ICAM-1 is involved in the mechanism of alcohol-induced liver injury: studies with knockout mice

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ICAM-1 is involved in the mechanism of alcohol-induced liver injury: studies with knockout mice. Am J Physiol Gastrointest Liver Physiol 280: G1289–G1295, 2001.—To test the hypothesis that leukocyte infiltration mediated by intercellular adhesion molecule (ICAM)-1 is involved in early alcohol-induced liver injury, male wild-type or ICAM-1 knockout mice were fed a high-fat liquid diet with either ethanol or isocaloric maltose-dextrin for 4 wk. There were no differences in mean urine alcohol concentrations between the groups fed ethanol. Alcohol administration significantly increased liver size and serum alanine aminotransferase levels in wild-type mice over high-fat controls, effects that were blunted significantly in ICAM-1 knockout mice. Dietary ethanol caused severe steatosis, mild inflammation, and focal necrosis in livers from wild-type mice. Furthermore, livers from wild-type mice fed ethanol showed significant increases in the number of infiltrating leukocytes, which were predominantly lymphocytes. These pathological changes were blunted significantly in ICAM-1 knockout mice. Tumor necrosis factor (TNF)-α mRNA expression was increased in wild-type mice fed ethanol but not in ICAM-1 knockout mice. These data demonstrate that ICAM-1 and infiltrating leukocytes play important roles in early alcohol-induced liver injury, most likely by mechanisms involving TNF-α.

Inflammatory cell infiltration is one characteristic histopathological change in alcoholic liver disease. Indeed, chronic enteral ethanol administration increases the number of infiltrating neutrophils in the liver in the Tsukamoto-French model (9, 14). Furthermore, ICAM-1 mRNA expression was increased in the liver from rats fed enteral ethanol chronically (19). Therefore, it has been speculated that ICAM-1 plays an important role in early alcohol-induced liver injury by facilitating adhesion of leukocytes, which produce toxic mediators; however, hard evidence for or against this hypothesis is still lacking. Activated Kupffer cells also generate a wide range of inflammatory mediators, including cytokines, eicosanoids, and reactive oxygen species. Indeed, early alcohol-induced liver injury is attenuated by inactivation of Kupffer cells (1). However, it is still unclear whether early alcohol-induced liver injury is caused directly by inflammatory mediators from Kupffer cells or from infiltrating leukocytes. Therefore, the purpose of this study was to evaluate the hypothesis that ICAM-1 and infiltrating neutrophils are involved in early alcohol-induced liver injury by studying ICAM-1 knockout mice fed enteral alcohol.

MATERIALS AND METHODS

Animals. Male wild-type (C57BL/6J) or ICAM-1 (129SV7 × C57BL/6J, backcrossed 12 times) knockout mice were obtained from Jackson Laboratory (Bar Harbor, ME). Animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care and received humane care in compliance with institutional guidelines. Specifically, 4-mo-old male mice (18–20 g body wt) were used in this study. Mice had free access to a chow diet and water ad libitum before the study.

Operative procedures and gastric cannulation. Mice were fasted for 24 h before surgery. A 45-cm PE-90 polyethylene tube (Becton Dickinson, Sparks, MD) with a small silicon tip (1.5 mm) on one end was used for gastric cannulation with aseptic surgical techniques (11, 25). Briefly, animals were anesthetized by inhalation of methoxyflurane and a vertical midline incision was made in the skin of the abdomen from the xiphoid cartilage extending to the midabdomen. A second small incision was made in the dorsal cervical area. A sub-

