Am J Physiol Gastrointest Liver Physiol 283: G537–G543, 2002.
First published April 24, 2002; 10.1152/ajpgi.00098.2002.

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

YOSHINORI HORIE,[1] YOSHIYUKI YAMAGISHI,[1] SHINZO KATO,[1] MIKIO KAJIHARA,[1] HIRONAO TAMAI,[1] D. NEIL GRANGER,[2] AND HIROMASA ISHII[1]
[1]*Department of Internal Medicine, School of Medicine, Keio University, Tokyo 160 8582, Japan; and* [2]*Department of Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71110*

Received 13 March 2002; accepted in final form 16 April 2002

Horie, Yoshinori, Yoshiyuki Yamagishi, Shinzo Kato, Mikio Kajihara, Hironao Tamai, D. Neil Granger, and Hiromasa Ishii. Role of ICAM-1 in chronic ethanol consumption-enhanced liver injury after gut ischemia-reperfusion in rats. *Am J Physiol Gastrointest Liver Physiol* 283: G537–G543, 2002. First published April 24, 2002; 10.1152/ajpgi.00098.2002.—Intercellular adhesion molecule-1 (ICAM-1) has been implicated in the hepatic microvascular dysfunction elicited by gut ischemia-reperfusion (I/R). Although the effects of chronic ethanol (EtOH) consumption on the liver are well known, it remains unclear whether this condition renders the hepatic microcirculation more vulnerable to the deleterious effects of gut and/or hepatic I/R. The objectives of this study were to determine whether chronic EtOH consumption alters the severity of gut I/R-induced hepatic microvascular dysfunction and hepatocellular injury and to determine whether ICAM-1 contributes to this response. Male Wistar rats, pair fed for 6 wk a liquid diet containing EtOH or an isocaloric control diet, were exposed to gut I/R. Intravital video microscopy was used to monitor leukocyte recruitment in the hepatic microcirculation, the number of nonperfused sinusoids (NPS), and plasma concentrations of endotoxin and tumor necrosis factor-α. Plasma alanine aminotransferase (ALT) levels were measured 6 h after the onset of reperfusion. In control rats, gut I/R elicited increases in the number of stationary leukocytes, NPS, and plasma endotoxin, tumor necrosis factor-α, and ALT. In EtOH-fed rats, the gut I/R-induced increases in NPS and leukostasis were blunted in the midzonal region, while exaggerated leukostasis was noted in the pericentral region and terminal hepatic venules. Chronic EtOH consumption also enhanced the gut I/R-induced increase in plasma endotoxin and ALT. The exaggerated responses to gut I/R normally seen in EtOH-fed rats were largely prevented by pretreatment with a blocking anti-ICAM-1 monoclonal antibody. In conclusion, these results suggest that chronic EtOH consumption enhances gut I/R-induced hepatic microvascular dysfunction and hepatocellular injury in the pericentral region and terminal hepatic venules via an enhanced hepatic expression of ICAM-1.

leukocyte adhesion; adhesion molecule; endotoxin; microcirculation; cytokine

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 β_2-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-

Address for reprint requests and other correspondence: Y. Horie, Dept. of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan (E-mail: yhorie@sc.itc.keio.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 Photonicus, Shizuoka, Japan). The liver was placed on an adjustable Plexiglas microscope stage with a nonfluorescent coverslip that allowed for observation of a 2-cm^2 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 (1 mg dissolved in 5 ml of 0.9% saline) 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 0, 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 ($2.1 \times 10^5 \mu m^2$). 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 B_4 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-

dospecy (an endotoxin-specific chromogeic *Limulus* reagent; Seikagaku, Tokyo, Japan) using an automated kinetic assay for endotoxin (35).

Statistics. The data were analyzed using standard statistical analyses, i.e., ANOVA and Scheffé's (post hoc) test. Values are means ± SE of five rats per group. Statistical significance was set at $P < 0.05$.

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. 1*A*) of the liver lobule and the entire liver lobule (sinusoids + THV; Fig. 1*B*) 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

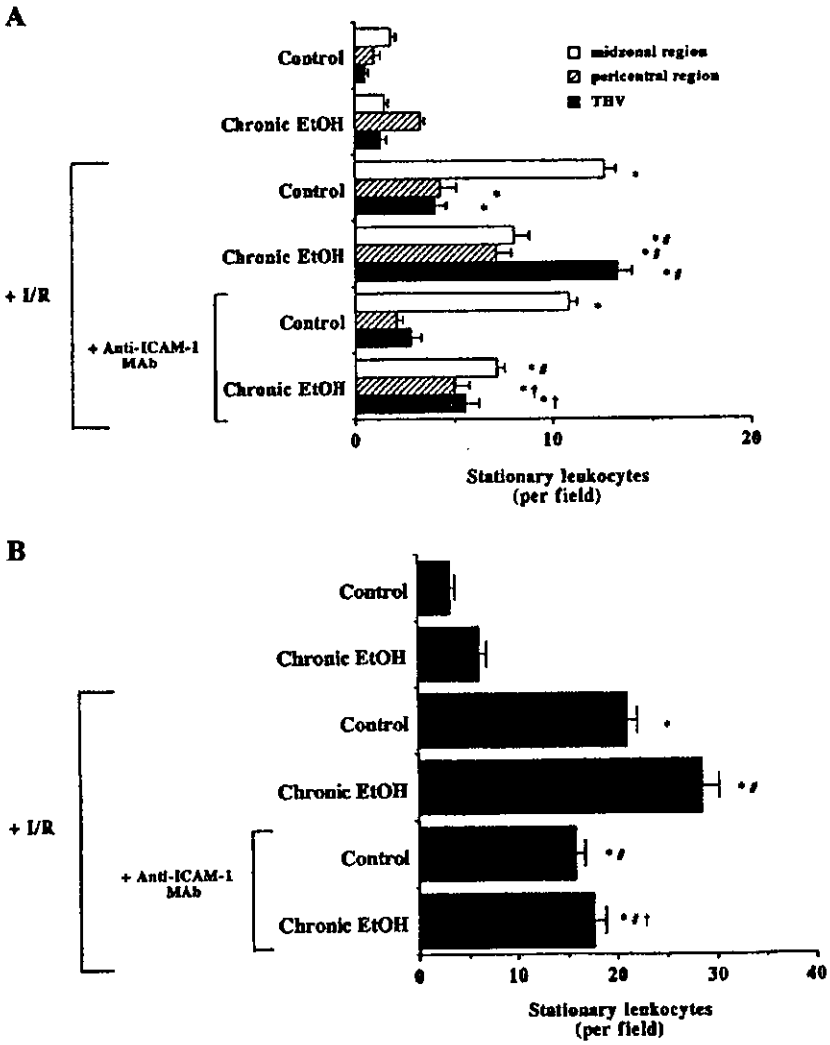

Fig. 1. Effects of chronic ethanol (EtOH) consumption on number of stationary leukocytes in each region (midzonal and pericentral regions; *A*) and entire (combined) liver lobule (*B*) after 30 min of gut ischemia and 60 min of reperfusion. Some animals were treated with a blocking antibody directed 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.

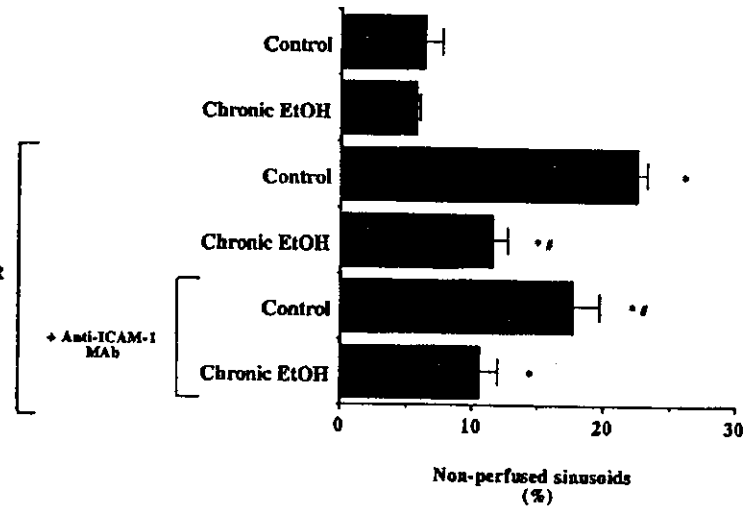

Fig. 2. Effects of chronic EtOH consumption on percentage of nonperfused sinusoids in mouse liver at 60 min after gut I/R. Some animals were treated with a blocking antibody directed against ICAM-1. Values are means ± SE of 5 animals. *$P < 0.05$ vs. control; #$P < 0.05$ vs. control + I/R.

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

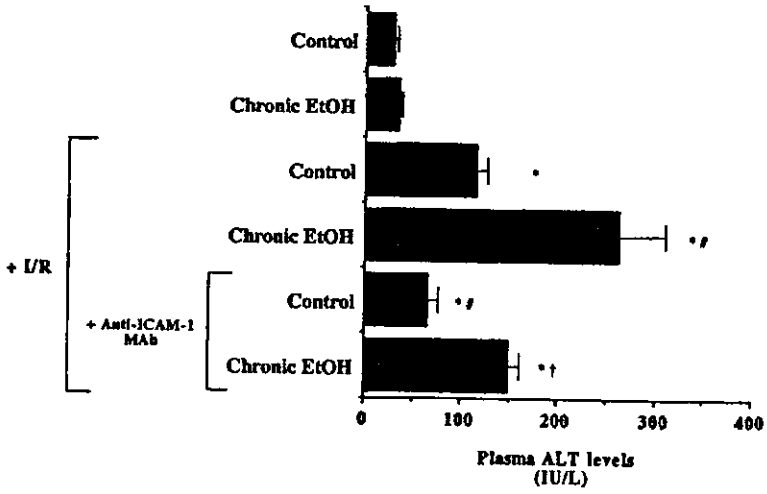

Fig. 3. Effects of chronic EtOH consumption on plasma alanine aminotransferase (ALT) levels at 6 h after gut I/R. Some animals were treated with a blocking antibody directed against ICAM-1. Values are means ± SE of 5 animals. *$P < 0.05$ vs. control; #$P < 0.05$ vs. control + I/R; †$P < 0.05$ vs. EtOH + I/R.

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

	Systemic	Portal
Control	9.5 ± 2.2	13.2 ± 2.1
EtOH	19.2 ± 4.5	22.5 ± 5.0
Control + I/R	26.2 ± 11.3*	47.0 ± 8.5*†
EtOH + I/R	93.2 ± 21.4*‡	104.0 ± 10.0*‡
Control + I/R + anti-ICAM-1 MAb	15.6 ± 4.6	31.4 ± 8.7*
EtOH + I/R + anti-ICAM-1 MAb	25.7 ± 7.9§	65.6 ± 9.7*

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.

