Role of ICAM-1 in chronic ethanol consumption-enhanced liver injury after gut ischemia-reperfusion in rats

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A LARGE BODY OF EVIDENCE implicates leukocytes as mediators of the microvascular dysfunction and tissue injury associated with reperfusion of ischemic organs. Several experimental strategies have been used to demonstrate the contribution of leukocytes to ischemia-reperfusion (I/R) injury, including polyclonal antibodies that render animals leukopenic (17, 19, 28), adhesion molecule-specific monoclonal antibodies (10, 17, 22, 31), and adhesion molecule-deficient mice (16, 20). The effectiveness of adhesion molecule-specific monoclonal antibodies (MAbs) and adhesion molecule deficiency in attenuating I/R-induced tissue injury has led to the widely held view that leukocyte-endothelial cell adhesion is a rate-determining step in the pathogenesis of this injury process.

Recent studies (11) have implicated intercellular adhesion molecule-1 (ICAM-1), a ligand for the β2-integrins (CD11/CD18) on leukocytes, as a key modulator of leukocyte-endothelial cell adhesion. ICAM-1 is expressed at low levels on resting vascular endothelium, and its expression is markedly upregulated by certain proinflammatory agents such as cytokines [e.g., tumor necrosis factor (TNF)-α] and endotoxin (8, 9, 12). Previous reports (13, 16, 17) from our laboratory described an attenuated leukocyte recruitment and hepatocellular dysfunction induced by gut I/R in rats receiving an adhesion molecule-specific MAb directed against CD11/CD18 or ICAM-1 as well as in adhesion molecule (CD11/CD18 or ICAM-1)-deficient mice. These observations implicate a key role for ICAM-1 in gut I/R-induced hepatic microvascular dysfunction and the accompanying liver (hepatocellular) injury.

Clinically, long-term alcohol consumption has been noted to significantly reduce the incidence of coronary artery disease (25). In the liver, however, chronic alcohol consumption often results in fat deposition (fatty liver) and organ failure, particularly when these fat-laden tissues are used as donor organs in liver transplantation. This important clinical problem has drawn attention to the relationship between ethanol (EtOH) consumption and reperfusion injury in the liver. Gut I/R and chronic consumption of EtOH are known to cause liver injury via mechanisms that involve oxida-
tive stress and microcirculatory disturbances, including leukocyte sequestration and sinusoidal malperfusion (18). Gut I/R is known to elevate plasma endotoxin levels (13), whereas chronic EtOH consumption has been reported to enhance the hepatic microcirculatory dysfunction and hepatocellular injury induced by endotoxin (1, 15, 26). On the basis of these observations, one might expect that chronic EtOH consumption would lead to an exaggerated liver injury response to gut I/R. This possibility is supported by reports describing enhanced I/R-induced hepatotoxicity (an increase in blood levels of liver enzymes) after EtOH consumption in a perfused liver model (38) as well as neutrophil accumulation in the gut wall after intestinal I/R (32). By contrast, an attenuation of I/R-induced cerebrovascular injury after pretreatment with EtOH has also been described (27). Furthermore, we recently reported that low-dose acute EtOH consumption affords protection against gut I/R-induced hepatic microvascular dysfunction in the midzonal region and subsequent hepatocellular injury, whereas high-dose EtOH enhances the hepatic leukosequestration, impaired sinusoidal perfusion, and hepatocellular injury caused by gut I/R (37).

Although the available literature suggests that the acute effects of EtOH on gut I/R-induced liver injury are deleterious, the responses of the liver to gut I/R in the face of chronic EtOH consumption remain unclear. Furthermore, the overall importance of leukocyte recruitment in the liver injury response to gut I/R in animals subjected to chronic EtOH consumption is not readily apparent from the literature. Hence, the overall objectives of this study were 1) to determine whether chronic EtOH consumption alters the severity of the hepatic microvascular dysfunction and hepatocellular injury induced by gut I/R and 2) to assess the contribution of ICAM-1-mediated leukocyte recruitment to this injury response.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing ~150 g were pair fed for 6 wk a liquid diet containing EtOH that provided 36% of the total dietary calories or an isocaloric control diet according to the method of Lieber and DeCarli (23). All rats were fasted for 18 h before the experiment. All experiments were performed according to the criteria outlined by the US National Research Council.

