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Hepatic fibrosis in rats fed a liquid diet with ethanol and carbonyl iron

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Abstract

It is known that iron acts as a co-factor to catalyze lipid peroxidation (LP) induced by an hepatotoxic compound, such as alcohol. To investigate the role of iron in the pathogenesis of alcoholic liver disease (ALD), we developed a new experimental rat model. Male Sprague–Dawley rats were pair-fed ad libitum a liquid high-fat diet containing ethanol (36% of total calories) or isocaloric carbohydrate with or without dietary carbonyl iron (0.5% w/v) for 12 weeks. Serum alanine aminotransferase (ALT) levels were greatly elevated in rats fed a high-fat diet containing both ethanol and iron (EtOH-Fe). Morphologically, slight fibrosis with fatty infiltration and occasional iron deposits were found in the liver of rats fed EtOH-Fe. Moreover, type 4 collagen was definitely stained in the liver of the EtOH-Fe-fed group. However, no evidence of fibrosis was seen in the liver of rats other than the EtOH-Fe-fed group. Furthermore, there was no evidence of secondary hemochromatosis in the rat fed EtOH-Fe or a high-fat diet containing iron. The hepatic content of hydroxyproline (HP) was significantly increased in EtOH-Fe-fed rats as compared to rats other than the EtOH-Fe-fed group. Similarly, microsomal malondialdehyde (MDA) levels were relatively elevated in EtOH-Fe-fed rats. These results demonstrate the evidence of a synergistic effect between alcohol and iron in producing alcoholic liver fibrosis through the enhancement of LP. This new rat model (Fukudai Model) may be useful for further studies in the pathogenesis of ALD.

Keywords: Lipid peroxidation (LP); Carbonyl iron; Alcoholic liver disease (ALD)

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1. Introduction

To study the pathogenesis of alcoholic liver disease (ALD), many investigators are still using a rat model feeding on a liquid diet containing ethanol [1]. Several studies have shown the evidence of alcoholic fatty liver histologically and the enhancement of lipid peroxidation (LP) biochemically, in rats that developed acute or chronically alcoholic-induced liver injury [2,3]. However, most of these studies failed to produce alcoholic liver fibrosis. Bacon et al. reported the evidence of iron-induced hepatic lipid peroxidation in an experimental rat model by feeding with a chronic high iron diet [4]. Meanwhile some studies have shown that the enhancement of LP is associated with liver fibrogenesis using a rat model with alcohol-induced liver injury [5,6]. Recently, it has been demonstrated that iron increases ethanol toxicity in the liver of rats fed a carbonyl iron-overloading and an alcohol-containing diet [7]. To assess the important role of iron in the pathogenesis of ALD and to provide the evidence for a synergistic effect between iron and alcohol, in producing hepatic fibrosis, we tried to develop a new experimental rat model, without secondary hemochromatosis, by feeding a liquid diet containing both carbonyl iron and alcohol.

2. Materials and methods

2.1. Animals and diets

Male Sprague–Dawley rats (140–150 g) were pair-fed ad libitum for 12 weeks a nutritionally adequate liquid high-fat diet containing ethanol (36% of the total caloric intake) or isocaloric carbohydrate [1] with or without dietary carbonyl iron (0.5% w/v) [4]. An intake volume of those liquid diets pair-fed to the rats isocalorically was 70–150 ml/day. Dietary carbonyl iron (FERRONYL) was purchased from ISP Chemicals Ltd., Tokyo, Japan. The animals were divided into four groups (5 rats in each group) as aforementioned and pair-fed with those liquid diets until sacrificed. All animals were humanely treated according to NIH guide for the care and use of laboratory animals.

2.2. Preparation of blood, tissue samples and microsomes

Animals were anesthetized with sodium pentobarbital before sacrifice according to a previous report [6]. A blood sample was taken from the inferior vena cava and the liver was quickly removed for histological study. Part of the remaining liver was homogenized for biochemical assay. Also, microsomes were isolated from the remaining tissue as described by the method of Ardies et al. [8].

