrophils were seen extravasated in the parenchymal tissue. On the other hand, ICAM-1-deficient mice had 65% fewer neutrophils in their livers after BDL compared with WT animals (Fig. 3A). More-
over, only 15% of these neutrophils (19 ± 5) extravasated into the parenchyma (Fig. 3A). Thus overall, there was a 90% reduction in the number of extravasated neutrophils in ICAM-1-deficient mice compared with WT animals. Neutrophils also accumulated in the portal venules and then extravasated into the surrounding portal tracts. Sham-operated WT or ICAM-1-deficient mice had only a few neutrophils in portal tracts (Fig. 3B). However, the total number of neutrophils in the portal tracts of BDL WT animals increased 145-fold compared with the sham-operated WT mice. Only 12% of these cells were observed inside the portal venules; all others were found extravasated (Figs. 2B and 3B). On the other hand, the total number of portal tract neutrophils and the number of extravasated neutrophils in this area was reduced by 77–80% in ICAM-1-deficient animals subjected to BDL (Fig. 3B). In contrast to the large number of neutrophils accumulating in portal venules and sinusoids (Fig. 3), only a few neutrophils were observed in postsinusoidal venules of BDL WT mice (7.0 ± 1.3 PMN/20 central venules) compared with sham-operated WT animals (2.0 ± 0.4 PMN/20 central venules). On the other hand, ICAM-1-deficient mice had similar numbers of neutrophils in postsinusoidal venules after BDL (2.3 ± 0.7 PMN/20 central venules) as in sham-operated controls (2.0 ± 1.4 PMN/20 central venules). A few neutrophils were extravasated only in the WT BDL mice (2.7 ± 1.2 PMN/20 central venules). A few neutrophils were extravasated only in the WT BDL mice (2.7 ± 1.2 PMN/20 central venules).

To assess the effect of hepatic neutrophil accumulation on BDL-induced liver injury, ALT levels were measured in plasma. Sham-operated WT mice had plasma ALT activities of 19 ± 9 U/l (Fig. 4A). After BDL, there was a 24-fold increase in the ALT levels in WT mice. On the other hand, plasma ALT levels were 65% lower in ICAM-1-deficient animals compared with WT mice (Fig. 4A). As a further cumulative indicator of liver cell injury, necrosis was estimated in liver sections stained with hematoxylin and eosin. No necrosis was seen in sham-operated WT or ICAM-1-deficient controls (Fig. 4B). There was no congestion in the sinusoids, and liver architecture looked normal. Three days after BDL in the WT mice, numerous necrotic foci were observed, and the total cell death in these livers was estimated to be 47 ± 11% of all hepatocytes.
To compare the extent of cholestasis in WT and ICAM-1-deficient mice, total bilirubin levels were measured in the plasma. Sham-operated controls had a plasma bilirubin level of 0.7 ± 0.1 mg/100 ml. Levels in the BDL WT mice were increased 20-fold compared with sham-operated controls (Fig. 5). Similarly, elevated plasma bilirubin levels were observed in BDL ICAM-1-deficient mice (Fig. 5). These data exclude that the protective effect observed in ICAM-1-deficient mice compared with WT animals was due to a difference in the severity of cholestasis.

Because we observed substantial neutrophil accumulation and extravasation in portal tracts and in sinusoids, we evaluated which group of neutrophils is most important for the injury. To cause tissue damage, neutrophils need to be fully activated. Under these conditions, they generate reactive oxygen such as hypochlorous acid and may cause lipid peroxidation. Therefore, we stained liver tissue for chlorotyrosine protein adducts as a footprint for hypochlorous acid formation and for 4-hydroxynonenal protein adducts as an indicator for lipid peroxidation. Sham-operated controls showed no positive staining for these adducts in their livers (Fig. 6, A and B). On the other hand, livers from BDL WT mice showed extensive staining for chlorotyrosine (Fig. 6C) and for 4-hydroxynonenal (Fig. 6D) protein adducts, especially in the necrotic foci of parenchymal tissue around the extravasated neutrophils. In contrast, very little staining was observed after BDL in the portal tracts in the WT mice (Fig. 6, E and F), centrilobular areas of WT mice (data not shown), or in the parenchymal tissue in the ICAM-1-deficient animals (Fig. 6, G and H).