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

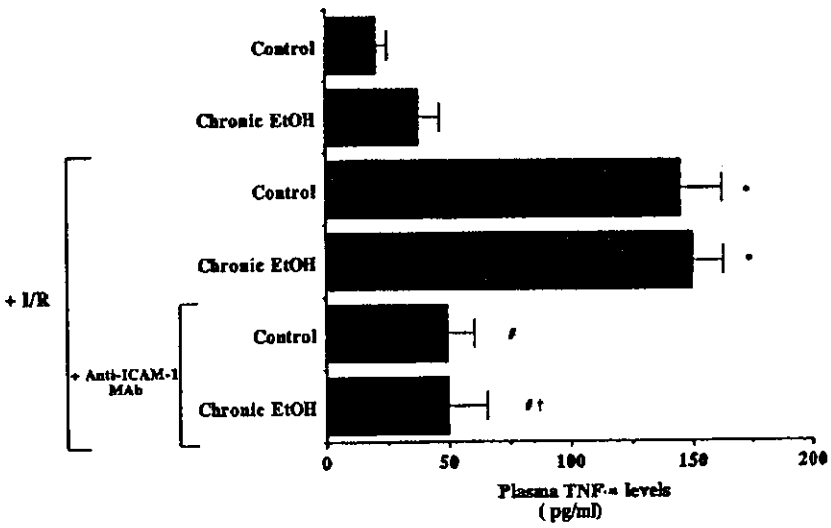

Fig. 4. Effects of chronic EtOH consumption on plasma tumor necrosis factor (TNF)-α concentration after 30 min of gut ischemia and 60 min of reperfusion. Some animals were treated with a blocking antibody directed against ICAM-1. Values are means ± SE of 5 animals. *$P < 0.05$ vs. control; #$P < 0.05$ vs. control + I/R; †$P < 0.05$ vs. EtOH + I/R.

endotoxin levels. Indeed, 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).

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, 21, 36). Our findings with an anti-ICAM-1 MAb implicate this endothelial cell adhesion molecule in the mechanism(s) responsible for the exaggerated hepatic inflammatory and injury responses to gut I/R that are seen in rats chronically fed EtOH. Pretreatment with the blocking anti-ICAM-1 MAb resulted in blunted inflammatory and microvascular responses in the liver as well as an attenuated increase in plasma ALT levels after gut I/R compared with control rats. These results suggest that ICAM-1 expressed in the liver contributes to the exaggerated responses to gut I/R in rats chronically fed EtOH. However, it remains unclear whether it is the ICAM-1 that is constitutively expressed in the liver or the newly expressed ICAM-1 (possibly in response to endotoxin) that mediates the responses noted in our study. ICAM-1 expression in the liver was reported to be enhanced in patients with alcoholic hepatitis (6). It has also been reported that soluble circulating ICAM-1 and E-selectin levels are higher in alcoholics, whereas serum vascular cell adhesion molecule-1 levels are similar to those in nonalcoholics (29). Because serum levels of endothelial cell adhesion molecules may reflect their expression on endothelial cells, the evidence from clinical studies supports the possibility that ICAM-1 expression is elevated in the livers of animals chronically fed EtOH. A role for ICAM-1 is further supported by reports describing that chronic EtOH consumption enhances endotoxin-induced leukostasis in the liver via an increased expression of LFA-1 and CD18 on leukocytes, which is a counterligand for ICAM-1 (4, 26). This enhanced expression of LFA-1 and CD18 may contribute to the enhancement of gut I/R-induced leukostasis in THV of rats chronically fed EtOH.

ICAM-1 expression can be induced by cytokines such as TNF-α (8, 9, 12). In the present study, plasma TNF-α levels were not different between control rats and rats chronically fed EtOH after gut I/R, whereas plasma TNF-α levels in rats chronically fed EtOH were almost twice the levels in control animals. This small increase in plasma TNF-α concentration may contribute to the enhanced expression of ICAM-1 in THV of rats chronically fed EtOH. However, most studies of TNF-α-induced expression of ICAM-1 have employed a single injection of a high dose of TNF-α. It remains unclear whether the small elevation in plasma TNF-α detected in our studies led to a functional increase in ICAM-1 expression in the liver. Reactive oxygen species are believed to rapidly increase the ability of endothelial ICAM-1 to bind neutrophils without detectable upregulation (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.

This study was supported by grants from the Japanese Ministry of Education and Science. D. N. Granger is supported by National Heart, Lung, and Blood Institute Grant HL-26441.

REFERENCES

1. **Arai M, Nakano S, Okuno F, Hirano Y, Sujita K, Kobayashi T, Ishii H, and Tsuchiya M.** Endotoxin-induced hypercoagulability: a possible aggravating factor of alcohol liver disease. *Hepatology* 9: 846–851, 1989.
2. **Arii S and Imamura M.** Physiological role of sinusoidal endothelial cells and Kupffer cells and their implication in the pathogenesis of liver injury. *J Hepatobiliary Pancreat Surg* 7: 40–48, 2000.
3. **Barlow CH, Harden WR, Harken AH, Simson MB, Haselgrove JC, Chance B, O'Connor M, and Austin G.** Fluorescence mapping of mitochondrial redox changes in heart and brain. *Crit Care Med* 7: 402–406, 1979.
4. **Bautista AP.** Chronic alcohol intoxication enhances the expression of CD18 adhesion molecules on rat neutrophils and release of a chemotactic factor by Kupffer cells. *Alcohol Clin Exp Res* 19: 285–290, 1995.
5. **Bautista AP.** Impact of alcohol on the ability of Kupffer cells to produce chemokines and its role in alcoholic liver disease. *J Gastroenterol Hepatol* 15: 349–356, 2000.
6. **Burra P, Hubscher SG, Shaw J, Elias E, and Adams DH.** Is the intercellular adhesion molecule-1/leukocyte function-associated antigen 1 pathway of leukocyte adhesion involved in the tissue damage of alcoholic hepatitis? *Gut* 33: 268–271, 1992.
7. **Diehl AM.** Cytokine regulation of liver injury and repair. *Immunol Rev* 174: 160–171, 2000.
8. **Eppihimer MJ, Russell J, Anderson DC, Wolitzky BA, and Granger DN.** Endothelial cell adhesion molecule expression in gene-targeted mice. *Am J Physiol Heart Circ Physiol* 273: H1903–H1908, 1997.
9. **Eppihimer MJ, Wolitzky B, Anderson DC, Labow MA, and Granger DN.** Heterogeneity of expression of E- and P-selectins in vivo. *Circ Res* 79: 560–569, 1996.
10. **Farhood A, McGuire GM, Manning AM, Miyasaka M, Smith CW, and Jaeschke H.** Intercellular adhesion molecule 1

(ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. *J Leukoc Biol* 57: 368–374, 1995.
11. **Granger DN, Kurose I, and Kvietys PR.** Modulation of leukocyte adherence and emigration during ischemia and reperfusion. In: *Physiology and Pathophysiology of Leukocyte Adhesion*, edited by Granger DN and Schmid Schönbein GW. New York: Oxford University Press, 1995, p. 323–338.
12. **Horie Y, Chervenak RP, Wolf R, Gerritsen ME, Anderson DC, Komatsu S, and Granger DN.** Lymphocytes mediate TNF-α-induced endothelial cell adhesion molecule expression: studies on severe combined immunodeficient and RAG-1 mutant mice. *J Immunol* 159: 5053–5062, 1997.
13. **Horie Y and Ishii H.** Liver dysfunction elicited by gut ischemia-reperfusion. *Pathophysiology* 8: 11–20, 2001.
14. **Horie Y, Kato S, Ohki E, Hamamatsu S, Fukumura D, Kurose I, Suzuki H, Suematsu M, Miura S, and Ishii H.** Effect of lipopolysaccharides on erythrocyte velocity in rat liver. *J Gastroenterol* 32: 783–790, 1997.
15. **Horie Y, Kimura H, Kato S, Ohki E, Tamai H, Yamagishi Y, and Ishii H.** Role of nitric oxide in endotoxin-induced hepatic microvascular dysfunction in rats chronically fed EtOH. *Alcohol Clin Exp Res* 24: 845–851, 2000.
16. **Horie Y, Wolf R, Anderson DC, and Granger DN.** Hepatic leukostasis and hypoxic stress in adhesion molecule-deficient mice after gut ischemia-reperfusion. *J Clin Invest* 99: 781–788, 1997.
17. **Horie Y, Wolf R, Miyasaka M, Anderson DC, and Granger DN.** Leukocyte adhesion and the hepatic microvascular responses to intestinal ischemia-reperfusion. *Gastroenterology* 111: 666–673, 1996.
18. **Ishii H, Kurose I, and Kato S.** Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. *J Gastroenterol Hepatol* 12: S272–S282, 1997.
19. **Jaeschke H, Farhood A, and Smith CW.** Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J* 4: 3355–3359, 1990.
20. **Kelly KJ, Williams WW Jr, Colvin RB, Meehan SM, Springer TA, Gutiérrez Ramos J, and Bonventre JV.** Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 97: 1056–1063, 1996.
21. **Kono H, Uesugi T, Froh M, Rusyn I, Bradford BU, and Thurman RG.** 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.
22. **Kurose I, Anderson DC, Miyasaka M, Tamatani T, Paulson JC, Todd RF, Rusche JR, and Granger DN.** Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. *Circ Res* 74: 336–343, 1994.
23. **Lieber CS and DeCarli LM.** Animal models of ethanol dependence and liver injury in rats and baboons. *Fed Proc* 35: 1232–1236, 1976.
24. **Menger MD, Richter S, Yamauchi J, and Vollmar B.** Role of microcirculation in hepatic ischemia/reperfusion injury. *Hepatogastroenterology* 46, *Suppl* 2: 1452–1457, 1999.
25. **Miyamae M, Diamond I, Weiner MW, Camacho SA, and Figueredo VM.** Regular alcohol consumption mimics cardiac preconditioning by protecting against ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 94: 3235–3259, 1997.
26. **Ohki E, Kato S, and Ohgo H, Mizukami T, Fukuda M, Tamai H, Okamura Y, Matsumoto M, Suzuki H, Yokoyama H, and Ishii H.** Effect of chronic ethanol feeding on endotoxin-induced hepatic injury: role of adhesion molecules on leukocytes and hepatic sinusoid. *Alcohol Clin Exp Res* 22, *Suppl* 3: 129S–132S, 1998.
27. **Phillis JW, Estevez AY, and O'Regan MH.** Protective effects of the free radical scavengers, dimethyl sulfoxide and ethanol, in cerebral ischemia in gerbils. *Neurosci Lett* 244: 109–111, 1998.
28. **Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, and Lucchesi BR.** Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 67: 1016–1023, 1983.
29. **Sacanella E, Estruch R, Badia E, Fernandez-Sola J, Nicolas JM, and Urbano Marquez A.** Chronic alcohol consumption increases serum levels of circulating endothelial cell/leucocyte adhesion molecules E-selectin and ICAM-1. *Alcohol Alcohol* 34: 678–684, 1999.
30. **Sellak H, Franzini E, Hakim J, and Pasquier C.** Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood* 83: 2669–2677, 1994.
31. **Simpson PJ, Todd RF, Fantone JC, Mickelson JK, Griffin JD, and Lucchesi BR.** Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. *J Clin Invest* 81: 624–629, 1988.
32. **Tabata T and Meyer AA.** Ethanol ingestion potentiates PMN migration into small intestine after ischemia. *J Surg Res* 58: 378–385, 1995.
33. **Tamai H, Kato S, Horie Y, Ohki E, Yokoyama H, and Ishii H.** Effect of acute ethanol administration on the intestinal absorption of endotoxin in rats. *Alcohol Clin Exp Res* 24: 390–394, 2000.
34. **Tamatani T, Kotani M, and Miyasaka M.** Characterization of the rat leukocyte integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. *Eur J Immunol* 21: 627–633, 1991.
35. **Tamura T, Arimoto Y, Tanaka S, Yoshida M, Obayashi T, and Kawai T.** Automated kinetic assay for endotoxin and 1-3-β-D-glucagon in human blood. *Clin Chim Acta* 226: 109–112, 1994.
36. **Thurman RG, Bradford BU, Iimuro Y, Knecht KT, Arteel GE, Yin M, Connor HD, Wall C, Raleigh JA, Frankenberg MV, Adachi Y, Forman DT, Brenner D, Kadiiska M, and Mason RP.** The role of gut-derived bacterial toxins and free radicals in alcohol-induced liver injury. *J Gastroenterol Hepatol* 13, *Suppl*: S39–S50, 1998.
37. **Yamagishi Y, Horie Y, Kajihara M, Kato S, Granger DN, and Ishii H.** Ethanol modulates gut ischemia/reperfusion-induced liver injury in rats. *Am J Physiol Gastrointest Liver Physiol* 282: G640–G646, 2002.
38. **Younes M, Wagner H, and Strubelt O.** Enhancement of acute ethanol hepatotoxicity under conditions of low oxygen supply and ischemia/reperfusion. The role of oxygen radicals. *Biochem Pharmacol* 38: 3573–3581, 1989.
39. **Zimmerman BJ, Holt JW, Paulson JC, Anderson DC, Miyasaka M, Tamatani T, Todd RF III, Rusche JR, and Granger DN.** Molecular determinants of lipid mediator-induced leukocyte adherence and emigration in rat mesenteric venules. *Am J Physiol Heart Circ Physiol* 266: H847–H853, 1994.