2.3. Histological study

Liver tissues were fixed in a 10% formalin solution, processed and stained with hematoxylin and eosin and Gordon's reticulin stains for light-microscopical examination. Part of the liver tissue was frozen and used for immunohistochemical staining of type 4 collagen. Frozen 5-μm liver sections were mounted on albumin-coated slides and fixed with cold acetone. Following elimination of endogenous peroxidase activity, these sections were stained using the monoclonal antibody against type 4

collagen (Dako Co., Denmark) at a final dilution of 1:50, according to the avidin-biotin peroxidase complex method. Peroxidase activity was developed in a buffer, containing 3,3-diaminobenzidine. All sections were subsequently counterstained with hematoxylin [9].

2.4. Biochemical assays

Serum alanine aminotransferase (ALT) levels were determined on a conventional computed biochemical analyzer. Serum and hepatic iron levels were measured as described by the method of Torrance and Bothwell [10]. To estimate collagen concentration in the liver, the release of hydroxyproline (HP) in the hydrolysate of the liver was determined with Ehrlich's reagent [11]. The measurement of microsomal malondialdehyde (MDA) was performed according to the method of Uchiyama and Mihara [12]. Protein concentration was measured by the method of Lowry et al. [13].

2.5. Statistical methods

All data are presented as the mean ± S.D. Significance of differences was assessed by Student's *t*-test.

3. Results

As shown in Table 1, serum ALT levels were significantly elevated in the high-fat diet containing both the ethanol- and iron (EtOH-Fe)-fed group as compared to rats other than the EtOH-Fe-fed group. Morphologically, slight fibrosis with fatty infiltration and occasional iron deposits were seen in the liver of the EtOH-Fe-fed group. Furthermore, type 4 collagen was clearly stained in the liver of the EtOH-Fe-fed group (Fig. 1D). In contrast, there was no evidence of fibrosis in the liver of the rats other than the EtOH-Fe-fed group (Fig. 1). Liver HP contents were significantly increased in EtOH-Fe-fed rats as compared with the rats other than the EtOH-Fe-fed

Table 1
Serum transaminase levels and serum and hepatic iron contents of rats

Groups[a]	ALT (IU/l)	Serum iron (μg/dl)	Liver iron (μg/g liver)
Control	28.0 ± 8.2[b]	156.8 ± 32.1	25.9 ± 5.7
Fe	70.4 ± 24.6	365.0 ± 74.4[d]	146.4 ± 36.0[f]
EtOH	62.4 ± 27.5	161.8 ± 32.3	26.1 ± 4.0
EtOH-Fe	180.2 ± 45.5[c]	400.4 ± 82.3[e]	101.8 ± 24.1[g]

[a] $N = 5$ rats/group.
[b] Data expressed as mean ± S.D. [c] $P < 0.01$: compared with the other groups.
[d] $P < 0.01$: compared with control and EtOH-fed rats.
[e] Not significant compared with Fe-fed rats.
[f] $P < 0.05$: compared with EtOH-Fe-fed rats.
[g] $P < 0.05$: compared with control and EtOH-fed rats.

