Hepatic Cytochrome P450 2E1 Level Rather Than Cecal Condition Contributes to Induction of Early Stage of the Alcoholic Liver Damage in Rats

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(Received September 10, 2008; Accepted February 23, 2009; Published online March 6, 2009)

Intestinal condition and ethanol toxicity have been discussed as predictors of alcoholic liver damage. In this study, we investigated the association of hepatic antioxidant enzymes and cecal condition, including intestinal bacteria estimated by terminal restriction fragment length polymorphism (T-RFLP), in the early stage of alcoholic fatty liver. Three liquid isocaloric diets, control (CT) diet, ethanol (ET) diet, or ethanol diet including modified beet fiber (MBF), were prepared and administered to rats for 4 weeks. At 4 weeks, the plasma alanine aminotransferase (ALT) levels in the ET group were higher and that in MBF groups tended to be higher than the CT group, but endotoxin was not detected in the portal vein in any of the samples. The hepatic thiobarbituric acid reactive substances (TBARS) concentration in the MBF group was higher than that in the CT group. Hepatic cytochrome P450 2E1 (CYP2E1) was induced in ethanol-fed rats. The hepatic catalase levels in the ET and MBF groups were lower than that in the CT group. The hepatic superoxide dismutase-2 (SOD-2) level did not differ among the groups. Plasma ALT level and/or hepatic TBARS level correlated positively with CYP2E1 and negatively with catalase level and SOD-2 level. Indicators of cecal condition, including the bacterial population estimated by T-RFLP, may not give any explanation for the hepatic damage. In conclusion, the hepatic CYP2E1 induced by ethanol, rather than the cecal conditions, may influence the early stage of alcoholic hepatic damage in rats; hepatic catalase may provide protective effects.

Key words —— cytochrome P450 2E1, ethanol, fatty liver, terminal restriction fragment length polymorphism, rat

INTRODUCTION

Consumption of alcohol induces physiological changes. Moderate consumption may induce some positive effects,[1,2] but massive consumption sometimes causes hepatic diseases such as fatty liver, alcoholic hepatitis, liver cirrhosis, and liver cancer.[3–6] In these cases, inflammatory events are involved in the development of alcoholic liver injury. Hepatic inflammation may be prevented by several endogenous factors including the antioxidant enzyme catalase and superoxide dismutases (SODs);[7,8] these enzymes possibly play a role in the prevention of alcoholic liver damage as well.

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Concerning alcoholic liver disease, endotoxin derived from intestinal Gram-negative bacteria has been hypothesized to activate the immune system via Kupffer cells, subsequently enhancing the inflammatory response.[9–11] Thus, it may be important to assess both the hepatic antioxidant system and cecal condition, including intestinal bacteria, to evaluate alcoholic liver damage. Additionally, as substrates of prebiotics, dietary fibers, such as beet fiber may improve alcoholic liver injuries because some dietary fibers can provide source of organic acids and change the cecal condition.[12,13]

For the analysis of human intestinal bacteria in the evaluation of cecal condition, culture methods have been established only for almost 20 to 30% of intestinal bacteria;[14,15] recently, molecular biological methods for the analyses of bacterial population have been introduced,[16–20] such as terminal restriction fragment length polymorphism (T-RFLP) and

denaturing gradient gel electrophoresis (DGGE). Although these techniques can detect wide-range bacterial populations including those that are difficult or impossible to culture, they have not been applied to the study of cecal bacteria in alcoholic liver diseases.

The aim of this study is to investigate the relationship of alcoholic liver damage with an ethanol-inducible enzyme, antioxidant enzymes, and cecal conditions, and also attempted to apply T-RFLP analysis to intestinal bacteria in alcohol-ingested rats.