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

Yoshinori Horie, Yoshiyuki Yamagishi, Shinzo Kato, Mikio Kajihara, Hironao Tamai, D. Neil Granger and Hiromasa Ishii

AJP - GI 283:537-543, 2002. First published Apr 24, 2002; doi:10.1152/ajpgi.00098.2002

Free Radical Biology & Medicine, Vol. 32, No. 6, pp. 487–491, 2002

0891-5849/02/$–see front matter

ELSEVIER

PII S0891-5849(02)00740-2

Serial Review: Alcohol, Oxidative Stress and Cell Injury
Guest Editor: Arthur Cederbaum

ROLE OF MITOCHONDRIA IN ALCOHOLIC LIVER INJURY

MASAYUKI ADACHI and HIROMASA ISHII
Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

(*Received* 10 *September* 2001; *Accepted* 27 *December* 2001)

Abstract—Oxidative stress and oxygen-derived free radicals are well known to play an important role in the pathogenesis of ethanol-associated liver injury. Active oxidants produced during ethanol metabolism induce mitochondrial membrane depolarization and permeability changes in cultured hepatocytes. These mitochondrial alterations (loss of $\Delta\Psi m$ and mitochondrial permeability transition [MPT]) are now recognized as a key step in apoptosis. In recent studies, including ours, the MPT has been identified as a key step for the induction of mitochondrial cytochrome c release and caspase activation by ethanol. In addition, chronic and/or acute ethanol modulates intracellular, especially mitochondrial, antioxidant levels, leading to the increased susceptibility to alcoholic liver injury induced by several apoptotic stimuli. In this review, we address the mechanism of mitochondrial alterations and liver injury induced by ethanol. © 2002 Elsevier Science Inc.

Keywords—Oxidative stress, Mitochondria, Apoptosis, Mitochondrial permeability transition, Glutathione, Cytochrome c, Free radicals

INTRODUCTION

Ethanol administration affects the generation of mitochondrial free radicals. Although a variety of experimental data suggests a role for active oxygen species in ethanol-induced mitochondrial dysfunction in hepatocytes, the detailed mechanism of ethanol-induced oxidative stress on mitochondria remains uncertain. Recent evidence implies that mitochondria have an important role in apoptosis. Mitochondria regulate apoptosis by releasing apoptosis-promoting factors into the cytosol. Although the apoptotic signal downstream of the mitochondria is relatively clear, the precise mechanism of apoptotic signals within the mitochondria has not been elucidated.

Apoptosis, as manifested by the histopathologic appearance of apoptotic bodies, is frequently observed in alcoholic liver disease. Several experimental reports have suggested that apoptosis occurs in animal models of alcoholic liver disease [1]. Hepatocyte apoptosis has also been reported to increase in alcoholic hepatitis patients [2]. The potential mechanism of hepatocyte apoptosis in alcoholic liver disease is thought to involve oxidative stress and inflammatory cytokines, such as tumor necrosis factor α [3]. Despite an increasing body of experimental and clinical evidence for the role of enhanced oxidative stress and oxygen-derived free radicals in alcoholic liver diseases [4,5], the precise role of oxidative injury in the mechanism of alcoholic liver injury remains unclear. Studies in our laboratory strongly suggest that active oxidants produced during ethanol metabolism

This article is part of a series of reviews on "Alcohol, Oxidative Stress and Cell Injury." The full list of papers may be found on the homepage of the journal.

Masayuki Adachi, M.D., graduated from Keio University School of Medicine in 1996. After a 1-year residency in Internal Medicine at Keio University Hospital, he was a postgraduate student under the direction of Prof. Hiromasa Ishii from 1997 to 2000. Now a staff physician at Keio University Hospital, his basic research foci are molecular mechanisms of liver injury, especially oxidative stress and mitochondria.

Hiromasa Ishii, M.D., Ph.D., is Professor and Chair, Department of Internal Medicine, Keio University School of Medicine. He was a Research Fellow and Visiting Instructor of Medicine, Mount Sinai School of Medicine (CUNY), and Senior Resident, Bronx VA Medical Center from 1969 to 1972. He was appointed as Assistant Professor (1973), Associate Professor (1979), and Professor and Chair (1994) of Keio University. He was Vice President of the International Society for Biomedical Research on Alcoholism (ISBRA) from 1994 to 1998, and became President of the 10th ISBRA meeting (2000). His specialty is Hepatology and Gastroenterology, especially alcoholic liver disease, alcohol and drug metabolism.

Address correspondence to: Hiromasa Ishii, M.D., Ph.D., Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan; Tel: +81 (3) 5363-3789; Fax: +81 (3) 3356-9654; E-Mail: hishii@sc.itc.keio.ac.jp.

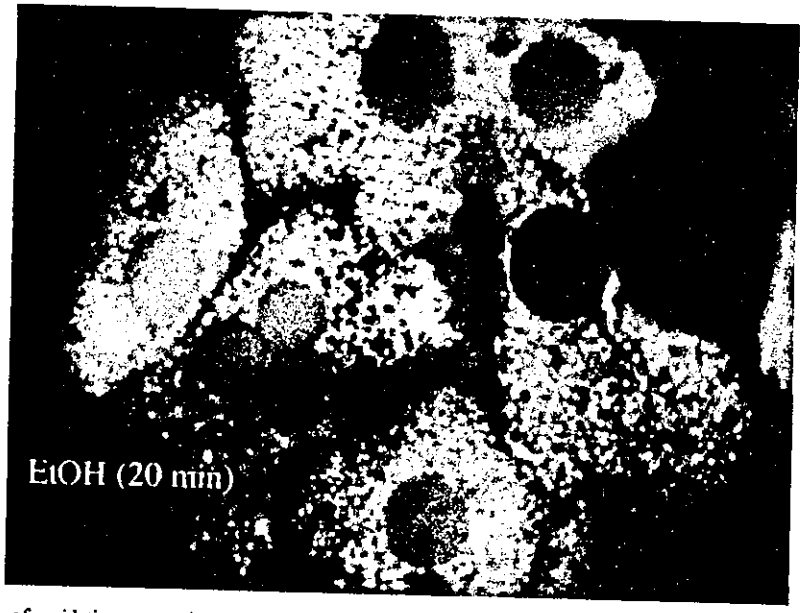

Fig. 1. Occurrence of oxidative stress in hepatocytes after ethanol administration. Dichlorofluorescein (DCF) fluorescence, a fluorochrome that indicates the excessive production of oxygen-derived free radicals, increased in cultured rat hepatocytes at 20 min after administration of ethanol (50 mM).

modulate mitochondrial energy synthesis and permeability changes in isolated and cultured hepatocytes [6–8]. Here, we will briefly summarize the evidence implicating mitochondria in ethanol-induced apoptosis.

ACUTE ETHANOL ADMINISTRATION INDUCES OXIDATIVE STRESS IN HEPATOCYTES

Oxidative stress can be defined as the exposure of the cell, tissue, or organism to an excess oxidant, particularly the superoxide anion (O_2^-) and its metabolites, which we will refer to as reactive oxygen species (ROS). Oxidative stress affects cellular integrity only when antioxidant mechanisms are no longer capable of coping with the generation of free radicals. Ethanol administration induces oxidative stress by either enhancing the production of oxygen reactive species and/or decreasing the level of endogenous antioxidants [4]. Therefore, many experimental studies have been conducted to ascertain the effects of acute and/or chronic ethanol administration on liver antioxidant enzymes and substrates.

Short-term ethanol administration has been shown to increase superoxide generation in liver mitochondria and in perfused rat liver [9]. The enhanced superoxide generation increases lipid peroxide and induces mitochondrial dysfunction in rats that have been subjected to acute ethanol exposure [10]. A previous study of ours demonstrated that levels of dichlorofluorescein (DCF) fluorescence, a fluorochrome that indicates the excessive production of oxygen-derived free radicals and lipid peroxides, actually increase in single cultured rat hepatocytes within 20 min of exposure to ethanol (Fig. 1). Ethanol can induce a hypermetabolic state in the liver that is characterized by enhanced mitochondrial respiration, which is driven by a large demand for the reoxidation of NADH that is produced during ethanol metabolism by cytosolic alcohol dehydrogenase. The decrease in the NAD+/NADH ratio induced by acute ethanol administration may favor mitochondrial superoxide generation by increasing the electron flow along the respiratory electron transport chain. Recent studies using HepG2 cells overexpressing CYP2E1 suggested that ethanol oxidation by CYP2E1 also contributes to ethanol-induced oxidative stress and cell injury [11]. As CYP2E1 activity is induced by the continuous alcohol intake, ethanol oxidation by CYP2E1 is implicated to be one of important sources of oxidative stress in alcoholics.