Intravital microscopy. The rats were anesthetized with pentobarbital sodium (35 mg/kg ip). The left carotid artery was cannulated, and the catheter was placed at the aortic arch for blood pressure monitoring. The left jugular vein was also cannulated for drug administration. After laparotomy, a lobe of the liver was observed with an inverted intravital microscope (model TMD-2S, Diaphot, Nikon, Tokyo, Japan) assisted by a silicone-intensified target camera (model C-2400-08, Hamamatsu Photonics, Shizuoka, Japan). The liver was placed on an adjustable Plexiglas microscope stage with a nonfluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The liver was carefully placed to minimize the influence of respiratory movements. The liver surface was moistened and covered with cotton gauze soaked with saline. Images of the microcirculation were observed from the surface of the liver through a ×20 fluorescent objective. The microfluorographs were recorded on videotape using a videocassette recorder (model S VHS-HQ, Victor).

Analysis of leukocyte accumulation and sinusoidal perfusion. Leukocytes were labeled in vivo with rhodamine-6G (0.2 ml/100 g body wt) using a previously described method (13, 17) that was based on a method in rat brain (3). It has recently been shown that rhodamine-6G selectively stains white blood cells and platelets but not endothelial cells (3). Thus the fluorochrome allows for differentiation between adherent leukocytes and endothelial cells. Rhodamine-6G (0.2 ml/100 g body wt) was injected before EtOH administration with subsequent injections every 30 min. Rhodamine-6G-associated fluorescence was visualized by epi-illumination at 510–560 nm with the use of a 590-nm emission filter. We selected one of the lobules with well-perfused sinusoids and the fewest stationary leukocytes. We chose the furthest lobule from the edge of the liver if all the conditions were thought to be equivalent. A microfluorograph of hepatic microcirculation, with rhodamine-6G-labeled leukocytes in the sinusoids, was continuously observed for 90 min after occlusion of the superior mesenteric artery (SMA) and recorded on a digital video recorder for 1 min at 30, 60, and 90 min. The number of stationary leukocytes was determined off-line during playback of videotape images. A leukocyte was considered stationary within the microcirculation (sinusoids) if it remained stationary for >10 s. The lobule with the fewest stationary leukocytes was selected for observation at the basal condition. stationary leukocytes were quantified in the midzonal and pericentral regions of the liver lobule and expressed as the number per field of view (2.1 × 10⁵μm²). The percentage of nonperfused sinusoids was calculated as the ratio of the number of nonperfused sinusoids to the total number of sinusoids per viewing field.

Experimental protocols. We observed the surface of the liver for 10 min before ligating the SMA to ensure that all parameters measured on-line were in a steady state. The SMA was then ligated with a snare created from polyethylene tubing for 0 (sham) or 30 min. After the ischemic period, the ligation was gently removed. Leukocyte accumulation and the number of nonperfused sinusoids were measured before ischemia, immediately after reperfusion, and every 15 min for 1 h thereafter.

In some experiments, the rats were given (15 min before control measurements) an MAb directed against ICAM-I [2 mg/kg body wt; 1A29, Upjohn Laboratories, Kalamazoo, MI (34)], and the same protocol was followed. The effective blocking dose used for the MAb was based on experiments that determined the minimal amount of MAb needed to maximally reduce the leukocyte adherence and emigration induced by leukotriene B₄ or platelet-activating factor in rat mesenteric venules (39). At the doses used, the MAb did not cause leukopenia.