MATERIALS AND METHODS

Preparation of Experimental Diets —— Beet fiber with fat-soluble components washed out[21] was obtained from Nippon Beet Sugar Manufacturing Co. Ltd. (Tokyo, Japan) as a dietary fiber source, and was termed "modified beet fiber" (MBF) in this study. Three liquid isocaloric diets were used in this study. The compositions of these diets and the preparation of the fluid diet has been published previously,[22,23] but vitamins and minerals were included based on the AIN-93G diet.[24] One diet was a high-fat liquid diet used as a control diet (CT diet); the others were high-fat, low-carbohydrate liquid diets containing ethanol. One of the ethanol diets was an ethanol control (ET diet), and in the other ethanol diet, MBF as a fiber source for alteration of the cecal condition was substituted for cellulose (MBF diet). The compositions and caloric distribution of the CT diet, including water, were as follows [in g/kg diet (percentage of energy)]: maltose dextrin, 131.6 (37.8); casein, 54.0 (15.5); corn oil, 67.5 (43.7); and cellulose, 22.5 (0). In the ethanol diets, 96 g of ethanol (34.5% of energy) replaced 120 g of maltose dextrin in the CT diet.

Experimental Procedure and Sampling —— All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee, National Agricultural Research Center for Hokkaido Region). Seven-week-old male Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats were maintained at a temperature of 24 ± 1°C, humidity of 40 ± 5%, and a 12 hr/12 hr light/dark cycle.

After acclimation for 1 week, the rats were divided into three groups ($n = 8$) and were administered the CT, ET, or MBF diet. During the first week, the ethanol content in the ET and MBF diets was increased gradually. The rats were then fed the above-described diets for the next 3 weeks. The weights of the diets administered were adjusted so that the caloric intake of the three groups did not differ. At 27 days, overnight fasting blood samples were collected in a heparinized capillary tube from the tail vein of the rats; plasma was obtained for estimating alanine aminotransferase (ALT) activity by centrifugation at $1500 \times g$ for 15 min at 4°C.

At the end of the feeding period, under diethylether anesthesia, the rats were killed after withdrawal of blood from the portal vein for determination of endotoxin. A piece of cecal content was harvested for the analysis of T-RFLP. The liver and cecum were collected, weighed, frozen in liquid nitrogen, and stored at −80°C until analyses.

Blood Analyses —— Alanine aminotransferase activity in the plasma was determined enzymatically using commercially available kits (Wako Pure Chemical Industries Ltd., Osaka, Japan). Endotoxin level in the whole blood from the portal vein was measured using Endotoxin Single Test Wako and its dedicated detector, or Toxinometer-MT-358 (Wako Pure Chemical Industries Ltd.).

Total Lipid (TL) in the Liver —— A piece of liver was freeze-dried and then extracted in a 2 : 1 (v/v) solution of chloroform: methanol with sonication for 15 min. Aliquot of the extract was evaporated, and the residuum weight was measured as TL.

Cecal Analysis —— Cecal ammonia concentration was measured by a commercially available kit (Wako Pure Chemical Industry Ltd.) according to the manufacturer's instructions.

Hepatic Thiobarbituric Acid-Reactive Substance Content —— A piece of the liver was homogenized in 1.15% chloride potassium with a polytron homogenizer and centrifuged at $1500 \times g$ for 10 min at 4°C. The supernatant was assayed for thiobarbituric acid-reactive substances (TBARS) content[25] and protein content by the method of Bradford *et al.*[26] with bovine serum albumin as the standard protein.

Immunoblot Analysis —— A piece of the liver was homogenized with sample buffer and subjected to immunoblot analysis as reported previously.[27] Intensities of the band were detected using specific antibodies of catalase (Lab Frontier, Seoul, Korea), SOD-2 (Lab Frontier), Cytochrome P450 2E1 (CYP2E1; BIOMOL International Inc., Plymous Meeting, PA, U.S.A.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz

Biotechnology Inc., Santa Cruz, CA, U.S.A.).