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cutaneous tunnel was exposed and 7-0 polypropylene sutures were passed 1 mm apart through the serosa and muscular layer of the stomach. After a small opening was made in the forestomach between sutures, the tip of the cannula was inserted 0.8 cm into the stomach. The tube was anchored to the stomach wall with Dacron felt, and the stomach was returned to the abdominal cavity. The small incision where the cannula exited through the abdominal wall was closed with 5-0 silk and tied around the cannula, resulting in tight fixation of the cannula to the abdominal wall. The abdominal wall and skin were closed with 5-0 silk. The mouse was placed in a prone position, an anchoring button was sutured to the muscles of the dorsal cervix, and the skin was closed around the button stem with 5-0 silk. The total surgical procedure took ~30 min. Gentamicin (8 mg/kg ip) and ampicillin (25 mg/kg ip) were administered postsurgically. The cannula exited through a flanged button (Instech Laboratories, Plymouth Meeting, PA) and a protective spring coil (Instech Laboratories) and was attached to a swivel (Lomir Biomedical, Notre Dame-de-L’Île-Perrot, PQ, Canada). The use of a spring coil and swivel protected the cannula and allowed free movement of animals in individual metabolic cages. The animals’ diet was delivered from syringes through the gastric cannula via infusion pumps. All animals were placed in a microorganism-free clean area during the experimental period.

Diet. A liquid diet described by Thompson and Reitz (22) supplemented with lipotropes as described by Morimoto et al. (18) was prepared fresh daily. It contained corn oil as fat (37% of total calories), protein (23%), minerals, and vitamins plus ethanol (35–40% of total calories) or isocaloric maltose-dextrin (control diet) as described elsewhere (24). Throughout the enteral feeding period, mice had free access to cellulose pellets as a source of fiber (Harlan Teklad, Madison, WI).

Experimental protocol. Mice were randomly divided into four experimental groups and fed either high-fat control or high-fat ethanol-containing diets intragastrically continuously for up to 4 wk. The diet (1.29–1.31 kcal/ml) was infused at a rate of 0.44 ml·g body wt^{-1}·day^{-1} with an infusion pump (Harvard Apparatus, Natick, MA). All animals received humane care in compliance with institutional guidelines, and severe alcohol intoxication was assessed carefully to evaluate the development of tolerance using a 0–3 behavioral scoring system (0, normal; 1, sluggish movement; 2, loss of consciousness; 3, loss of consciousness) (11, 25). The number of hepatocytes was also counted in each field and the number of leukocytes was expressed per 400 hepatocytes. Pathology was scored in a blinded manner by one of the authors and by an expert in rodent liver pathology.

Statistics. ANOVA or Student’s t-test was used for determination of statistical significance as appropriate. For comparison of pathological scores, the Mann-Whitney rank-sum test was used. A P value <0.05 was selected before the study as the level of significance.

RESULTS

Body weight gain and liver weight-to-body weight ratio. Diets were initiated 1 wk after surgery to allow for complete recovery, and all mice were healthy during the enteral feeding period. Steady weight gains were observed during 4 wk of continuous enteral feeding of liquid diets with or without ethanol, indicating adequate nutrition (Table 1). Liver weight-to-body weight ratios in wild-type mice fed ethanol (8.2 ± 0.3%) were significantly greater than in wild-type mice fed the high-fat control diet (4.9 ± 0.2%; Table 1). However, the ratios in the ICAM-1 knockout mice fed ethanol (6.3 ± 0.3%) were not different from those fed the high-fat control diet (4.8 ± 0.2%) and significantly less than in wild-type mice fed ethanol.

Ethanol concentrations in urine. Representative plots of daily urine alcohol concentrations in mice given ethanol are depicted in Fig. 1. As reported previously from this laboratory (11, 25) with the Tsukamoto-
French enteral protocol in mice, urine alcohol levels also fluctuated in a cyclic pattern from 0 to >500 mg/dl in this study, even though ethanol was infused continuously. Similar patterns were observed here in wild-type and ICAM-1 knockout mice. Mean urine alcohol concentrations over 4 wk were 228 ± 19 mg/dl in wild-type and 229 ± 24 mg/dl in ICAM-1 knockout mice (Table 1). There were no significant differences in mean urine alcohol concentrations between the groups studied. Furthermore, there were no differences in the behavioral score between wild-type and ICAM-1 knockout mice fed ethanol.