Liver enzyme, endotoxin, and TNF assays. At 60 min after the onset of reperfusion, the rats were removed from the microscope stage and the abdomen was closed. Blood (plasma samples for measurement of endotoxin and TNF-α levels were collected from the inferior vena cava at a point proximal to the hepatic vein at 1 h after the onset of reperfusion. For measurement of endotoxin levels, blood samples were also collected from the portal vein. Samples for plasma alanine aminotransferase (ALT) measurement were obtained at 6 h after the onset of reperfusion. Plasma ALT activity was determined by conventional ultraviolet methods, as previously described (14). Plasma TNF-α concentration was determined in a microtiter plate using a TNF-α immunoassay kit (BioSource International, Camarillo, CA) based on enzyme-linked immunosorbent assay. According to our previous report (33), plasma endotoxin levels were measured by en-
RESULTS

Figure 1 illustrates the effects of anti-ICAM-1 MAb treatment on gut I/R-induced leukostasis in sinusoids of the midzonal and pericentral regions and the terminal hepatic venule (THV; Fig. 1A) of the liver lobule and the entire liver lobule (sinusoids + THV; Fig. 1B) in the presence or absence of chronic EtOH consumption. In control rats, gut I/R elicited increases in the number of stationary leukocytes in hepatic sinusoids and THV. In EtOH-fed rats, the gut I/R-induced leukostasis was blunted in the periportal and midzonal regions (12.6 ± 0.6 and 8.0 ± 0.8 per field in control and EtOH-fed rats, respectively), while exaggerated leukostasis was noted in the pericentral region (4.3 ± 0.8 and 7.1 ± 0.8 per field in control and EtOH-fed rats, respectively) and THV (4.0 ± 0.6 and 13.3 ± 0.7 per field in control and EtOH-fed rats, respectively). Although the leukostasis elicited by gut I/R in control rats was attenuated by pretreatment with a blocking anti-ICAM-1 MAb, the exaggerated leukostasis in EtOH-fed rats was largely prevented by pretreatment with the blocking anti-ICAM-1 MAb (5.0 ± 0.7 and 5.5 ± 0.7 per field in pericentral region and THV, respectively).

Figure 2 summarizes the effects of anti-ICAM-1 MAb treatment on the gut I/R-induced increase in the percentage of nonperfused sinusoids (NPS) in the presence or absence of chronic EtOH consumption. In control rats, gut I/R elicited a significant increase in NPS. However, this response was blunted in EtOH-fed rats (22.5 ± 0.8 and 11.6 ± 1.1% for control and EtOH-fed rats, respectively, P < 0.01). Although the gut I/R-induced increase in NPS was attenuated by pretreatment with the blocking anti-ICAM-1 MAb in control rats, it did not affect the response in EtOH-fed rats.

Figure 3 shows the effects of anti-ICAM-1 MAb treatment on plasma ALT levels after gut I/R in the

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**Fig. 1.** Effects of chronic ethanol (EtOH) consumption on number of stationary leukocytes in each region (midzonal and pericentral regions; A) and entire (combined) liver lobule (B) after 30 min of gut ischemia and 60 min of reperfusion. Some animals were treated with a blocking antibody directed against intercellular adhesion molecule-1 (ICAM-1). Values are means ± SE of 5 animals. THV, terminal hepatic venule; MAb, monoclonal antibody. *P < 0.05 vs. control; †P < 0.05 vs. control + ischemia-reperfusion (I/R); ‡P < 0.05 vs. EtOH + I/R.
presence or absence of chronic EtOH consumption. In control rats, gut I/R led to an elevated plasma ALT level. Chronic EtOH consumption enhanced the gut I/R-induced increase in plasma ALT levels (115 ± 12 and 263 ± 48 IU/l for control and EtOH-fed rats, respectively). The increase in plasma ALT levels elicited by gut I/R in control and EtOH-fed rats was significantly attenuated by pretreatment with the blocking anti-ICAM-1 MAb.

Table 1 shows the effects of anti-ICAM-1 MAb treatment on plasma systemic and portal endotoxin levels after gut I/R in the presence or absence of chronic EtOH consumption. Gut I/R caused a slight elevation of plasma systemic and portal endotoxin levels in control rats, whereas chronic EtOH consumption enhanced the gut I/R-induced increase in plasma systemic and portal endotoxin levels (26.3 ± 11.3, 93.2 ± 21.4, 47.0 ± 8.5, and 104.0 ± 10.0 pg/ml for systemic control, systemic EtOH, portal control, and portal EtOH, respectively). The exaggerated elevation of plasma systemic endotoxin levels in EtOH-fed rats was largely prevented by pretreatment with the anti-ICAM-1 MAb (25.7 ± 7.9 pg endotoxin/ml), whereas pretreatment with the anti-ICAM-1 MAb caused a small reduction of the exaggerated increase in plasma portal endotoxin levels in EtOH-fed rats (there was no significant reduction but no significant difference between that in control and EtOH-fed rats after pretreatment with the anti-ICAM-1 MAb).