T-RFLP Analysis —— Literature was reviewed with regard to T-RFLP.[17,18] Genome DNA from intestinal bacteria was obtained using QIAmp® DNA Stool Mini Kit[28] according to the manufacturer's instruction. The genome DNA was amplified by polymerase chain reaction (PCR) in 50 μl of reaction solution with 10 pM of the primers of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) labeled with 6-FAM and 1492R (5′-GGTTACCTTGTTACGACT-3′), 100 ng of genome DNA, 1.25 U of Ex Taq (Takara Shuzo, Tokyo, Japan), 5 μl of 10 × Ex Taq buffer, and 4 μl of 2.5 mM dNTP mixture. The PCR condition was as follows: 95°C for 3 min, followed by 30 cycles consisting of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1.5 min, with final extension of 72°C for 10 min. Amplified DNA solution was added with 2.5 volumes of ethanol and 1/12 volumes of 3 M sodium acetate (pH 5.2), kept at −80°C for 30 min, and centrifuged at 20400 × *g* for 15 min at 4°C. The pellet was washed with 70% ethanol twice, dried in air, and resolved in 20 μl of water. In 10 μl of total volume, 2.5 μl of the DNA solution was digested with 20 U of *Hha I* restriction enzyme (Takara Shuzo). With 0.5 μl of 1200 G LIZ size standard and 11 μl of deionized formamide (Applied Biosystems, Tokyo, Japan), 2.5 μl of the digested DNA was applied to ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) after heating at 95°C for 2 min. Fragment size was estimated using GeneMapper® software ver. 3.7 (Applied Biosystems).

Statistical Analysis —— The respective intensities of the immunoblots were normalized to those of GAPDH, and the values are represented by comparison with that in the CT group. The respective positions of clusters or peaks were compared with those in previous reports,[16,17] and *Bacteroides* and relatives were presumed as approx. 100 bp, *Clostridium* cluster IV as approx. 390 bp, *Clostridium* subcluster XIVa as approx. 186 and 1040 bp, *gammapropiobacteria* as approx. 369 bp, and *Lactobacillus gallinarum* (*L. gallinarum*) as 174 bp. Two sample data for T-RFLP in the MBF group were omitted because of insufficient separation and no detection of major peaks. Bacterial population evaluated by T-RFLP was expressed by the relative areas of the respective peaks as a ratio of each area to the total area. Data are expressed as mean ± standard error of the mean (SEM). Significance was estimated by Fisher's protected least significant difference (PLSD) method ($p < 0.05$).

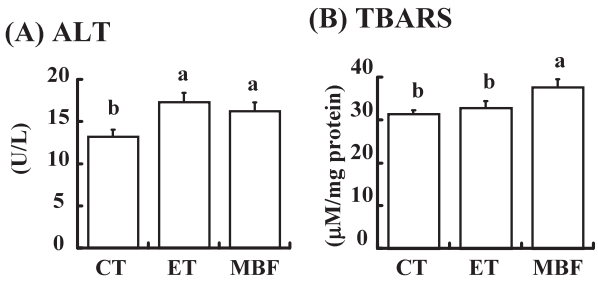

Fig. 1. (A) Plasma ALT Level and (B) Hepatic TBARS Concentration in the Rats Fed the CT, ET, and Ethanol Diet Containing MBF Diet for 4 Weeks

Values are mean ± SEM of eight rats. Values not sharing a common letter significantly differ ($p < 0.05$).

RESULTS

Analyses of Blood, Cecal Content, and Liver

As shown in Fig. 1, the plasma ALT levels in the CT, ET, and MBF groups were 13.1 ± 0.8 (in U/l), 17.2 ± 1.1, and 16.2 ± 1.1, respectively, and those in the ET and MBF groups were 1.3-fold and 1.2-fold higher than that in the CT group. In this study, blood endotoxin level in the portal vein was less than the detection limit (< 5 pg/ml) in all samples.

The liver was heavier in both the ethanol groups compared with the CT group (Table 1). The total lipid contents in the whole liver in the ET and MBF groups were more than that in the CT group. The hepatic TBARS level was higher in the MBF group than in the CT and ET groups (Fig. 1B).