Serum transaminase levels and pathological evaluation. Serum ALT levels were 30 ± 3 IU/l in wild-type mice fed a high-fat control diet for 4 wk; however, values were increased significantly about fourfold by enteral ethanol (Table 1). In contrast, values were blunted significantly by 50% in ICAM-1 knockout mice fed ethanol.

Figure 2 shows representative photomicrographs of livers from wild-type and ICAM-1 knockout mice. After 4 wk of high-fat control diet, no pathological changes were observed in livers from both wild-type (Fig. 2A) and ICAM-1 knockout (Fig. 2B) mice. However, wild-type mice fed enteral ethanol for 4 wk developed severe steatosis in the pericentral to midzonal regions as well as focal inflammation and necrosis (Fig. 2C) with total pathology scores of 4.6 ± 0.6 (Table 2). In contrast, these pathological changes were significantly less in ICAM-1 knockout mice fed ethanol (Fig. 2D; total pathology score 1.9 ± 0.4). The predominant pathological change observed in this study was steatosis. Furthermore, infiltrating inflammatory cells, sinusoidal congestion (Fig. 2E), and focal necrosis (Fig. 2F) were observed in wild-type mice fed ethanol. In contrast, these pathological changes were not observed in ICAM-1 knockout mice fed ethanol (Fig. 2, G and H). A macro- or microvesicular pattern of fat accumulation was observed mainly in the pericentral to midzonal regions, except for one to three layers of hepatocytes around central veins as observed in other studies with enteral alcohol in mice (11, 25).

Table 1. Effect of chronic enteral ethanol on routine parameters in wild-type and ICAM-1 knockout mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild Type</th>
<th>ICAM-1 Knockout</th>
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<tbody>
<tr>
<td></td>
<td>High-fat control diet</td>
<td>High-fat ethanol diet</td>
</tr>
<tr>
<td>Weight gain, g/day</td>
<td>0.19 ± 0.03</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Mean urine alcohol concentration, mg/dl</td>
<td>N/A</td>
<td>228 ± 19</td>
</tr>
<tr>
<td>Liver weight-to-body weight ratio, %</td>
<td>4.9 ± 0.2</td>
<td>8.2 ± 0.3*</td>
</tr>
<tr>
<td>Serum ALT, IU/l</td>
<td>30 ± 3</td>
<td>124 ± 6*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 per group. Urine alcohol concentrations and serum alanine aminotransferase (ALT) levels were determined as described in MATERIALS AND METHODS. *P < 0.05 compared with wild-type mice fed high-fat control diet; †P < 0.05 compared with wild-type mice fed high-fat ethanol-containing diet by 2-way ANOVA using Bonferroni’s post hoc test.

Fig. 1. Representative plot of daily urine alcohol concentrations of ethanol-fed mice. Urine alcohol concentrations were measured daily as described in MATERIALS AND METHODS. Typical urine alcohol concentrations in wild-type (A) and intercellular adhesion molecule (ICAM)-1 knockout (B) mice fed enteral ethanol are shown.

Fig. 2. Representative photomicrographs of livers from wild-type and ICAM-1 knockout mice.
tered around necrotic hepatocytes. These clusters were composed mostly of lymphocytes and included a few PMNs (Figs. 2E and 3A).

MPO activity in the liver was minimal in mice fed the control diet, and there were no differences between wild-type and ICAM-1 knockout mice (Fig. 4). MPO activity was increased significantly about two-fold in wild-type mice fed enteral alcohol for 4 wk. In contrast, this increase was not observed in ICAM-1 knockout mice, consistent with the observation that infiltrating neutrophils were not increased in the knockout mice after enteral alcohol feeding.