Ethanol also enhances oxidative stress by reducing the level of antioxidants. Superoxide anions produced in mitochondria can be catalyzed by mitochondrial active MnSOD to hydrogen peroxide. GSH peroxidase acts to reduce the hydrogen peroxide, preventing the subsequent production of more reactive radicals. Since the mitochondria of liver cells do not contain catalase, the ability of the mitochondrial GSH system to reduce hydrogen peroxide is probably the main mechanism for protecting mitochondria against oxidative stress. Chronic ethanol exposure is reported to induce a selective decrease in the mitochondrial pool of glutathione because of impaired uptake of glutathione from cytosol to mitochondrial ma-

trix [12,13]. The decreased levels of glutathione cause greater susceptibility of chronically alcohol-fed liver to oxidative stress or TNF-α [14]. By contrast, there is still no agreement on the effects of acute ethanol intoxication on the hepatic glutathione level. In acute models of ethanol intoxication, we have previously reported that acute ethanol treatment (< 100 mM) did not alter cellular glutathione level in rat hepatocytes until 30 min after treatment [6]; however, a later report showed that acute ethanol intoxication decreased the liver glutathione level [15]. In support of this important role of glutathione as a cytoprotective factor, maintaining cellular glutathione levels is attempted to prevent the development of alcoholic liver injury. We have reported that the depletion of hepatocyte glutathione leads to enhanced oxidative stress in mitochondria and cell membranes exposed to ethanol and treatment with the glutathione precursor N-acetyl-L-cysteine prevented these alterations [6]. In addition, treatment with S-adenosyl-L-methionine is reported to prevent alcoholic liver injury by restoring increased glutathione levels in the liver [16].

MITOCHONDRIAL MEMBRANE DEPOLARIZATION AND PERMEABILITY TRANSITION

The mitochondrial transmembrane potential ($\Delta\Psi$m) results from the asymmetric distribution of protons and other ions on both sides of the inner mitochondrial membrane, giving rise to a chemical and electric gradient that is essential for mitochondrial function. The inner side of the inner mitochondrial membrane is negatively charged. Alterations in the mitochondrial membrane potential ($\Delta\Psi$m) and membrane permeability are now thought to be a central regulatory mechanism of cell viability [17]. We have examined the alteration of mitochondrial membrane potential in rat hepatocytes exposed to ethanol using Rh123 as an indicator of mitochondrial membrane potential [6]. Acute ethanol administration significantly decreased $\Delta\Psi$m in hepatocytes within 30 min, suggesting that mitochondrial depolarization is actually observed as an early event of ethanol-induced hepatocyte injury. In addition to the disruption in $\Delta\Psi$ m, the mitochondria exhibit an increase in membrane permeability. This phenomenon is known as the mitochondrial membrane permeability transition (MPT). MPT is induced by the opening of the mitochondrial megachannel, also known as the PT pore [18,19]. The PT pore is thought to be a multiprotein complex located at the point of contact between the inner and outer mitochondrial membranes. This complex consists of several proteins, including a voltage-dependent anion channel (VDAC), an adenine nucleotide translocator (ANT), and a cyclophillin D (CyP-D). The open/closed state of the PT pore is regulated by the condition of the mitochondrial matrix, and is influenced by a variety of factors, including the electrical membrane potential, thiols, oxidants, pH, and calcium concentration. MPT occurs as a consequence of the reversible opening of the PT pore. When the PT pore opens, rapid ion movement causes extensive mitochondrial swelling and loss of the mitochondrial membrane potential. MPT after acute ethanol intoxication was observed using laser confocal microscopy (Fig. 2). Acute ethanol-induced MPT in cultured hepatocytes is associated with an increase in oxidative stress and a decrease in $\Delta\Psi$m, both of which are inhibited by antioxidants or an inhibitor of alcohol dehydrogenase [6,8]. Thus, oxidative stress via ethanol metabolism is thought to trigger $\Delta\Psi$m loss and MPT in hepatocytes exposed to acute ethanol.

ROLE OF MITOCHONDRIA IN ETHANOL-INDUCED HEPATOCYTE APOPTOSIS

Mitochondria are central to the life of eukaryotic cells and also play a key role in apoptosis [20,21]. Mitochondrial depolarization and permeability transitions are central to apoptotic cell death. Inhibitors of the PT pore complex, such as bongkrekic acid and cyclosporin A, inhibit MPT and the loss of $\Delta\Psi$m, thereby preventing apoptosis. Thus, the opening of the PT pore and the loss of $\Delta\Psi$m are responsible for several types of apoptotic cell death.

The mitochondria-mediated pathway of apoptosis does not simply involve a "loss of function," resulting in a bioenergic defect; the pathway is also affected by the release of apoptosis-inducing factors. These apoptosis-inducing factors, such as cytochrome c and apoptosis-inducing factor (AIF), are released from the intermembrane space of mitochondria into the cytosol. Once cytochrome c has been released, caspase-9 is activated, and subsequently cleaves caspase-3 to its activated form. Caspase-3 is a key factor in apoptosis. The mitochondrial pathway of apoptosis is also controlled and regulated by the Bcl-2 family proteins. This protein family can be divided into antiapoptotic (Bcl-2, Bcl-XL, Mcl-1, Bcl-w, A1) and proapoptotic (Bax, Bak, Bcl-Xs, Bid, Bad, Bik, Bim, Blk) members [20]. The antiapoptotic Bcl-2 family proteins inhibit the release of cytochrome c from mitochondria in several apoptotic models. On the other hand, the proapoptotic Bcl-2 family proteins enhance the release of cytochrome c.

We recently reported that acute ethanol exposure induces the release of cytochrome c from mitochondria to the cytosol, activating caspase-9 and caspase-3 and leading to apoptosis. These apoptotic changes are inhibited by the addition of antioxidants, suggesting that oxidative stress is involved in the release of cytochrome c that precedes apoptosis in hepatocytes exposed to acute ethanol [6,8]. We have also shown that inhibitors of the PT

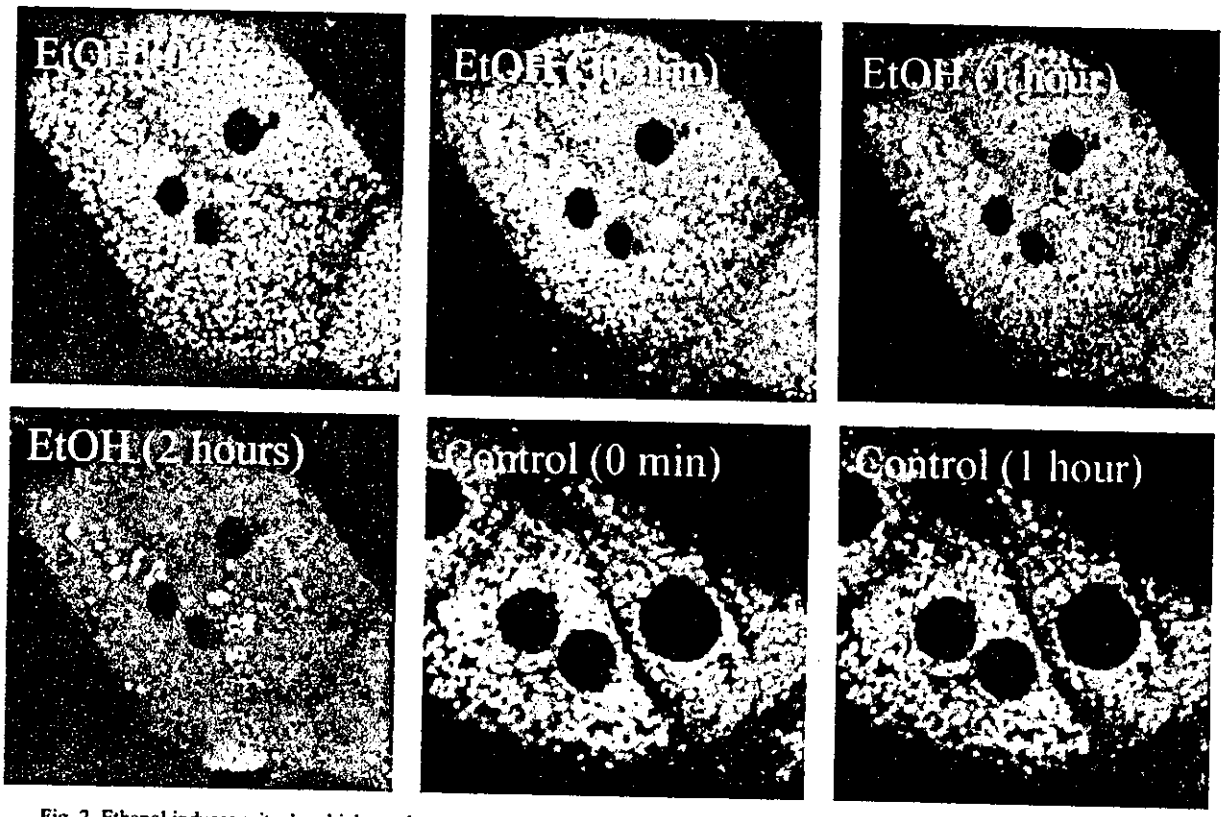

Fig. 2. Ethanol induces mitochondrial membrane permeability transition in cultured hepatocytes. Cultured hepatocytes were preloaded with calcein-AM and $CoCl_2$. Hepatocytes were incubated with ethanol (50 mmol/l). The green calcein fluorescence was observed under a laser scanning confocal microscope. Note mitochondrial calcein fluorescence decreased wit time. (This figure is cited from reference [8] with permission.)

pore complex, such as cyclosporin A, also inhibit MPT, the loss of $\Delta\Psi m$, and subsequent apoptosis. Thus, ethanol-induced hepatocyte apoptosis is dependent on the induction of MPT. These results suggest that when MPT is triggered by acute ethanol, the mitochondria play a role in the induction of apoptosis via the release of cytochrome c and that acute ethanol-induced hepatocyte apoptosis is dependent on the activation of caspase. Furthermore, mitochondria from chronic ethanol-fed rats were demonstrated to be more sensitive to MPT induced by various apoptotic stimuli [22]. This also suggests that mitochondria play important roles in the development of alcohol liver injury.

The precise mechanism by which cytochrome c is released from mitochondria remains controversial. An important point is whether apoptotic cytochrome c can pass through the PT pore. Only the molecules of less than 1.5 kD are able to pass through the PT pore, and the molecular weight of cytochrome c is about 15 kD. One potential mechanism for the release of cytochrome c from mitochondria involves the swelling and rupture of mitochondrial outer membrane as a result of the opening of the PT pore in the inner membrane [17,18]. The opening of the PT pore is thought to induce the influx of water and solutes, followed by mitochondrial swelling and the rupture of its outer membrane, resulting in the release of cytochrome c. This model accounts for some, but not all mechanisms of apoptotic cell death; the mitochondria remain morphologically normal in many types of apoptosis. In a second model, a new channel is formed, enabling the passage of cytochrome c through the mitochondrial membrane. A recent report suggests that the Bcl-2 family proteins play an important role in this mitochondrial event [23]. Nevertheless, the mechanisms of cytochrome c release in ethanol-induced hepatocyte apoptosis must be further investigated.

CONCLUSION

This review suggests a putative role for ethanol-induced oxidative stress in the process of mitochondrial dysfunction and cell death. Acute ethanol intoxication induces oxidative stress and mitochondrial dysfunction in primary cultured hepatocytes. These mitochondrial alterations (loss of $\Delta\Psi m$ and MPT) are now recognized as key steps in apoptosis. We have demonstrated that acute ethanol-associated oxidative stress induces a change in mitochondrial permeability, the release of cy-

tochrome c, caspase activation, and apoptosis in cultured hepatocytes. Oxidative stress occurs as the consequence of an imbalance in oxidant production and antioxidant defense. The development of new and effective strategies to diminish the production of oxidants and/or enhance intracellular and extracellular antioxidant defenses in the liver offers great promise for the prevention and treatment of liver disease.