The cecal contents in both ethanol-ingested groups were more than three times higher than that in the CT group. As shown in Table 1, the pH of cecal content in the MBF group was lower than the other groups, and the cecal ammonia concentrations did not differ among the groups.

Immunoblot Analysis of the Liver

As shown in Fig. 2, the catalase level was significantly lower in the MBF group and tended to be lower in the ET group than in the CT group (Fig. 2A). The hepatic SOD-2 level did not differ among the groups (Fig. 2B). The hepatic CYP2E1 levels in the ET and MBF groups were five or more times higher than that in the CT group (Fig. 2C). The CYP2E1 level correlated positively with plasma ALT activity ($r = 0.445$, $p < 0.05$), and plasma ALT activity correlated negatively with hepatic catalase ($r = -0.407$, $p < 0.05$) and SOD-2

Table 1. Liver Weight and TL Content, and Cecal Content, its pH, and Ammonia Concentration in Rats Fed CT, ET, or MBF-containing Ethanol Diet for 4 Weeks[a)]

	Liver		Cecal		
	weight[b)] (g/100 g BW)	TL (mg)	content (g)	pH	$NH_4^{+c)}$ (mM/g)
CT	2.97 ± 0.04^b	431 ± 41^b	3.4 ± 0.2^b	7.54 ± 0.10^b	116 ± 9
ET	3.51 ± 0.04^a	538 ± 23^a	11.3 ± 0.3^a	7.53 ± 0.03^b	88 ± 10
MBF	3.44 ± 0.07^a	551 ± 21^a	10.7 ± 0.5^a	7.26 ± 0.09^a	94 ± 7

a) Values are mean ± SEM of eight rats. Values not sharing a common superscript within the same column significantly differ ($p < 0.05$). *b*) Relative value to 100 g of body weight. *c*) Ammonia nitrogen expressed in millimoles per gram of cecal wet content.

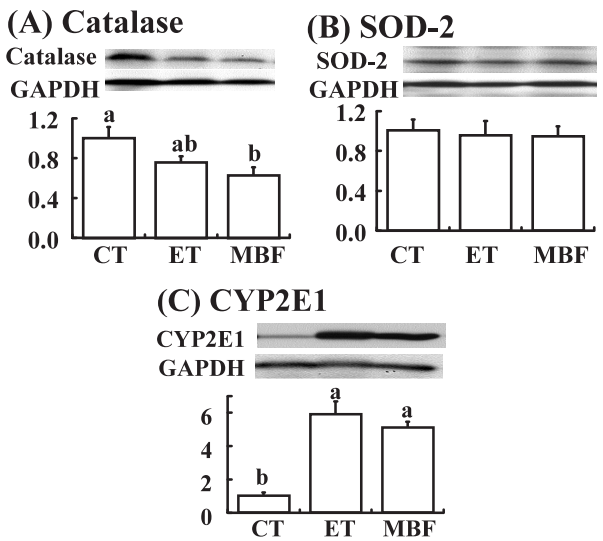

Fig. 2. Abundances of Hepatic (A) Catalase, (B) SOD-2, and (C) CYP2E1 in the Rats Fed the CT, ET, and the Ethanol Diet Containing MBF Diet for 4 Weeks

Abundance of each protein is standardized by that of GAPDH and expressed in arbitrary units against the value of the CT group as one. Values are mean ± SEM of eight rats. Values not sharing a common letter significantly differ ($p < 0.05$).

levels ($r = -0.555$, $p < 0.01$). Hepatic TBARS level had negative correlation with catalase level ($r = -0.486$, $p < 0.02$).