Figure 4 summarizes the effects of anti-ICAM-1 MAb treatment on the gut I/R-induced increase in plasma TNF-α levels in the presence or absence of chronic EtOH consumption. In control rats, gut I/R elicited a significant increase in plasma TNF-α levels. Although chronic EtOH consumption did not affect gut I/R-induced increases in plasma TNF-α levels, anti-ICAM-1 MAb treatment reduced plasma TNF-α in control and EtOH-fed rats.

DISCUSSION
Several novel aspects of this study extend the existing body of knowledge on the hepatic microvascular and parenchymal cell responses to gut I/R in rats

Fig. 2. Effects of chronic EtOH consumption on percentage of nonperfused sinusoids in mouse liver at 60 min after gut I/R. Some animals were treated with a blocking antibody directed against ICAM-1. Values are means ± SE of 5 animals. *P < 0.05 vs. control; †P < 0.05 vs. control + I/R.

Fig. 3. Effects of chronic EtOH consumption on plasma alanine aminotransferase (ALT) levels at 6 h after gut I/R. Some animals were treated with a blocking antibody directed against ICAM-1. Values are means ± SE of 5 animals. *P < 0.05 vs. control; †P < 0.05 vs. control + I/R; ‡P < 0.05 vs. EtOH + I/R.
chronically fed EtOH. Our study represents the first systematic evaluation of the effects of gut I/R on the liver of rats chronically fed EtOH. This work also provides supportive evidence with a blocking anti-ICAM-1 MAb that leukocyte-endothelial cell adhesion is an important determinant of the exaggerated microvascular dysfunction and tissue injury observed after gut I/R in the liver of rats chronically fed EtOH.

Reperfusion of the ischemic intestine in control rats results in accumulation of adherent leukocytes in sinusoids and THV, reduction in the number of perfused sinusoids, and release of liver enzymes (ALT) into the bloodstream. In control rats, the gut I/R-induced leukostasis in the pericentral region and THV was not noted after pretreatment with the anti-ICAM-1 MAb. This pretreatment also attenuated the gut I/R-induced increase in plasma ALT and TNF-α levels. Overall, the findings are consistent with our previous studies (13, 16, 17). An interesting finding in the present study is that the gut I/R-induced increase in plasma endotoxin level was not seen in control rats after pretreatment with the anti-ICAM-1 MAb. Gut I/R was reported to result in elevated plasma endotoxin, which appears to be derived from the gut. The anti-ICAM-1 MAb has been reported to blunt mesenteric I/R injury (22). Taken together, these results and evidence in the literature suggest that the anti-ICAM-1 MAb reduces blood endotoxin levels by protecting the intestinal mucosal barrier from I/R injury, thereby preventing the subsequent hepatic microvascular dysfunction and hepatocellular injury. Because endotoxin is a potent stimulant for ICAM-1 expression (2, 9), the reduction of plasma endotoxin levels by the anti-ICAM-1 MAb might result in a blunted expression of ICAM-1 in the liver.