Acknowledgements — This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture of Japan and by a grant from Keio University, School of Medicine.

REFERENCES

[1] Benedetti, A.; Brunelli, E.; Risicato, R.; Cilluffo, T.; Jezequel, A. M.; Orlandi, F. S. Subcellular changes and apoptosis induced by ethanol in rat liver. *J. Hepatol.* **29**:137–143; 1988.

[2] Natori, S.; Rust, C.; Stadheim, L. M.; Srinivasan, A.; Burgart, L. J.; Gores, G. J. Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. *J. Hepatol.* **34**:248–253; 2001.

[3] Nanji, A. A. Apoptosis and alcoholic liver disease. *Semin. Liver. Dis.* **18**:187–190; 1998.

[4] Ishii, H.; Kurose, I.; Kato, S. Pathogenesis of alcoholic liver disease with particular emphasis on oxidative stress. *J. Gastroenterol. Hepatol.* **12**(Suppl.):S272–S282; 1997.

[5] Kaplowitz, N.; Tsukamoto, H. Oxidative stress and liver disease. *Prog. Liver Dis.* **14**:131–159; 1996.

[6] Kurose, I.; Higuchi, H.; Kato, S.; Miura, S.; Watanabe, N.; Kamegaya, Y.; Tomita, K.; Takaishi, M.; Horie, Y.; Fukuda, M.; Mizukami, K.; Ishii, H. Oxidative stress on mitochondria and cell membrane of cultured rat hepatocytes and perfused liver exposed to ethanol. *Gastroenterology* **112**:1331–1343; 1997.

[7] Kurose, I.; Higuchi, H.; Miura, S.; Saito, H.; Watanabe, N.; Hokari, R.; Hirokawa, M.; Takaishi, M.; Zeki, S.; Nakamura, T.; Ebinuma, H.; Kato, S.; Ishii, H. Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication. *Hepatology* **25**:368–378; 1997.

[8] Higuchi, H.; Adachi, M.; Miura, S.; Gores, G. J.; Ishii, H. The mitochondrial permeability transition contributes to acute ethanol-induced apoptosis in rat hepatocytes. *Hepatology* **34**:320–328; 2001.

[9] Bautista, A. P.; Spitzer, J. J. Acute ethanol intoxication stimulates superoxide anion production by in situ perfused rat liver. *Hepatology* **15**:892–898; 1992.

[10] Masini, A.; Ceccarelli, D.; Gallesi, D.; Giovannini, F.; Trenti, T. Lipid hydroperoxide induced mitochondrial dysfunction following acute ethanol intoxication in rats: the critical role for mitochondrial reduced glutathione. *Biochem. Pharmacol.* **47**:217–224; 1994.

[11] Wu, D.; Cederbaum, A. I. Ethanol-induced apoptosis to stable HepG2 cell lines expressing human cytochrome P-4502E1. *Alcohol Clin. Exp. Res.* **23**:67–76; 1999.

[12] Fernandez-Checa, J. C.; Garcia-Ruiz, C.; Ookhtens, M.; Kaplowitz, N. Impaired uptake of GSH by hepatic mitochondria from chronic ethanol-fed rats. Tracer kinetic studies in vitro and in vivo and susceptibility to oxidative stress. *J. Clin. Invest.* **87**:397–405; 1991.

[13] Takeshi, H.; Kaplowitz, N.; Kamimura, T.; Tsukamoto, H.; Fernandez-Checa, J. C. Hepatic mitochondrial GSH depletion and progression of experimental alcoholic liver disease in rats. *Hepatology* **16**:423–1428; 1992.

[14] Colell, A.; Garcia-Ruiz, C.; Miranda, M.; Ardite, E.; Mari, M.; Morales, A.; Corrales, F.; Kaplowitz, N.; Fernandez-Checa, J. C. Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. *Gastroenterology* **115**:1541–1551; 1998.

[15] Speisky, H.; MacDonald, A.; Giles, G.; Orrego, H.; Israel, Y. Increased loss and decreased synthesis of hepatic glutathione after acute ethanol administration. *Biochem. J.* **225**:565–572; 1985.

[16] Vendemiale, G.; Altomare, E.; Trizio, T.; LeGrazie, C.; Di Padova, C.; Salerno, M. T.; Carrieri, V. Effects of oral S-adenosyl-L-methionine on hepatic glutathione in patients with liver disease. *Scand. J. Gastroenterol.* **18**:307–318; 1989.

[17] Susin, S. A.; Zamzami, N.; Kroemer, G. Mitochondria as a regulator of apoptosis: doubt no more. *Biochim. Biophys. Acta* **1366**:151–165; 1998.

[18] Bernardi, P. The permeability transition pore. Control points of cyclosporin A-sensitive mitochondrial channel involved in cell death. *Biochem. Biophys. Acta* **1275**:5–9; 1996.

[19] Zoratti, M.; Szabo, I. The mitochondrial permeability transition. *Biochem. Biophys. Acta* **1241**:139–176; 1995.

[20] Green, D. R.; Reed, J. C. Mitochondria and apoptosis. *Science* **281**:1309–1312; 1998.

[21] Kroemer, G.; Zamzami, N.; Susin, S. A. Mitochondrial control of apoptosis. *Immunol. Today* **18**:44–51; 1997.

[22] Pastorino, J. G.; Marcinevicuite, A.; Cahill, A.; Hoek, J. B. Potentiation by chronic ethanol treatment of the mitochondrial permeability transition. *J. Biol. Chem.* **265**:405–409; 1999.

[23] Narita, M.; Shimizu, S.; Ito, T.; Chittenden, T.; Lutz, R. J.; Matsuda, H.; Tsujimoto, Y. Bax interacts with permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc. Natl. Acad. Sci. USA* **95**:14681–14686; 1998.

Journal of Gastroenterology and Hepatology (2003) 18, 851–857

DRUGS AND THE LIVER

Clinical usefulness of edaravone for acute liver injury

SHINICHIRO TADA,* NOBUHIRO NAKAMOTO,* KAORI KAMEYAMA,† SATOSHI TSUNEMATSU,‡ NAOKI KUMAGAI,‡ HIDETSUGU SAITO* AND HIROMASA ISHII*

*Departments of *Internal Medicine and †Pathology, School of Medicine, Keio University and ‡Center for Liver Diseases, Kitasato Institute Hospital, Tokyo, Japan*

Abstract

Background and Aims: Edaravone, a newly synthesized radical scavenger, has shown an excellent effect on treating stroke patients. The effect of edaravone on carbon tetrachloride (CCl_4)-induced acute liver injury was examined.

Methods: Six rats were injected with CCl_4 alone and six rats were intravenously injected with edaravone immediately after and 3 h after injection of CCl_4. Another six rats were injected with olive oil alone. The animals were killed at 24 h after the CCl_4 injection.

Results: Injection of CCl_4 was followed by a marked increase in serum alanine aminotranferase (ALT) level (CCl_4, 1630.6 ± 606.8 IU/L; olive oil, 21.0 ± 2.6 IU/L; $P < 0.001$), lactate dehydrogenase (LDH) level (CCl_4, 5068.0 ± 2956.4 IU/L; olive oil, 203.6 ± 30.5 IU/L; $P < 0.005$), and total bilirubin (TB) level (CCl_4, 0.88 ± 0.48 mg/dL; olive oil, 0.37 ± 0.05 mg/dL; $P < 0.01$), whereas in the edaravone-treated rats, the ALT (119.4 ± 113.5 IU/L, $P < 0.001$), LDH (369.7 ± 288.2 IU/L, $P < 0.005$), and TB values (0.29 ± 0.16 mg/dL, $P < 0.01$) were significantly decreased. Histological examination of the liver by hematoxylin and eosin and oil red O staining showed a marked reduction of steatosis in the CCl_4 and edaravone-treated rats compared with the CCl_4-injected rats. Significant inhibition of hepatocytic apoptosis was demonstrated by the terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) method in the edaravone-treated rats.

Conclusions: These results suggest that edaravone has a marked preventive effect on oxidative stress-induced acute liver injury.

Key words: acute liver injury, apoptosis, carbon tetrachloride, free radicals, steatosis.

INTRODUCTION

Reactive oxygen species are generated by oxidative stress within the liver as a result of injuries such as drug-induced hepatitis,[1] alcoholic liver damage,[2] ischemic injury, or particularly, chemotherapeutic agents.[3] Free radicals formed as a result of oxidative stress injure the cell membrane of hepatocytes by lipid peroxidation or other means.[4–7] Free radicals have been shown to damage proteins, lipids, carbohydrates, and also DNA.[4–6,8] When free radicals exceed the capacity of the endogenous antioxidant system, cell damage occurs via oxidative stress.[6,7] However, no clinically successful and useful treatment for liver injury caused by oxidative stress, and subsequent cell death or apoptosis, that is based on the approach to the pathogenic mechanism described has ever been developed.

It is well known that intraperitoneal injection of carbon tetrachloride (CCl_4) causes acute liver injury in rats.[9] Carbon tetrachloride induces centrilobular necrosis and fatty change in the liver.[7,10] Its toxicity is mediated by cytochrome p450-induced metabolites, which consist of a highly reactive trichloromethyl radical and a subsequent form of trichloromethylperoxyl radical, which initiates lipid peroxidation by withdrawing allylic hydrogens from polyunsaturated fatty acids. Intracellu-

Correspondence: Dr Hidetsugu Saito, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Toyko 160-8582, Japan. E-mail: hsaito@sc.itc.keio.ac.jp

Address requests for reprints to: Dr Hiromasa Ishii, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Toyko 160-8582, Japan.

*The first two authors contributed to this study equally.

Accepted for publication 17 December 2002.

larly generated free radicals then induce hepatocyte membrane damage.[8,10] These effects in turn induce inflammatory cytokine production by immune cells, such as Kuppfer cells, and the cytokines then induce injury of the entire liver.[11,12]

Hepatic antioxidants have been demonstrated to decrease the progression of liver injury generated by a single injection of CCl_4. Intracellular or endogenous vitamin E is a well-known lipid-soluble antioxidant whose levels are important in protecting against oxidative damage, and is also a potent treatment modality. However, the remarkable lipophilicity of vitamin E causes slow incorporation into tissue, suggesting that vitamin E cannot be used clinically in acute hepatic injury.[13–15] Edaravone (3-methyl-1-phenyl-pyrazolin-5-one), a newly synthesized antioxidant, inhibits both non-enzymatic peroxidation and lipoxigenase activity[16] and prevents the oxidative damage of vascular endothelial cells caused by hydroperoxyeicosatetraenoic acid *in vitro*.[17] Furthermore, testing in various experimental animal models has suggested that edaravone has protective effects against cerebral and myocardial ischemia–reperfusion injuries.[18–21] These effects of edaravone on ischemia–reperfusion injury are thought to be attributable to its antioxidant property.[16]

These observations suggest that edaravone may be effective in inhibiting CCl_4-induced acute liver damage. In the present study, the effect of edaravone on acute liver injury caused by CCl_4 in rats was investigated, and the possibility of using edaravone to treat acute liver damage was considered.