T-RFLP Analysis

The results of T-RFLP analysis are shown in Table 2. The ratio of *Bacteroides* and relatives was higher in the ET group and tended to be higher in the MBF group than in the CT group. The respective ratio of *L. gallinarum* in the MBF group was higher than those in the CT and ET groups. The ratio of *Clostridium* subcluster XIVa in the ET group was higher than those in the CT and MBF groups. The ratios of *gamma-proteobacteria* and *Clostridium* cluster IV did not differ among the groups. The ratio of *L. gallinarum* correlated with TBARS concentration positively ($r = 0.562$, $p < 0.02$) and catalase level negatively ($r = -0.530$, $p < 0.03$). The ratio of *Bacteroides* and relatives correlated with the cecal content ($r = 0.534$, $p < 0.01$). The cecal pH correlated with the ratios of *Clostridium* cluster IV ($r = -0.568$, $p < 0.01$) and with that of *L. gallinarum* weakly ($r = -0.471$, $p < 0.06$).

DISCUSSION

In the present study, we showed that hepatic CYP2E1 is associated with induction of the early stage of the alcoholic liver injury in rats, whereas obvious involvement of intestinal bacteria in this event was not observed. Previous studies reported that hepatic CYP2E1[3,29)] and/or plasma endotoxin derived from intestinal bacteria[9–11)] were associated with alcoholic hepatitis. In this study, the CYP2E1 levels increased in the ethanol-ingested rats with positive correlation with the ALT level, but the endotoxin level in the portal vein was below the limit of detection. These results indicate that the CYP2E1 induced by ethanol rather than endotoxin was important for early liver damage. Although alcoholic hepatic damage may not be so severe despite abundant CYP2E1 levels in this study, it has been shown that chronic exposure of ethanol did not necessarily cause severe liver disease in another study.[30)] Other factors, such as supplement of iron,[3,29)] are possibly involved in induction of experimental alcoholic hepatitis.

Furthermore, it was shown that hepatic catalase is partly associated with hepatic protection in this liver injury. Accumulation of hepatic lipid and increases in plasma ALT level and/or hepatic TBARS level were observed in ethanol-ingested rats. These are typical indicators for alcoholic liver damage,[30–32)] and these indicators negatively correlated with hepatic catalase levels. These results suggest that hepatic damage was induced by the in-

Table 2. Distribution of Intestinal Bacteria Estimated with Terminal Restriction Fragment Length Polymorphism in Rats Fed CT, ET, or MBF-containing Ethanol Diet for 4 Weeks[a)]

	Bacteroides[b)]	*Clostridium*		GPB[c)]	*L. gallinarum*
		IV	XIVa		
CT	25.6 ± 3.0^{b} (8)	7.5 ± 1.5 (8)	14.4 ± 2.2^{b} (8)	4.05 ± 1.30 (8)	2.0 ± 0.4^{b} (7)
ET	35.8 ± 1.4^{a} (8)	7.2 ± 1.1 (8)	23.9 ± 2.7^{a} (8)	3.41 ± 0.46 (7)	1.4 ± 0.2^{b} (4)
MBF	$32.0 \pm 3.1^{a,b}$ (6)	11.1 ± 2.3 (6)	9.6 ± 2.8^{b} (6)	1.12 ± 0.15 (2)	13.7 ± 2.1^{a} (6)

a) Values are expressed as percent of each area to the total area and mean ± SEM. The numbers in case arc mean frequency of detection of the T-RF peak in eight rats (CT and ET groups) and in six rats (MBF group). Values not sharing a common superscript within the same column significantly differ ($p < 0.05$). *b*) *Bacteroides* and relatives. *c*) *Gammaproteobacteria*.

gestion of ethanol in this study and hepatic catalase may be involved in hepatic protection in the early stage of alcoholic liver damage. Hepatic catalase and SODs are antioxidant enzymes, and we have previously reported that the acute phase of hepatic damage induced by intraperitoneal injection of galactosamine was improved in the rats expressing higher hepatic catalase and SODs mRNA.[7)] These findings also suggest that some antioxidant enzymes are associated with protection of the liver from injury. In some cases, pattern of SOD-2 level may be similar to that of catalase level in alcoholic liver disease,[30,31)] and in other cases, including this study, these patterns were not same.[32)] The main transcriptional factors for expression of each enzyme may differ in this study, as well as in previous reports.[33,34)]