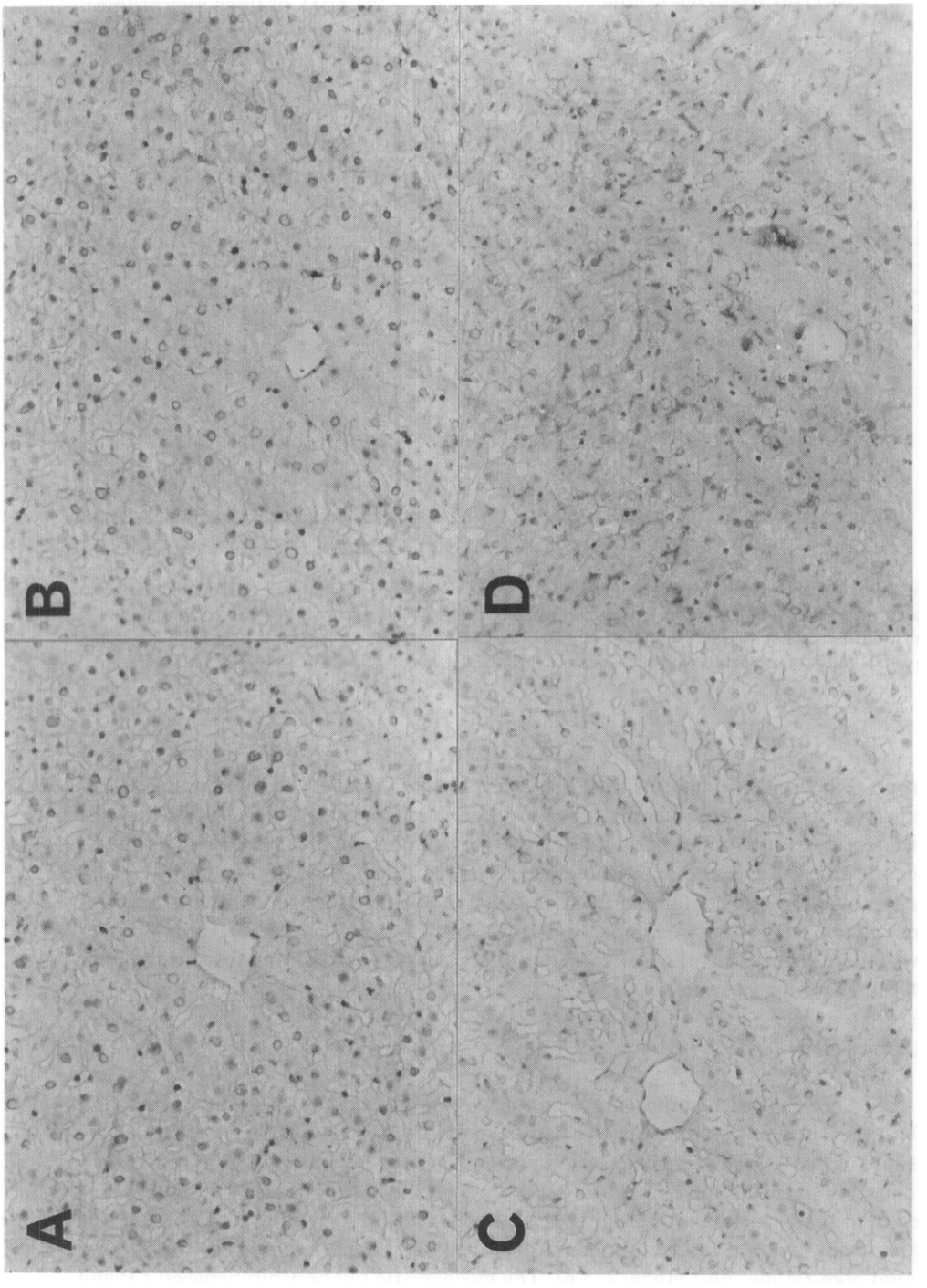

Fig. 1. Liver tissues from rats pair-fed with (A) high-fat diet alone (Control), (B) high-fat diet containing carbonyl iron (Fe), (C) high-fat diet containing ethanol (EtOH), and (D) high-fat diet containing both ethanol and carbonyl iron (EtOH-Fe) for 3 months, respectively (type 4 collagen stain; original magnification × 100).