METHODS

Reagents

The edaravone was a gift from Mitsubishi Wellpharma (Tokyo, Japan). The CCl_4 and olive oil were purchased from Kanto Kagaku (Tokyo, Japan). The proteinase K, phosphate-buffered saline (PBS), cacodylate, Tris HCl solution, bovine serum albumin and cobalt chloride ($CoCl_2$) were purchased from Sigma Chemical (St. Louis, MO, USA). The TdT and Biotin-16-dUTP were obtained from Roche Applied Science (Basel, Switzerland).

Animals and treatment

The animal experiment was approved by the Animal Experimentation Committee of the School of Medicine, Keio University. Male Wistar rats (6 weeks old, 200–250 g in weight) obtained from Clea Japan, (Tokyo, Japan) were used. Acute liver injury was induced by intraperitoneal CCl_4 injection in the form of 50% CCl_4 solution in olive oil at a dose of 2 mL/kg animal weight. A total of 18 rats were used and divided into three groups: a group with olive oil alone, a group injected with CCl_4 and saline, and a group injected with CCl_4 and edaravone. The edaravone (3 mg/kg) and saline were injected via the penile vein immediately after and 3 h after injection of CCl_4. The animals were killed under ether anesthesia at 24 h after CCl_4 injection, and at that time blood was collected from the hearts and the liver was isolated.

Histology

The excised livers were immediately fixed with 10% neutral-buffered formalin and, after embedding in paraffin, they were cut into 5 μm sections. After hematoxylin and eosin (HE) staining and oil red O staining, these sections were examined with a light microscope.

TUNEL method

Paraffin blocks were cut into 4 μm sections. Following deparaffinization, the sections were treated with proteinase K in PBS (10 μg/mL) for 15 min at 37°C, and after washing with PBS, these sections were incubated with 0.3% H_2O_2 for 20 min at room temperature to block endogenous peroxidase activity. The slides were washed with PBS and distilled water and then immersed in TdT buffer (200 mmol potassium cacodylate, 25 mmol Tris HCL pH 6.5, 0.25 mg/mL bovine serum albumin, 1 mmol $CoCl_2$, 0.01 mmol biotin-16-dUTP, 1120 U/mL TdT) for 60 min at 37°C. After washing with PBS, the sections were reacted with VECSTATIN Elite ABC KIT (Vector Laboratories, Burlingame, CA, USA) and incubated for 30 min at 37°C. These sections were stained with 0.025% 3–3′-diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl buffer. The nuclei were stained with Mayer hematoxylin solution, and the sections were examined with a light microscope.

Serum alanine aminotransferase, total bilirubin and lactate dehydrogenase measurements

The serum alanine aminotranferase (ALT), total bilirubin (TB), and lactate dehydrogenase (LDH) values of the killed animals were determined. The value was measured by using Dri-chem 3500i (Fuji Film, Tokyo, Japan).

Statistical analysis

Data are expressed as means ± standard deviation. The differences between the means of each of the two groups were evaluated by the Kruskal–Wallis test and significance was determined when $P < 0.05$.

RESULTS

Serum alanine aminotransferase, lactate dehydrogenase and total bilirubin values

The serum values of ALT, LDH and TB were analyzed to evaluate liver injury. In the CCl_4 alone group, the

Table 1 Serum ALT and LDH activities and TB levels 24 h after injection of CCL_4

Experimental groups	*n*	ALT (IU/L)	LDH (IU/L)	TB (mg/dL)
Olive oil (1.0 mL/kg)	6	21.0 ± 2.7	203.7 ± 30.6‡	0.4 ± 0.1
CCl_4 (1.0 mL/kg) + saline	6	1630.6 ± 606.9*	5068.0 ± 2956.5*	0.9 ± 0.5§
CCl_4 + edaravone (3 mg/kg)	6	119.4 ± 113.5†	369.7 ± 288.2‡	0.3 ± 0.2¶

*$P < 0.001$ *vs* olive oil group. †$P < 0.001$ *vs* CCl_4 + saline group. ‡$P < 0.005$ *vs* CCl_4 + saline group. §$P < 0.01$ *vs* olive oil group. ¶$P < 0.01$ *vs* CCl_4 + saline group. ALT, alanine aminotranferase; CCL_4, carbon tetrachloride; LDH, lactate dehydrogenase; TB, total bilirubin.

serum ALT and LDH values 24 h after the injection of CCl_4 were significantly higher than in the control group (olive oil alone). The increases in serum ALT, LDH, and TB values were significantly reduced by administration of edaravone (Table 1).

Histological examinations

The CCl_4 administration induced marked fatty degeneration in the liver (Fig. 1a) 24 h after intraperitoneal injection. Little inflammatory cell infiltration was noted. Most of the hepatocytes in acinar zones 2 were distended by microvesicular fat droplets, and these cells had pyknotic, centrally located nuclei and rarefied cytoplasm. The steatosis induced by CCl_4 was markedly reduced by the two intravenous doses of edaravone (Fig. 1b), and a single injection failed to prevent the fatty change (data not shown). Oil red O staining, which detects fat deposits, revealed diffuse fatty degeneration throughout the liver in the CCl_4 alone group, but zones 2 and 3 were more severely injured by fatty degeneration than zone 1 (Fig. 2a). The fat deposits around the central vein area were larger than in zone 1 (Fig. 2b). By contrast, edaravone administration significantly reduced the fatty change, but fatty degeneration disappeared in a spotty manner (Fig. 2c). The patchy fat deposits were mainly present in zone 2 and 3 (Fig. 2d), and few were present in zone 1 (Fig. 2e). The nuclei of the cells exhibiting fatty degeneration seemed to be condensed (Fig. 3a, mainly in zone 2), and slight cell infiltration was observed in zone 3. This nuclear condensation was demonstrated by the TUNEL method to represent apoptotic cell death. Several TUNEL-positive cells were observed around the central vein in the CCl_4-treated mice 24 h after administration (Fig. 3b). In contrast, very few TUNEL-positive cells were found in the liver of the edaravone-treated rats at the same point in time (Fig. 3c).

Many TUNEL-positive cells were observed in zones 2 and 3 at 48 h after the initial injection of CCl_4 (Fig. 4a), and the numbers of cells were significantly reduced by the two doses of edaravone. A marked reduction in TUNEL-positive cells was demonstrated in zone 3 of the edaravone group 48 h after administration of CCl_4 (Fig. 4b), compared with the CCl_4 group (Fig. 4a). These results suggest that edaravone significantly attenuated CCl_4-induced hepatocyte apoptosis.

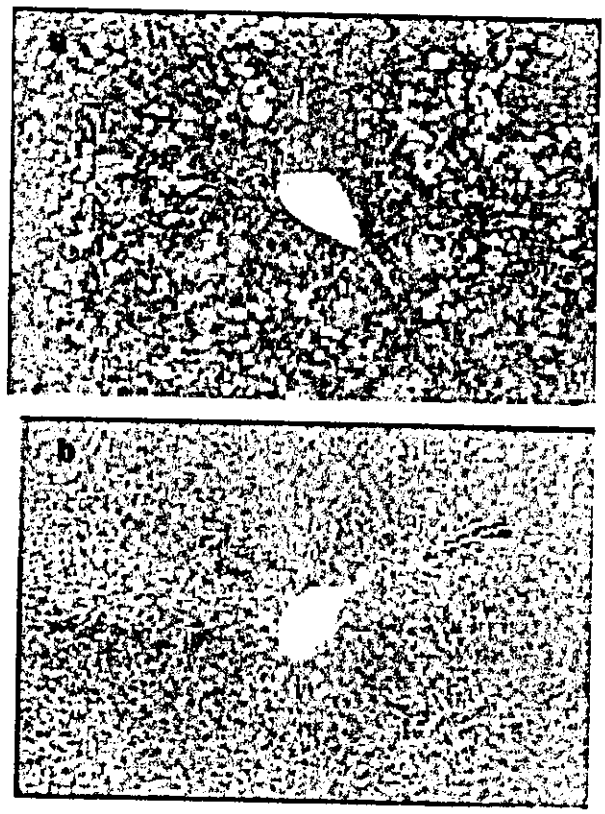

Figure 1 Effect of edaravone on carbon tetrachloride (CCl_4)-induced acute liver injury. Rats were injected with edaravone or saline immediately after and 3 h after CCl_4 injection. Liver sections were stained with HE. (a) Histological features of the liver in the CCl_4 group (at 24 h). Microvesicular fat deposits were observed, particularly in acinar zone 2 (×200). (b) Histological features of the liver in the edaravone group (at 24 h). A dramatic decrease in fatty droplets was seen (×200).

DISCUSSION

The results of the present study showed that edaravone attenuates CCl_4-induced acute liver injury in rats. The most important aspects of this study are that edaravone was administered just after injection of CCl_4 and that the effective dosage of the agent was a clinical dose.

