Iron Chelator Deferoxamine Reduces Preneoplastic Lesions in Liver Induced by Choline-Deficient L-Amino Acid-Defined Diet in Rats

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The aim of this study was to investigate whether an iron chelator, deferoxamine (DFO) can prevent lipid peroxidation, resulting in reduced liver injury as well as reducing preneoplastic lesions induced by a choline-deficient L-amino acid-defined (CDAA) diet. CDAA diet administration resulted in an increased serum ALT level (367 ± 58) after two weeks, but simultaneous DFO treatment for two weeks reduced this elevation of ALT as well as malondialdehyde (MDA) production in the liver. Feeding rats a CDAA diet for 12 weeks led to the development of severe liver fibrosis and preneoplastic lesions detected as enzyme-altered lesions. DFO treatment prevented the expression of activated stellate cells, resulting in the reduction of liver fibrosis as well as reducing the development of preneoplastic lesions. These results indicate that iron chelation can reduce the development of preneoplastic lesions in a CDAA diet model.

KEY WORDS: preneoplastic lesion; enzyme-altered lesion; liver fibrosis; free radical; iron.

A cellular pool of transient iron, chelatable by deferoxamine (DFO) and distinct from ferritin is required to catalyze the generation of active oxygen species (1–3). Oxidative stress can initiate membrane lipid peroxidation related to the loss of cell viability not only in cultured hepatocytes (4–6) but also in vivo as occurs alcoholic or drug-induced liver injury (7, 8). This can be protected against by an iron chelator, deferoxamine (9, 10).

On the other hand, some reports suggest that hepatoacellular carcinoma in the rat liver induced by prolonged exposure to a diet with a low concentration of choline without any carcinogens is presumably the result of oxidative stress (11, 12). A modified choline-devoid diet—the choline deficient L-amino acid defined (CDAA) diet—has been reported to be more carcinogenic due to a lack of oligopeptides and antioxidant minerals, resulting in more active fibrosis (13, 14), which is related to the development of preneoplastic lesions detected as glutathione S-transferase placental form (GSTP)-positive lesions (15).

The hepatic stellate cell (Ito cell, fat-storing cell) is considered to be the main collagen-producing cell under pathological conditions (16, 17), and it seems to be activated by liver injury, including lipid peroxidation (18).

In the present study, we induced the development of liver cirrhosis with a CDAA diet and, using this...
model, we investigated the effect of an iron chelator, deferoxamine, on lipid peroxidation, liver fibrosis (stellate cell activation), and preneoplastic lesions. Our results showed that the chelation of hepatic iron by deferoxamine reduced the liver injury caused by lipid peroxidation, leading to the reduction of preneoplastic lesions in the less fibrotic liver.

**MATERIALS AND METHODS**

**Animals.** Male Wistar rats (180–200 g) were obtained from Nippon SLC Co., Ltd. (Shizuoka, Japan). The animals were fed *ad libitum* for one week before use and kept in a climate-controlled room with a 12-hr dark–light cycle.

**Experimental Protocol.** For the short-term experiment, rats were fed a CDAA diet for two weeks with administration of 0, 200, or 400 mg/kg DFO (Ciba Pharmaceutical Co., Summit, New Jersey) once a day by intraperitoneal injection of a 250 mg/ml solution in 0.9% NaCl. FeCl3 (125 mg/kg) dissolved in 0.9% NaCl was administered simultaneously with 400 mg/kg DFO by intraperitoneal injection for two weeks. All rats were killed under ether anesthesia 12 hr after the final DFO injection.

For the long-term experiment, rats were fed a CDAA diet for 12 weeks (82 days) with the administration of 0, 200, or 400 mg/kg DFO three times a week [Monday (days 1, 8, 15, etc), Wednesday (days 3, 10, 17, etc), and Friday (days 5, 12, 19, etc)]. Each experimental group was composed of six rats, and three rats per cage were fed the CDAA diet or a choline-supplemented l-amino acid defined (CSAA) diet as a control with or without DFO injection, as previously described (19). To equalize the total food intake in all groups, additional food was not supplied until all food in all groups had been consumed.