To confirm the reduced inflammatory response in ICAM-1-deficient BDL animals, mRNA expression of several cytokines was evaluated by real-time RT-PCR. Compared with WT sham-operated animals, liver tissue of WT BDL animals had 3.6-fold higher TNF-α mRNA levels, 21.7-fold higher IL-6 mRNA levels, and 18-fold higher IL-10 mRNA levels (Fig. 7). On the other hand, all hepatic cytokine mRNA levels in ICAM-1-deficient animals were at the level of sham-operated controls (TNF-α) or were still slightly elevated but not significantly different from controls (IL-6, IL-10) (Fig. 7).

**DISCUSSION**

The objectives of the present study were to evaluate the functional importance of ICAM-1 in the acute inflammatory injury after BDL and to determine the site of neutrophil extravasation relevant for parenchymal cell injury. Our data showed that after BDL, high numbers of neutrophils accumulated in hepatic sinusoids and portal venules, but not in post-sinusoidal venules, and then extravasated in an ICAM-1-dependent way into the surrounding parenchymal tissue and portal tracts, respectively. However, our data indicate that neutrophils extravasating from the sinusoids, and not portal venules, are responsible for the aggravation of liver injury through the release of reactive oxygen species.

Chlorotyrosine and 4-hydroxynonenal protein adducts are indicators for reactive oxygen formation in vivo. Positive staining for these adducts in livers of BDL WT animals, especially around necrotic foci, suggests that a substantial oxidant stress occurred in these livers. Because chlorotyrosine adducts are specific for neutrophil-derived hypochlorous acid (7, 19), our data indicate that the oxidant stress was mainly generated by extravasated neutrophils. On the other hand, chlorotyrosine and 4-hydroxynonenal protein adducts and liver injury were substantially reduced in ICAM-1-deficient animals subjected to BDL. These observations support the conclusion that the neutrophil-derived oxidant stress was a major factor in the mechanism of liver injury. Although the number of neutrophils present in the liver of ICAM-1-deficient animals was substantially lower than in WT animals, the most dramatic effect was the 90% reduction in the number of extravasated neutrophils. Thus our data support the hypothesis that ICAM-1 deficiency protected because of the inhibition of neutrophil extravasation into the parenchyma. These findings are consistent with other models of neutrophil-induced liver injury, e.g., endotoxemia, in which anti-ICAM-1 antibodies prevented neutrophil-induced liver injury due to inhibition of extravasation (9). In general, transmigration through an intact endothelial cell layer requires ICAM-1-β2-integrin interactions (49). In con-
trast, in models with severe sinusoidal endothelial cell damage, e.g., ischemia-reperfusion, blocking ICAM-1 is only moderately protective (11, 43) mainly because neutrophils have direct access to hepatocytes and the neutrophil-hepatocyte interactions are only partially dependent on ICAM-1 (41). Thus we conclude that in the BDL model, ICAM-1 is required for extravasation from sinusoids into the parenchyma. The lack of extravasated neutrophils almost eliminated the neutrophil-induced oxidant stress and prevented the aggravation of liver injury.