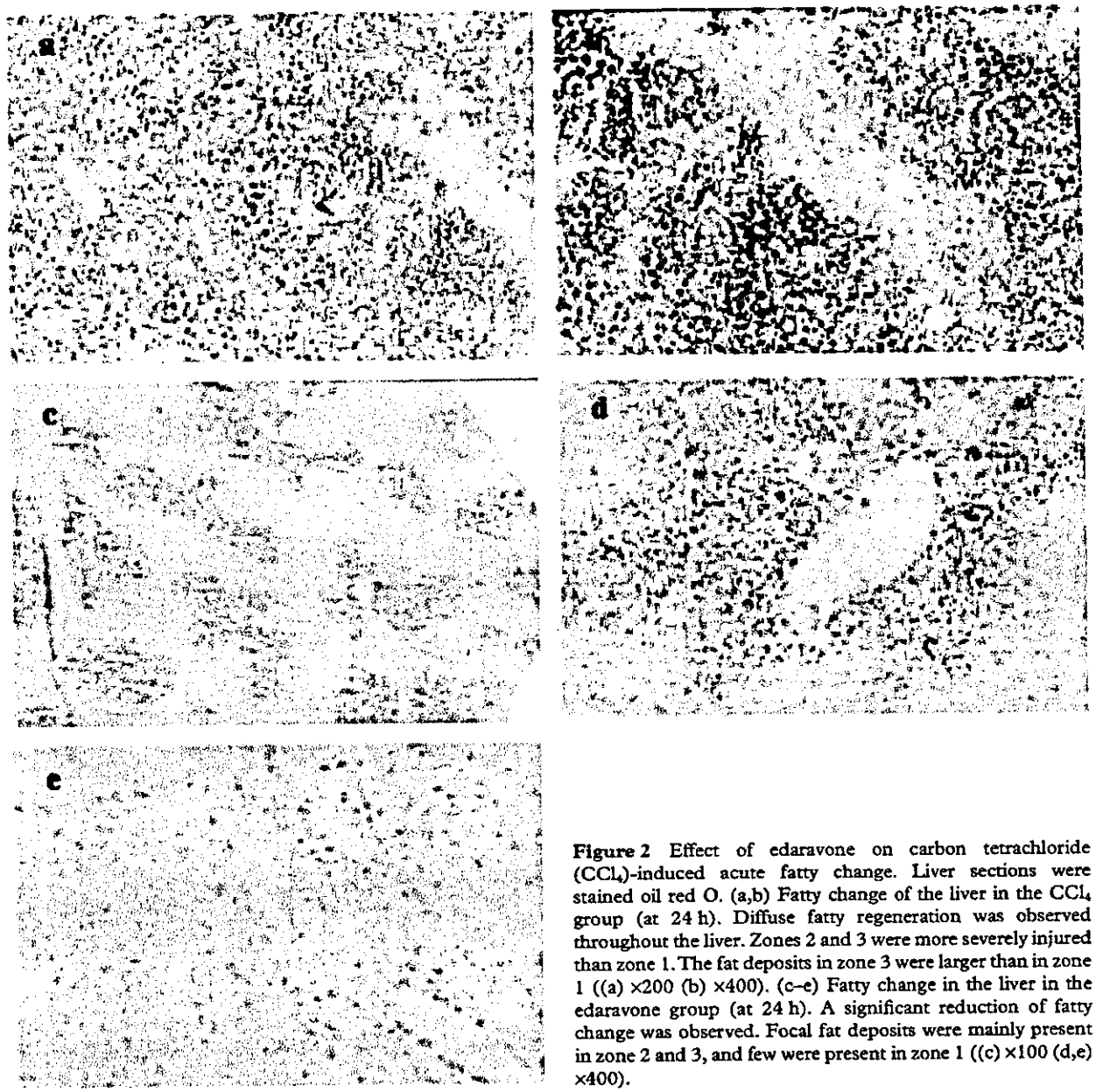

Figure 2 Effect of edaravone on carbon tetrachloride (CCl_4)-induced acute fatty change. Liver sections were stained oil red O. (a,b) Fatty change of the liver in the CCl_4 group (at 24 h). Diffuse fatty regeneration was observed throughout the liver. Zones 2 and 3 were more severely injured than zone 1. The fat deposits in zone 3 were larger than in zone 1 ((a) ×200 (b) ×400). (c–e) Fatty change in the liver in the edaravone group (at 24 h). A significant reduction of fatty change was observed. Focal fat deposits were mainly present in zone 2 and 3, and few were present in zone 1 ((c) ×100 (d,e) ×400).

Edaravone has been reported to have a great antistroke effect in both cerebral ischemia animal models[18–20] and in cerebral ischemia patients.[22] Moreover, edaravone has already been used clinically to treat stroke patients, and an excellent effect has been demonstrated.[22] These effects may be attributable to the free-radical-scavenging ability of edaravone and the fact that it possesses good pharmacological features, such as being lipophilic, readily transferable to tissue, and has effective tissue levels that are maintained after injection,[21] whereas the poor performance of vitamin E in these regards is a drawback. It was also reported that the distribution of edaravone to tissue after a single intravenous administration was rapid and that the concentration of edaravone in the liver at 5 min was high, corresponding to 5–7-fold the concentration in the brain, which was one of the target organs of edaravone.[23] These features of edaravone have played a major role in the results of the present experiment. This is the first study to demonstrate that edaravone inhibits CCl_4-induced acute liver injury in rats.

Carbon tetrachloride causes lipid peroxidation, which is followed by loss of membrane integrity and hepatocellular toxicity, and the hepatic toxicity of CCl_4 is thought to depend on its metabolism. Carbon tetrachloride is dehalogenated by cytochrome p-450 to form the trichloromethyl free radical ($CCl_3\cdot$), which readily reacts with O_2 to generate the trichloromethylperoxy

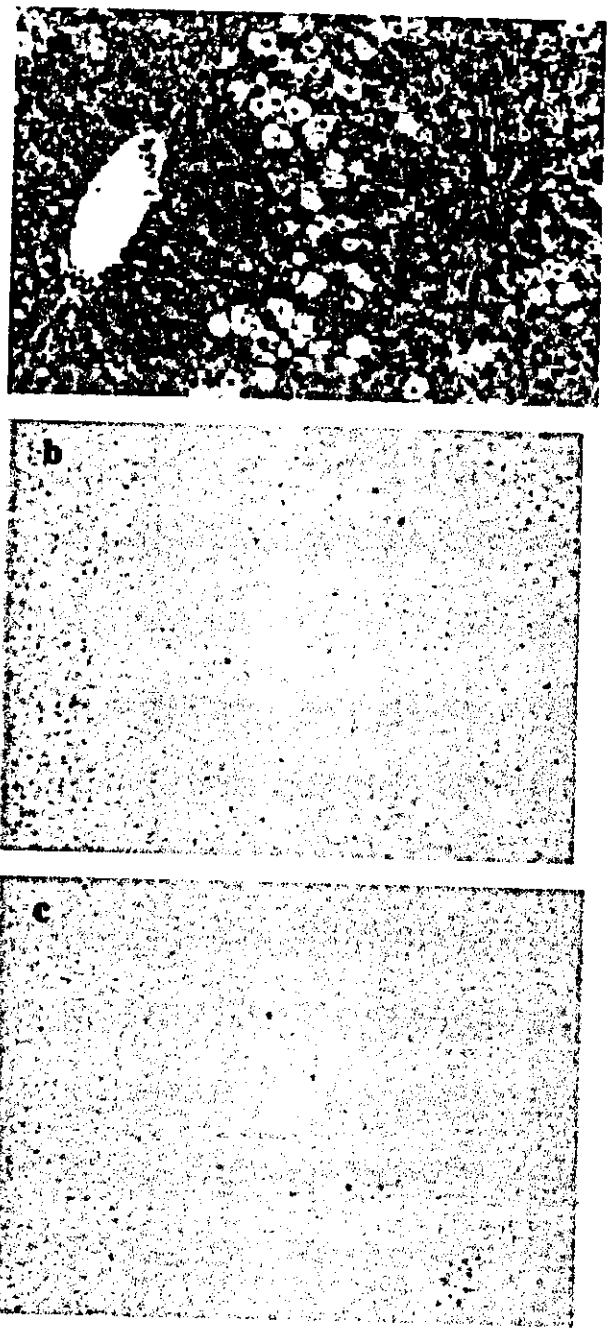

Figure 3 Effect of edaravone on carbon tetrachloride (CCl_4)-induced hepatocyte apoptosis detected by TUNEL assay. (a) HE staining of the liver in the CCl_4 group (at 24 h). The nuclei of the cells exhibiting fatty degeneration seemed to be condensed mainly in zone 2 (×400). (b) TUNEL assay of the liver in the CCl_4 group (at 24 h). TUNEL-positive nuclei were observed around the central vein (×200). (c) TUNEL staining of the liver in the edaravone group (at 24 h). Very few TUNEL-positive cells were found in the liver (×200).

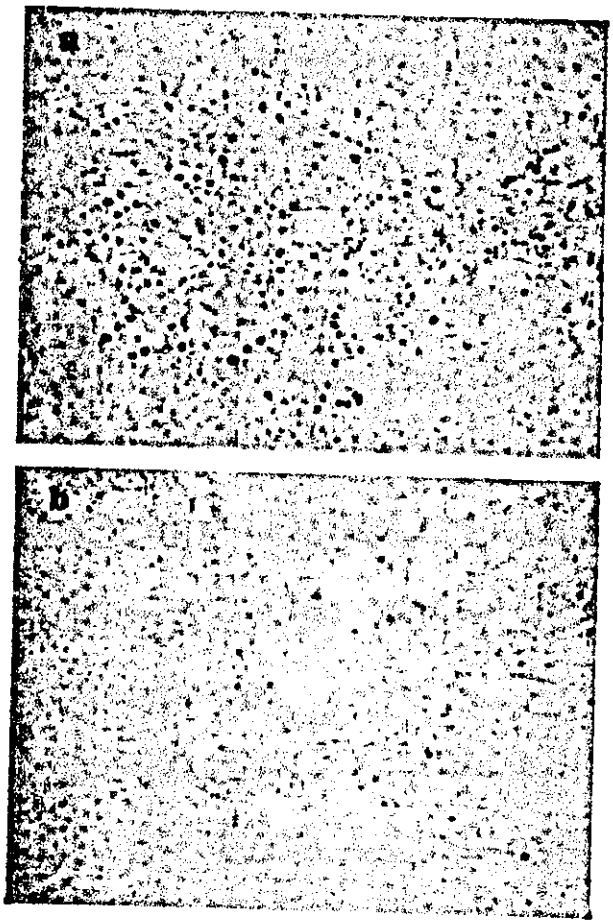

Figure 4 Effect of edaravone on carbon tetrachloride (CCl_4)-induced hepatocyte apoptosis detected by TUNEL assay. (a) TUNEL assay of the liver in the CCl_4 treated group (at 48 h). Many TUNEL-positive nuclei were observed in zones 2 and 3 (×200). (b) TUNEL assay of the liver in the edaravone group (at 48 h). The number of apoptotic nuclei was significantly reduced (×200).

radical ($CCl_3O_2\cdot$).[8,24] This reactive free radical is capable of removing hydrogen atoms from unsaturated lipids to form carbon-centered radicals. These lipid radicals inflict cell damage through direct reaction with cell constituents or mitochondrial membrane phospholipids. Several reports have indicated that high doses of certain supplements, such as vitamin E, can protect against CCl_4-induced acute liver injury,[13–15,25] and that most of these supplements have a radical-scavenging effect. However, the hepatoprotective effects of the agents in these studies have been limited, whereas in the present experiment edaravone showed significant hepatoprotective activity. Edaravone can scavenge hydroxyl radicals and peroxyl radicals, but can not scavenge superoxide.[26] Thus, in the present study, edaravone could scavenge peroxyl radicals derived from CCl_4 and could effectively inhibit CCl_4-induced liver injury.

It has been reported that edaravone has had little effect on cytochrome p-450.[27] These data suggested that

edaravone did not inhibit the expression and function of CYP2E1, which metabolizes CCl_4 and mediates CCl_4 liver injury, and that the hepatoprotective effect of edaravone on CCl_4-induced liver injury can be attributed to its antioxidant property.

In the present study, rats were injected with 3 mg/kg edaravone. This dose was chosen on the basis of previous reports showing a preventive effect of edaravone on ischemia-induced brain damage.[16,18,28] In fact, edaravone has already been approved by the Japanese Ministry of Health, Labour and Welfare. It is administered to stroke patients clinically at the same dose used in the present study and has shown great efficacy,[22] suggesting the possibility of expanding its indications to other diseases, such as liver diseases.

The histological features shown in the present study correspond to those in certain human diseases in which oxidative stress is a major pathophysiological mechanism of liver injury. Mitochondrial injury is always accompanied by oxidative stress and secondary apoptosis. A representative liver injury caused by oxidative stress is alcoholic liver injury.[2,29] Antioxidants, such as metallothionein,[30] superoxide dismutase,[31,32] and glutathione,[33] are considered potent pharmaceutical agents for the treatment of alcoholic liver injury. However, the agent tested in the present study has already been used clinically, and the present study demonstrated that edaravone at the clinical dosage adequately attenuated the liver injury. Other pathological conditions may also be suitable targets for edaravone therapy. Acetaminophen-induced hepatotoxicity,[34,35] acute bile-duct obstruction,[36] cholestasis,[37] iron-overload,[38] circulatory disturbance,[39,40] and even hepatitis C virus infection[41] induce oxidative stress in the liver, and these conditions should all be treatment targets of edaravone.