**Table 2. Pathology scores in wild-type and ICAM-1 knockout mice fed enteral diets**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>ICAM-1 Knockout</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>High-fat control diet</td>
<td>High-fat ethanol diet</td>
</tr>
<tr>
<td></td>
<td>High-fat ethanol diet</td>
<td>High-fat ethanol diet</td>
</tr>
<tr>
<td>Steatosis</td>
<td>0</td>
<td>2.9 ± 0.3*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.3 ± 0.3†</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.4 ± 0.1†</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0</td>
<td>0.8 ± 0.1*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.3 ± 0.1†</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>4.6 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.9 ± 0.4†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 per group. Pathological changes were scored as described in MATERIALS AND METHODS. *P < 0.05 compared with wild-type mice fed high-fat control diet; †P < 0.05 compared with wild-type mice fed high-fat ethanol-containing diet by the Mann-Whitney rank-sum test.

TNF-α mRNA expression in liver. TNF-α mRNA expression was increased in the liver by enteral ethanol feeding in wild-type mice; however, this elevation was prevented in ICAM-1 knockout mice fed ethanol (Fig. 5).
DISCUSSION

Studies with knockout mice in mechanism of alcohol-induced liver injury. Long-term intragastric enteral feeding in the rat has recently been adapted to the mouse (11, 25). With this protocol, it was demonstrated that TNF-α was involved in early alcohol-induced liver injury via tumor necrosis factor receptor 1 (TNFR-1) (25). Furthermore, in studies using CYP2E1 knockout mice and p47phox knockout (NADPH oxidase deficient) mice (13), NADPH oxidase in the Kupffer cells, but not CYP2E1 in the parenchymal cell, was identified as the predominant source of oxidants in the early alcohol-induced liver injury (see Fig. 6). Thus the intragastric enteral feeding model with the knockout mouse provides a powerful new tool to study the mechanism of alcohol-induced liver injury. However, this model is not without its difficulties. For example, during continuous intragastric feeding, careful observation for intoxication and nursing care were required for maximal success, because mice did not recover from severe alcohol intoxication as well as rats. Accordingly, animals given ethanol were observed frequently for signs of severe alcohol intoxication. With care, however, continuous intragastric feeding has been successful for up to 4 mo.

In the present study, wild-type and ICAM-1 knockout mice received the same diet and the same amount of ethanol. Under these conditions, they achieved steady weight gains (Table 1), as observed in several other studies using mice (11, 25); however, hepatic pathology was much greater in wild-type than ICAM-1 knockout mice (Table 2). Therefore, it is concluded that this adhesion molecule is involved in the mechanism of early alcohol-induced liver injury and that nutritional complications cannot explain these results.

Role of TNF-α and ICAM-1 in early alcohol-induced liver injury. Chronic alcohol consumption increases gut-derived endotoxin levels in the portal circulation, which is a key factor in alcoholic liver disease (9, 14). Gut-derived endotoxin can activate Kupffer cells, which produces reactive oxygen species leading to activation of redox-sensitive transcriptional factors such

![Fig. 3. Effect of chronic enteral ethanol on the transmigration of leukocytes into the liver. Liver tissues were stained with Giemsa stain. A: representative photomicrograph from a wild-type mouse given ethanol diet. Note the cluster of white blood cells around necrotic hepatocytes (arrowheads). These clusters were composed mostly of lymphocytes and included a few polymorphonuclear neutrophils (PMNs; arrows). Original magnification, ×400. B: number of extravasated PMNs and lymphocytes in the liver from control and ethanol-fed mice. Values were determined as described in MATERIALS AND METHODS. Data represent means ± SE (n = 6). *P < 0.05 compared with wild-type mice given high-fat control diet, #P < 0.05 compared with wild-type mice given ethanol-containing diet by ANOVA with Bonferroni’s post hoc test.](image)