Another interesting finding in the present study is that chronic EtOH consumption exaggerated the gut I/R-induced leukostasis in the liver and the subsequent hepatocellular injury (ALT elevation). A growing body of literature is based on the use of animals chronically fed EtOH to study the pathogenesis of alcoholic liver injury per se as well as the influence of chronic EtOH feeding on stimulus-induced liver inflammation (1, 5, 7, 15, 26). For example, chronic EtOH consumption has been reported to enhance the hepatic microcirculatory disturbances and liver injury induced by endotoxin (1, 15, 26). The findings of the present study support the possibility that elevated plasma levels of endotoxin also contribute to the exaggerated inflammatory and tissue injury responses seen in the liver after gut I/R in rats chronically fed EtOH. Because endotoxin levels are also elevated in otherwise normal rats (i.e., those not fed EtOH) after gut I/R, it is also possible that endotoxin contributes to the pathogenesis of gut I/R-induced liver injury. In the present study, the portal endotoxin level was higher in rats chronically fed EtOH than in control rats. This result suggests that intestinal mucosal permeability was increased in EtOH-fed rats after gut I/R. However, the systemic endotoxin level was much lower than the portal endotoxin level in control rats, in contrast to no significant difference between systemic and portal endotoxin levels in EtOH-fed rats after gut I/R. This result suggests that clearance of endotoxin in EtOH-fed rats was impaired. Thus an increase in intestinal mucosal permeability and a reduction of endotoxin clearance in EtOH-fed rats can be involved in the enhancement of plasma

Table 1. Systemic and portal endotoxin levels in control and EtOH-fed rats

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<thead>
<tr>
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<th>Systemic</th>
<th>Portal</th>
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<tbody>
<tr>
<td>Control</td>
<td>9.5 ± 2.2</td>
<td>13.2 ± 2.1</td>
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<tr>
<td>EtOH</td>
<td>19.2 ± 4.5</td>
<td>22.5 ± 5.0</td>
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<tr>
<td>Control + I/R</td>
<td>26.2 ± 11.3*</td>
<td>47.0 ± 8.5*†</td>
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<tr>
<td>EtOH + I/R</td>
<td>53.2 ± 21.4‡</td>
<td>104.0 ± 10.0*‡</td>
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<tr>
<td>Control + I/R + anti-ICAM-1 MAb</td>
<td>15.6 ± 4.6</td>
<td>31.4 ± 8.7*</td>
</tr>
<tr>
<td>EtOH + I/R + anti-ICAM-1 MAb</td>
<td>25.7 ± 7.98</td>
<td>65.6 ± 9.7*</td>
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Values are means ± SE in pg/ml. EtOH, ethanol; I/R, ischemia-reperfusion; ICAM-1, intercellular adhesion molecule-1; MAb, monoclonal antibody. *P < 0.05 vs. without I/R; †P < 0.05 vs. systemic; ‡P < 0.05 vs. control + I/R; §P < 0.05 vs. same feeding group + I/R.
endCAM-1 MAb caused a small reduction of the exaggerated increase in plasma portal endotoxin levels in EtOH-fed rats (there was no significant reduction but no significant difference between that in control and EtOH-fed rats after pretreatment with the anti-ICAM-1 MAb).

Although chronic EtOH consumption enhanced the gut I/R-induced increase in plasma endotoxin levels, it did not affect the gut I/R-induced increase in plasma TNF-α levels. However, chronic EtOH consumption enhanced the gut I/R-induced increase in plasma ALT activities with a parallel increase in leukostasis in the liver. It is similar to the findings in our acute EtOH model (37) that pretreatment with high-dose EtOH administration markedly enhanced the gut I/R-induced increase in plasma endotoxin levels but not the gut I/R-induced increase in plasma TNF-α levels. These results suggest that leukostasis per se or leukocyte-derived oxidants may play a more important role in the gut I/R-induced liver (hepatocellular) injury than cytokines. Another likely interpretation is that cytokines other than TNF-α are involved in the enhanced responses after gut I/R in rats chronically fed EtOH.

The expression of ICAM-1 has been shown in a variety of liver diseases (2, 24). Increased ICAM-1 expression has been observed on hepatocytes and on endothelial cells lining hepatic sinusoids in several inflammatory liver diseases. The role of ICAM-1 in alcoholic liver injury has recently received attention (2, 30). Because it is widely accepted that reactive oxygen species play an important role in hepatic reperfusion injury and alcoholic liver disease (18, 36), it is possible that chronic EtOH consumption-induced enhancement of free radical formation after gut I/R may contribute to the gut I/R-induced leukostasis in the liver by activating constitutively expressed ICAM-1.

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