TUNEL-positive cells were observed in zones 2 and 3 of the liver of the CCl_4-injected rats. Although the CCl_4-induced liver injury was thought to represent necrotic cell death,[42] CCl_4 has recently been reported to also induce hepatocyte apoptosis.[10,43,44] The mechanism of apoptosis induced by CCl_4 has not been clarified. However, CCl_4 inactivates mitochondrial cytochrome oxidase, which is thought to play a key role in apoptosis. Indeed, some reports have suggested that CCl_4 injection induces apoptosis and necrosis in the liver simultaneously. In the present study, edaravone significantly reduced the increase in serum ALT, LDH and TB values, and TUNEL staining showed that edaravone significantly decreased the number of apoptotic hepatocytes. These findings indicated that edaravone suppresses both the apoptosis and necrosis induced by CCl_4 injection, and therefore suggest that both the apoptosis and necrosis induced by the CCl_4 might be mediated by free radicals, although hepatic apoptosis and necrosis might not be individually induced. Some cytokines have been postulated to be a cause of hepatic necrosis. The CCl_4-induced liver injury is associated with increased levels of tumor necrosis factor (TNF)-α and Czaja *et al.* reported that the injury could be significantly prevented by treatment with anti-TNF-α antibodies.[45] Tumor necrosis factor-α is also established as an inducer of hepatocyte apotosis.[45] Thus, the mechanism of apoptosis induced by CCl_4 administration may be complicated. In the present study, the fact that edaravone attenuated both apoptosis and necrosis indicated that edaravone not only scavenges free radicals but also inhibits cytokine production. Further study is necessary to clarify these mechanisms.

In conclusion, our results show evidence of a significant inhibitory effect of edaravone on acute liver injury, and suggest that this agent has therapeutic potential for use as a hepatoprotective agent in acute liver damage after ischemic change.

REFERENCES

1 Liu SL, Degli Esposti S, Yao T, Diehl AM, Zern MA. Vitamin E therapy of acute CCl4-induced hepatic injury in mice is associated with inhibition of nuclear factor kappa B binding. *Hepatology* 1995; 22: 1474–81.

2 Lieber CS. Alcoholic liver injury: pathogenesis and therapy in 2001. *Pathol. Biol. (Paris)* 2001; 49: 738–52.

3 Inglott FS, Mathie RT. Nitric oxide and hepatic ischemia-reperfusion injury. *Hepatogastroenterology* 2000; 47: 1722–5.

4 Comporti M. Lipid peroxidation and cellular damage in toxic liver injury. *Lab. Invest.* 1985; 53: 599–623.

5 Halliwell B, Gutteridge J. Oxygen radicals, transition metals and disease. *Biochem. J.* 1984; 219: 1–14.

6 Cheeseman KH, Saalter TF. An introduction to free radical biochemistry. *Br. Med. Bull.* 1993; 49: 481–93.

7 Salter TF. Free radical mechanisms in tissue injury. *Biochem. J.* 1984; 222: 1–5.

8 Brattin WJ, Glende EA, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. *Free Radic. Biol. Med.* 1985; 1: 27–8.

9 Berger ML, Bhatt H, Combes B, Estabrook R. CCl4-induced toxicity in isolated hepatocytes: the importance of direct solvent injury. *Hepatology* 1996; 6: 36–45.

10 Shi J, Aisaki K, Ikawa Y, Wake K. Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. *Am. J. Pathol.* 1998; 153: 515–25.

11 Andus T, Bauer J, Geruk W. Effects of cytekines on the liver. *Hepatology* 1991; 13: 364–75.

12 Tilg H, Vogel W, Wiedermann CJ *et al.* Circulating interleukin-1 and tumor necrosis factor antagonists in liver. *Hepatology* 1993; 18: 1132–8.

13 Parola M, Leonarduzzi G, Biasi F *et al.* Vitamin E dietary supplementation protects against carbon tetrachloride-induced chronic liver damage and cirrhosis. *Hepatology* 1992; 16: 1014–21.

14 Naziroglu M, Cay M, Ustundag B, Aksakal M, Yekeler H. Protective effects of vitamin E on carbon tetrachloride-induced liver damage in rats. *Cell Biochem. Funct.* 1999; 17: 184–8.

15 Campo GM, Squadrito F, Ceccarelli S *et al.* Reduction of carbon tetrachloride-induced rat liver injury by IRFI 042, a novel dual Vitamin E-like antioxidant. *Free Radic. Res.* 2001; 34: 379–93.

16 Watanabe T, Yuki S, Egawa M, Nishi H. Protective effects of MCI-186 on cerebral ischemia: Possible involvement of free radical scavenging and antioxidant actions. *J. Pharmacol. Exp. Ther.* 1994; 268: 1597–604.

17 Watanabe T, Morita I, Nishi H, Murota S. Preventive effect of MCI-186 on 15-HPETE induced vascular endot-

helial cell injury *in vitro*. *Prostaglandins Leukot. Essent. Fatty Acids* 1989; 33: 81–7.

18 Abe K, Yuki S, Kogure K. Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger. *Stroke* 1988; 19: 480–5.

19 Nishi H, Watanabe T, Sakurai H, Yuki S, Ishibashi A. Effect of MCI-186 on brain edema in rats. *Stroke* 1989; 20: 1236–40.

20 Oishi T, Itoh Y, Nishibori M, Watanabe T, Nishi H, Saeki K. Effect of MCI-186 on ischemia-induced changes in monoamine metabolism in rat brain. *Stroke* 1989; 20: 1557–64.

21 Minhaz U, Tanaka M, Tsukamoto H *et al.* Effect of MCI-186 on postischemic reperfusion injury in isolated rat heart. *Free Radic. Res.* 1996; 24: 361–7.

22 Houkin K, Nakayama N, Kamada K, Noujou T, Abe H, Kashiwabe T. Neuroprotective effect of the free radical scavenger MCI-186 in patients with cerebral infarction: clinical evaluation using magnetic resonance imaging and spectroscopy. *J. Stroke Cere. Dis.* 1998; 7: 315–22.

23 Komatsu T, Nakai H, Masaki K *et al.* [Pharmacokinetic studies of 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) in rats (1).] *Yakubutsudoutai* 1996; 11: 463–80 (In Japanese with abstract in English).

24 Chvapil M, Ryan JN, Zukosky CF. Effect of zinc on lipid peroxidation in liver microsomes and mitochondria. *Proc. Soc. Exp. Biol. Med.* 1972; 141: 150–3.

25 Arosio B, Gagliano N, Fusaro LMP *et al.* Aloe-Emodin quinone pretreatment reduces acute liver injury induced by carbon tetrachrolide. *Pharmacol. Toxicol.* 2000; 87: 229–33.

26 Watanabe T, Yuki S, Saito K *et al.* Pharmacological studies of MCI-186, a new drug for acute stroke. *Jpn. Pharmacol. Ther.* 1997; 25: 181–8.

27 Takamatsu Y, Ono A, Tatsuno J, Yamamoto M. Pharmacokinetic studies of 3-methyl-1-phenyl-2-pyrazolin-5-one in rats—Effect of MCI-186 on drug metabolizing enzymes in rat liver. *Jpn. Pharmacol. Ther.* 1997; 25 (Suppl.): S1765–71.

28 Mizuo A, Umemura K, Nakashima M. Inhibitory effect of MCI-186, a free radical scavenger, on cerebral ischemia following rat middle cerebral artery occlusion. *Gen. Pharmacol.* 1998; 30: 575–8.

29 Albano E. Free radical mechanisms in immune reactions associated with alcoholic liver disease. *Free Radic. Biol. Med.* 2002; 32: 110–14.

30 Zhou Z, Sun X, James KY. Metallothionein protection against alcoholic liver injury through inhibition of oxidative stress. *Exp. Biol. Med.* 2002; 227: 214–22.

31 Wheeler MD, Nakagami M, Bradford BU *et al.* Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. *J. Biol. Chem.* 2001; 276: 36 664–72.

32 Wheeler MD, Kono H, Yin M *et al.* Delivery of the Cu/Zn-superoxide dismutase gene with adenovirus reduces early alcohol-induced liver injury in rats. *Gastroenterology* 2001; 120: 1241–50.

33 Sakurai K, Stoyanovsky DA, Fujimoto Y, Cederbaum AI. Mitochondrial permeability transition induced by 1-hydroxyethyl radical. *Free Radic. Biol. Med.* 2000; 28: 273–80.

34 Knight TR, Kurtz A, Bajt ML, Hinson JA, Jaeschke H. Vascular and hepatocellular peroxynitrate formation during acetaminophen toxicity: role of mitochondrial oxidant stress. *Toxicol. Sci.* 2001; 62: 212–20.

35 Liu J, Liu Y, Hartley D *et al.* Metallothionein-I/II knockout mice are sensitive to acetamonophen induced hepatotoxicity. *J. Pharmacol. Exp. Ther.* 1999; 289: 580–6.

36 Baron V, Muriel P. Role of glutathione, lipid peroxidation and antioxidants on acute bile-duct obstruction in the rat. *Biochim. Biophys. Acta* 1999; 1472: 173–80.

37 Zhong Z, Froh M, Wheeler MD, Smutney O, Lehmann TG, Thurman RG. Viral gene delivery of superoxide dismutase attenuates experimental cholestasis-induced liver fibrosis in the rat. *Gene Ther.* 2002; 9: 183–91.

38 Junge B, Carrion Y, Bosco C *et al.* Effects of iron overload and lindane intoxication in relation to oxidative stress, Kupffer cell function, and liver injury in the rat. *Toxicol. Appl. Pharmacol.* 2001; 170: 23–8.

39 Kusmic C, Boggi U, Bellini R *et al.* Oxidative stress in fulminant hepatic failure: comparison of two pig models with and without liver necrosis. *Hepatogastroenterology* 2001; 48: 762–9.

40 Nguyen WD, Kim DH, Alam HB, Provido HS, Kirkpatrick JR. Polyethylene glycol-superoxide dismutase inhibits lipid peroxidation in hepatic ischemia/reperfusion injury. *Crit. Care* 1999; 3: 127–30.

41 Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; 122: 366–75.

42 Zimmerman HJ. *Hepatotoxicty, the Adverse Effects of Drugs and Other Chemicals on the Liver*. New York: Appleton-Century-Crofts, 1978.

43 Sun F, Hamagawa E, Tsutsui C, Ono Y, Ogiri Y, Kojo S. Evaluation of oxidative stress during apoptosis and necrosis caused by carbon tetrachloride in rat liver. *Biochim. Biophys. Acta* 2001; 1535: 186–91.

44 Yasuda M, Okabe T, Itoh J *et al.* Differentiation of necrotic cell death with or without lysosomal activation: application of acute liver injury models induced by carbon tetrachloride and Dimethylnitrosamine. *J. Histochem. Cytochem.* 2000; 48: 1331–9.

45 Czaja MJ, Xu J, Alt E. Prevention of carbon tetrachrolide-induced rat liver injury by soluble tumor necrosis factor receptor. *Gastroenterology* 1995; 108: 1849–54.