![Fig. 4. Effect of enteral ethanol on myeloperoxidase activity in the liver from wild-type and ICAM-1 knockout (KO) mice. Myeloperoxidase activity in the liver as a marker for activated neutrophils was determined as described in MATERIALS AND METHODS. Data represent means ± SE (n = 6). *P < 0.05 compared with wild-type mice given high-fat control diet, #P < 0.05 compared with wild-type mice given ethanol-containing diet by ANOVA with Bonferroni’s post hoc test.](image)
as nuclear factor (NF)-kB. NF-kB regulates production of inflammatory cytokines such as TNF-α, which is involved in pathogenesis of early alcohol-induced liver injury (Refs. 10, 14; see Fig. 6). Indeed, anti-TNF-α antibody diminished the number of infiltrating neutrophils and the amount of necrosis in the liver in the enteral model in the rat (10). Furthermore, liver injury observed in wild-type mice was nearly completely prevented in TNFR-1 knockout mouse (25). Moreover, TNF-α mRNA expression and liver injury were blunted nearly completely in a NADPH oxidase-deficient mouse fed alcohol (13). One important effect of TNF-α is stimulation of endothelial cells to synthesize adhesion molecules such as ICAM-1. ICAM-1 and other adhesion molecules expressed constitutively on the surface of endothelial cells facilitate adhesion to CD11/CD18 integrins expressed on the surface of leukocytes, leading to recruitment and adhesion of leukocytes into the liver (5, 6). Thus ICAM-1 could be involved in pathogenesis of alcoholic liver disease. Indeed, ethanol increases ICAM-1 expression on the sinusoidal endothelial cell in the Tsukamoto-French model (9, 10).

Furthermore, pathological changes observed in the liver from wild-type mice fed enteral ethanol were prevented in ICAM-1 knockout mice (Fig. 2). Subsequently, the number of infiltrating leukocytes and amount of the marker enzyme MPO were increased in the liver after 4 wk of enteral alcohol feeding in wild-type mice. These increases were blunted significantly in ICAM-1 knockout mice, as expected (Figs. 3 and 4). Interestingly, the increase of TNF-α mRNA expression in wild-type mice caused by ethanol was blunted in ICAM-1 knockout mice (Fig. 5). The mechanism underlying this suppression of TNF-α is inexplicable without the role of infiltrating leukocytes. One possibility is that TNF-α was produced mainly by infiltrating leukocytes. Alternatively, it is also possible that chemoattractant molecules released by infiltrating leukocytes activate Kupffer cells and endothelial cells to produce TNF-α.

Hepatic steatosis is one of the most characteristic findings in drinkers. In this study, hepatic steatosis was blunted significantly in ICAM-1 knockout mice fed ethanol. However, it is not clear how a deficiency of ICAM-1 expression would reduce fatty liver caused by ethanol. One possibility involves TNF-α. It is known that TNF-α stimulates lipid synthesis in the liver (8) and causes peripheral lipolysis that increases circulating...
ing levels of free fatty acids (7). In this experiment, TNF-α production was blunted in ICAM-1 knockout mice fed ethanol, consistent with the hypothesis that reduced hepatic steatosis is the result of a decrease in production of TNF-α. Furthermore, it was previously shown that chronic enteral ethanol treatment caused hypoxia in liver tissue in vivo (2) and it is also known that blocking ICAM-1 expression improves microcirculatory blood flow and oxygenation (23). In this study, sinusoidal congestion observed in wild-type mice fed ethanol was undetectable in ICAM-1 knockout mice. Thus it is also possible that improved microcirculation and oxygenation may increase fat metabolism, leading to a reduction of fat accumulation.

Conclusion. It was clear from previous studies that Kupffer cells play an important role in initiation of early alcohol-induced liver injury (1, 25). On the basis of recent evidence from studies using knockout mice, it is concluded that free radicals from NADPH oxidase in the Kupffer cell cause NF-κB activation, which induces ICAM-1 expression on the sinusoidal endothelial cell via TNF-α (see Fig. 6). Furthermore, the data presented here support the hypothesis that ICAM-1 and infiltrating leukocytes are also involved in the pathogenesis of alcohol-induced liver injury in addition to a role for the Kupffer cell. Thus alcohol-induced liver injury involves several organs (gut, liver, adipose tissue) as well as several critical cell types in the liver (Kupffer cells, endothelial cells, infiltrating leukocytes, and the ultimate target, parenchymal cells). Obviously, progress can be made best by studies using in vivo models in which these elements are all present.

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REFERENCES