In addition to sinusoids, neutrophils adhere in portal and postsinusoidal venules and extravasate from these locations. There is an ongoing controversy regarding the pathophysiological role of portal or postsinusoidal venular adherence of neutrophils in various models (5, 31, 51, 52). In the BDL model, the number of extravasated neutrophils in portal venules is substantially higher than that observed during endotoxemia or ischemia-reperfusion. However, very few neutrophils accumulated in postsinusoidal venules. Because neutrophils require a chemotactic signal to transmigrate (30), the enhanced formation of CXC chemokines in the portal tract may be responsible for the portal neutrophil accumulation and extravasation (45). Absence of relevant neutrophil accumulation in central venules and transmigration suggest the absence of a chemotactic signal in this region. However, despite extravasation of neutrophils in the periportal area, we found no evidence for chlorotyrosine or hydroxynonenal protein adduct formation in the vicinity of these extravasated neutrophils in the portal tract. In addition, there was no specific periportal hepatocellular necrosis in this model. Therefore, we conclude that adhesion and transmigration of neutrophils in portal veins of animals with BDL is ICAM-1 dependent. However, the extravasated neutrophils did not generate an oxidant stress and did not appear to cause any injury. The reason for the lack of neutrophil cytotoxicity in the periportal region is unclear at present. Because neutrophils actually need to adhere to a target cell for full activation in vivo, i.e., reactive oxygen formation and degranulation (30, 48), one can hypothesize that the extravasated neutrophils in the periportal area do not recognize a specific target to attack. Further studies are required to investigate this important question. Nevertheless, our present data provide further evidence for the hypothesis that, in the liver, neutrophils relevant for the injury are accumulating in and extravasating from sinusoids, not from portal or postsinusoidal venules.

Overall, fewer neutrophils were seen in sinusoids of ICAM-1-deficient mice than in WT animals after BDL. However, a number of previous investigations demonstrated that neutrophil accumulation in sinusoids is independent of cellular adhesion molecules including ICAM-1 (9, 11, 26, 51, 55). Although our findings in the BDL model appear to contradict these previous reports, it is important to recognize that a neutrophil-dependent inflammatory response and injury is mostly a self-aggravating process (25). Once the process is initiated, additional mediators are generated during the injury process that will promote neutrophil recruitment into the liver during the late phase of injury. Lipid peroxidation products, such as 4-hydroxynonenal, are potent chemotactic factors for neutrophils (6). Thus the inhibition of transmigration in ICAM-1-deficient mice during the initial phase of injury prevented the neutrophil-induced oxidant stress and generation of chemotactic factors, which resulted in the reduced further recruitment of neutrophils. The conclusion that the overall inflammatory response was attenuated in ICAM-1-deficient animals 3 days after BDL is also supported by the almost complete elimination of cytokine formation in these mice. Thus the reduced number of sinusoidal neutrophils was due to the protective effect of ICAM-1 deficiency and the reduced aggravation of the inflammatory response rather than the direct inhibition of neutrophil adhesion in sinusoids.

It is well established that Kupffer cells are activated and generate proinflammatory cytokines after BDL (4, 37). However, the cytotoxic potential of Kupffer cells, i.e., superoxide formation, is actually impaired (50). In addition, only very few mononuclear cells accumulated in the liver after 3 days of BDL (15), suggesting that monocyte-derived tissue macrophages do not play an important role in the cytotoxicity at that early time point. Moreover, chlorotyrosine adduct formation, a specific marker for neutrophil-induced oxidant stress, correlated well with 4-hydroxynonenal protein adducts, a general marker of oxidant stress. Therefore, we can conclude that mainly neutrophils, and not Kupffer cells or tissue macrophages, were responsible for generation of reactive oxygen species in the liver 3 days after BDL. The potential role of NK and T cells in the early cholestatic injury has not been investigated in detail. However, the function and tumoricidal activity of NK and T cells is suppressed rather than activated as early as 3 days after BDL (12, 34, 44). These findings suggest that NK and T cells are unlikely to be relevant for the acute inflammatory injury after BDL.

In summary, the present study demonstrated the functional importance of ICAM-1 in the neutrophil-induced injury after BDL in mice. The mechanism of protection involves predominantly the inhibition of neutrophil extravasation into the parenchyma, which prevents the neutrophil-induced oxidant stress and injury. As a consequence, the self-aggravating inflammatory injury process is dramatically attenuated. In addition, ICAM-1 is involved in the adherence and transmigration of neutrophils from portal venules. In contrast to neutrophils extravasating from sinusoids, neutrophils in the portal tract did not show evidence for oxidant stress and did not contribute to the inflammatory liver injury. Thus blocking ICAM-1 expression or function could be a potentially promising therapeutic strategy for the treatment of acute cholestatic liver injury.

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