All rats were killed under ether anesthesia on day 83, 12 hr after the final DFO injection. Blood was drained from the aortic bifurcation, allowed to clot at room temperature, then centrifuged for 15 min at 3000 g. After draining the blood, the liver was excised and immediately frozen for the assessment described below or fixed in neutralized 10% formalin for 24 hr and embedded in paraffin for immunohistochemical examination other than GSTP staining.

**Serum Markers.** After 2 or 12 weeks, serum ALT, AST, and iron concentrations were measured as described elsewhere (9).

**Histology and Immunohistochemical Examination.** Sections of the right lobe of all rat livers 5-μm thick were processed routinely for hematoxylin and eosin (H&E) and Azan-Mallory staining. α-Smooth muscle actin for the detection of activated stellate (Ito) cells and glutathione S-transferase placental form (GSTP) for the detection of enzyme-altered (preneoplastic) lesions were immunohistochemically examined by the avidin–biotin–peroxidase complex method as previously described (15, 19). Anti-α-smooth muscle actin and anti-rat GSTP antibodies (MBL Inc., Nagoya, Japan) were employed. Monospecific antisera to malondialdehyde (20) was a kind gift from Koji Uchida (Nagoya University, Nagoya, Japan).

For morphometric semiquantitative analysis of the activated stellate cells in the liver fibrosis induced by CDAA diet administration with or without DFO, we assessed the mean value of α-SMA-positive cells in six ocular fields per specimen as a percent area at 40 × magnification using an image analysis system (Personal Image Analysis System LA-555; Pias, Ltd., Osaka, Japan). As vessels were stained by the anti-α-SMA monoclonal antibody, the mean value for three specimens (six ocular fields per specimen) of untreated rat livers was subtracted from each experimental specimen, and α-SMA-positive cells were expressed as a percentage of the total area of the specimen as previously described (21).

For morphometric semiquantitative analysis of GSTP-positive lesions, three sections from three major lobes of each rat liver were fixed in an ice-cold 19:1 mixture of dehydrated ethyl alcohol and glacial acetic acid for 3 hr. This was followed by overnight incubation in 99.5% ethyl alcohol at 4°C and embedding in paraffin. After this assessment was performed as described above and findings expressed as a percentage of the total area of the specimen, as previously reported (15). For the immunostaining of MDA in the liver, livers of three rats fed CDAA for 12 weeks were perfused through the portal vein with 1 liter of 4% paraformaldehyde–5% sucrose, pH 7.4) containing 50 mM BHT and 1 mM EDTA. Tissue segments for immunostaining were rinsed in 0.1 M sodium phosphate, pH 7.4, 4% sucrose, 0.15 mM CaCl2, 50 mM BHT, and 1 mM EDTA overnight at 4°C; dehydrated through a graded series of ethanol concentrations; and embedded in paraffin as previously described (22).

**Assay of Lipid Peroxidation, Iron, and Hydroxyproline Content in Liver.** Hepatic lipid peroxidation was quantified by measuring MDA in the liver using the modified method described by Yagi (23) as previously described elsewhere (9). Hepatic iron concentration was measured as also previously described (24). Hydroxyproline content was determined by a modification of the method of Kivirikko as previously reported (14, 15).

**Probes.** The following probes were used in this study. Type I α2-procollagen cDNA, used as described previously (25), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (26) were purchased from the American Type Culture Collection.

**Northern Blot Analysis.** Northern blot analysis was performed after poly(A)+ RNA selection as previously described (14).

**Statistical Analysis.** All results are given as mean ± SD. The statistical significance of observed differences was assessed using one-way ANOVA. Differences were considered statistically significant at the 0.05 level.

**Ethical Considerations.** This experiment was reviewed by the Ethics Committee for Animal Experiments of Yamaguchi University School of Medicine and was carried out under the control of the Guidelines for Animal Experiments of Yamaguchi University School of Medicine and Law No. 105 and Notification No. 6 of the Japanese government.

**RESULTS**

**Short-Term Experiment: Effect of DFO on CDAA Diet-Induced Liver Injury After Two Weeks**

Table 1 shows the dose-dependent protective effect of DFO on CDAA diet-induced liver injury after two