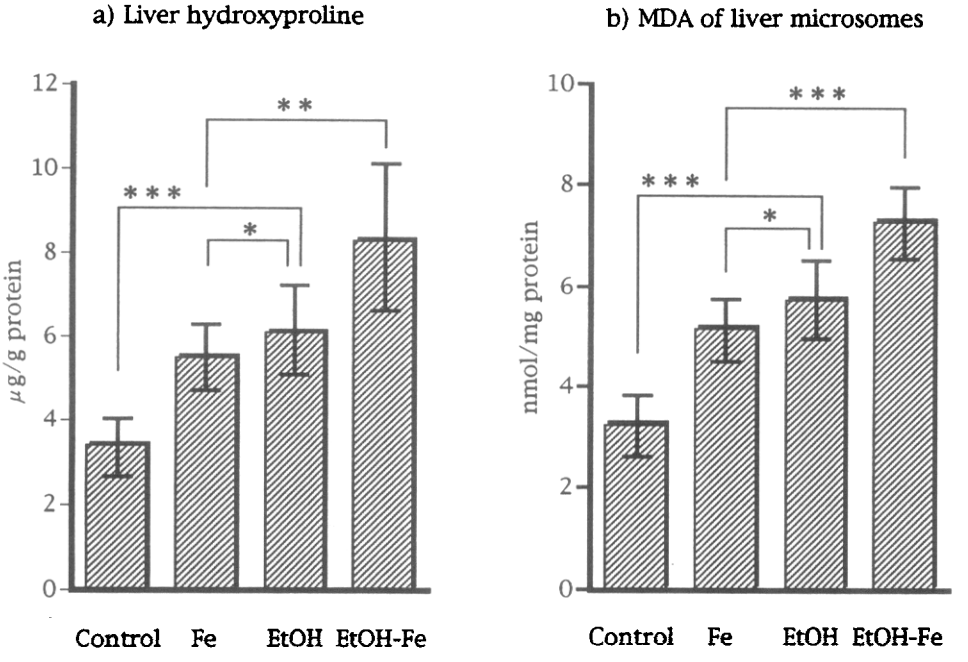

Fig. 2. (a) Hepatic hydroxyproline contents in rats. Note significant elevation in EtOH-Fe-fed rats compared with Fe-fed animals and in EtOH-fed rats compared with the Controls. (b) Microsomal MDAs in the liver from rats. Also note significant increase in EtOH-Fe-fed rats compared with Fe-fed animals and in EtOH-fed rats compared with the Controls. *Not significant; **$P < 0.05$; ***$P < 0.01$.

group (Fig. 2A). Also, microsomal MDA levels were relatively elevated in EtOH-Fe-fed rats (Fig. 2B). There were significant differences ($P < 0.01$) between the high-fat diet alone (Control) and the high-fat diet containing ethanol (EtOH)-fed rats on hepatic HP contents and microsomal MDA levels (Figs. 2A,B). In rats fed EtOH-Fe or the high-fat diet containing iron (Fe), serum and hepatic iron contents were increased about 3–5-fold as compared to non-iron-fed groups as seen in Table 1. However, there was no histological evidence of secondary hemochromatosis in the liver of EtOH-Fe- or Fe-fed rats.

4. Discussion

Since a liquid diet containing ethanol was developed for a rat by Lieber and DeCarli [1], many studies for the pathogenesis of ALD have been performed by using this diet [3]. However, most of these studies have only produced, at most, alcoholic fatty liver and have failed to develop alcoholic liver fibrosis. Recently, it was reported that the enhancement of LP is associated with liver fibrogenesis, from the experiment, by a rat model (Tsukamoto-French rat model) fed intragastrically on a liquid high-fat diet plus ethanol [5,6]. The mechanistic relationship between LP and

fibrogenesis was proposed by Chojkier and colleagues in a study in which increased collagen gene expression was demonstrated in vitro by fibroblasts exposed to ascorbic acid [14]. It was concluded that this stimulatory effect was mediated by formation of aldehydic metabolic products (e.g. MDA) of LP [14]. On the other hand, Bacon et al. reported the evidence of iron-induced hepatic lipid peroxidation in a rat model chronically fed an iron-overloaded diet [4] and Stal et al. demonstrated that iron increases ethanol toxicity in the liver of rats, without producing hepatic fibrosis, which were fed on dietary carbonyl iron (3%w/v; iron-overloading diet) and a diet containing alcohol separately [7]. In the present study, to increase the LP in alcohol-induced liver injury in rats, we added a small amount of carbonyl iron to a liquid diet containing ethanol, and fed this liquid diet containing both ethanol and carbonyl iron (0.5%w/v) continually to rats until they died. Consequentially, we obtained the histological confirmation of hepatic fibrogenesis in the liver of rats through immunohistochemical staining of type 4 collagen and succeeded in producing alcoholic liver fibrosis in the rat model by using this special diet. Additionally, secondary hemochromatosis was not definitely shown in the liver of EtOH-Fe- or Fe-fed rats in spite of increasing levels of serum and liver iron. It was also shown that hepatic HP contents and microsomal MDA levels were significantly elevated in EtOH-Fe-fed rats, providing the biochemical confirmation of liver fibrogenesis and enhanced LP. These observations demonstrate that there is evidence for a synergistic effect between alcohol and iron in producing alcoholic liver fibrosis through the acceleration of LP. Also the evidence of a similar phenomenon was recently reported in a study by using the aforementioned (Tsukamoto-French) rat model [15]. This new rat model may become more useful for further studies in the pathogenesis of ALD than the conventional rat model utilizing a liquid high-fat diet containing ethanol alone.

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