In the MBF-fed rats, hepatic oxidative damage, expressed as TBARS level, was observed, which was possibly due to accumulation of hepatic lipid and reduction of catalase content because a previous report suggested that accumulation of hepatic lipid contents was involved in reduction of catalase levels.[30)] Contrary to the reduction in catalase levels in the ethanol-ingested rats, some studies had indicated no reduction in catalase activity in ethanol-treatment although experimental desings had been different from this study.[31,35,36)] Considering these data, protective role of catalase in hepatic damages should be investigated in further studies.

It has been reported that the analytical technique T-RFLP can reveal bacterial population including the non-cultivable bacteria,[16–18)] and we had expected that this technique was valuable for assessment of cecal bacteria associated with alcoholic liver injury, but this technique was not enough to explain the association of the intestinal bacteria with hepatic symptom in this study. This technique can detect bacteria that are present as 0.1% in a sample, which is more sensitive than DGGE although T-RFLP may detect bacterial peaks as rather genera than species.[37)] Meanwhile, we cannot deny the involvement of minor species of intestinal bacteria in the hepatic damage in this study. So, other techniques suitable for the detection of bacterial species, such as DGGE,[37)] may be also useful for the analysis of intestinal bacteria in alcoholic liver disease. In either case, these molecular biological techniques are developing and further data may be needed for some cosequences. For example, concerning reproducibility, there were the relationships of *L. gallinarum* with the catalase and TBRAS levels in this study, and this bacterium possibly plays a key role in hepatic injury; however, similar relationship was not observed in our other studies (data not shown).

On the other hand, the results of T-RFLP may reveal changes in cecal bacteria associated with the cecal condition on the ground of the correlations between bacterial population and indicators of the cecal condition. For example, the increases in *L. gallinarum* or *Clostridium* cluster IV may be involved in the lowering of cecal content pH; *Bacteroides* and relatives were predominant, and their population increased depending on the cecal size. These bacteria are suggested to be predominant bacteria in human feces,[18)] and the present results of intestinal bacteria may also represent considerable part of cecal bacteria. These results and the previous findings suggest that T-RFLP could give some information about cecal conditions, and this analytical method is possibly useful in the evaluation of severe alcoholic hepatitis enhanced by endotoxin.[9–11)]

Additionally, we had hypothesized that some dietary fibers might act as a prebiotic for alcoholic liver diseases because of improvement in the intestinal condition,[12,13)] and we examined the prebiotic effect of MBF in this study. Actually, the MBF diet probably improved the cecal condition because cecal pH was reduced and *Lactobacillus* was suggested to be increased in the MBF group. As

mentioned above, however, the cecal condition may make a little contribution to early stage of hepatic damage, and the prebiotic effect on more advanced damage still remains unclear.

In conclusion, hepatic CYP2E1 level, rather than cecal condition, may be involved in the induction of the early stage of alcoholic liver injury in rats, and hepatic catalase is likely to be associated with improvement of this injury. Although effects of cecal condition and changes in bacterial content based on T-RFLP were not observed to correlate with liver injury, T-RFLP analysis could give information regarding cecal condition. This analytical technique is possibly beneficial for the estimation of intestinal condition in more severe alcoholic liver injury.

Acknowledgements This work was supported in part by a Grant-in-Aid from The Ministry of Agriculture, Forestry and Fisheries of Japan (Bio-Recycle Research Project; Rural Bio-Mass Research Project, BUM-Cm1210). We are thankful to Dr. Hayashi (Maebashi Institute of Technology, Gunma, Japan) and Dr. Hosono (Nihon University, Kanagawa, Japan) for suggestion of technical literatures. We thank Ms. Minako Saito for her skillful assistance.

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