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Immunohistochemical and Biochemical Studies in 4-aminopyrazolopyrimidine (4-APP)-induced Fatty Liver

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Four-aminopyrazolopyrimidine (4-APP)-induced fatty livers were studied immunohistochemically and biochemically. Immunohistochemical localization of glutathione-peroxidase (GSH-PO) in control rat liver was predominantly observed in the hepatocytes of portal zones of the hepatic lobules. After 3 days of 4-APP administration, the intensity of GSH-PO staining was weaker than that of control. Especially, this tendency was predominantly observed in portal zones of the hepatic lobules. Biochemcally, lipid peroxide levels measured by thiobarbituric acid method in the liver homogenates were markedly increased following 4-APP administration. However, glutathione-peroxidase (GSH-PO) activity in the same homogenates was decreased. Based on our data, we strongly suggested that pathogenesis of 4-APP induced-fatty liver may be due to uncontrolled free radical formation, peroxidation of lipids, and an associated lipid accumulation in the liver.

Key words: 4-aminopyrazolopyrimidine (4-APP), Fatty liver, Glutathione-peroxidase (GSH-PO), Lipid peroxidation

INTRODUCTION

Pathogenesis of the fatty livers induced by certain kinds of chemical agents such as carbon tetrachloride [11] and ethanol [5] has been well identified as due to free radical production in the cellular components and followed by the lipid peroxidation. Concomitantly with triglyceride accumulation in the livers of the rats receiving carbon tetrachloride or ethanol, liver peroxide values are also increased. On the other hand, glutathione-peroxidase (GSH-PO) has been well recognized to effectively reduce the organic hydroperoxides induced by liver injury [3, 14].

In the present study, in order to confirm the relationship between fatty changes and lipid peroxidation in the liver, we made immunohistochemical and biochemical analysis of the livers of rats administered with 4-aminopyrazolopyrimidine (4-APP) as agent, which inhibits the secretion of triglycerides from liver [1, 7, 12].

MATERIALS AND METHODS

Animals

Male Crj: CD(SD) IGS rats were purchased from Charles River Japan Inc. (Atsugi, Japan) at the age of seven weeks. The animals were kept in a barrier-maintained animal room, which was maintained at a temperature of 22 ± 2 ℃ with a relative humidity of 55 ± 15 %. The room was ventilated twenty-one times per hr and provided with 12 hr of light (from 8:00 to 20:00). Solid food (CE-2, CLEA Japan Inc.) and tap water were given *ad libitum*. The animals were kept for one-week acclimation period under laboratory conditions.

Experiments

Group 1 consisted of untreated controls. In group 2, animals were injected intraperitoneally with 2 mg of 4-aminopyrazolopyrimidine (4-APP, Sigma Chemical Co. St. Louis) once daily for 3 days. Saline was used as the control for 4-APP. Each rat was sacri-

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ficed under deep anesthesia. Then, the livers were removed immediately.

The animals were cared for according to the principles outlined in the guide for the care and use of laboratory animals prepared by the Japanese Association for Laboratory Animal Science and our institution.

Immunohistochemical studies

Small tissue blocks of liver were fixed in periodate-lysine-4 % paraformaldehyde solution [8] for 18 to 20 hr at 4 ℃ under constant agitation. The fixed tissues were then washed in 0.01M phosphate buffered saline (PBS), pH 7.4, containing sucrose from 10 to eventurally 20 % overnight at 4 ℃ Subsequently, 6 μm frozen sections were prepared from these tissues in a cryostat, and were placed on glass slides. Then, the sections were incubated with the anti-GSH-PO IgG Fab fragment labeled with horseradish peroxidase (HRPO, Sigma Chemical Co., St. Louis) for 1 hr. After the incubation was completed, the sections were incubated in Graham-Karnovsky's reaction medium [6] which contained 3,3'-diaminobenzidine (DAB, Wako pure Chemical Co., Osaka) and 0.005 % hydrogen peroxide as the substrate for 5 to 10min. Then the sections were counterstained for nuclei with 1 % methyl green dissolved in veronal acetate buffer, pH 4.2.

Measurement of lipid peroxides

One gram of fresh liver was homogenized with Potter type homgenizer in 10 ml of 0.25 M sucrose solution containing 1 mM $MgCl_2$ and 0.7 mM 2-mercaptethanol. The homogenates were centrifuged at 700 xg for 10 min and pellets were removed. Then, lipid peroxide level (TBA value) of homogenates was determined by the thiobarbituric acid (TBA) method according to Yagi [13] and expressed in terms of malondialdehyde (nmol/g wet weight of liver).

Measurement of GSH-PO activity

One hundred microliters of obtained homogenate was pre-incubated with 2.5 ml of reaction medium containing 0.1 M Tris/borate buffer (pH 8.5), 3 mM EDTA/Tris, 0.12 mM NADPH (Oriental Yeast Co., Tokyo), 0.25 mM reduced glutathione (Sigma Chemical Co., St. Louis) and 2.5 μg of glutathione reductase (Sigma Chemical Co., St. Louis) for 2 min at 37 ℃. The reaction was then started by the additional of 0.1 ml of cumene hydroperoxide (Mathen Colemen and Bell Manufacturing Chemist, USA). The decreases in the absorbance at 340 nm was measured after a 5 min incubation using a Hitachi spectrophotometer model 210. One unit of enzyme oxidized 1 μmol NADPH to NADP/min at 37 ℃.

Statistical analysis

The data were expressed as mean ± SD. Homogeneity of variance was tested by Bartlett's methods, and when the assumption of homogeneity of variance was met, one-way layout analysis of variance was performed. When a significant difference was observed, Dunnett's multiple comparative test was performed between the control group and the experimental group.

RESULTS

Immunohistochemical staining

In control rat liver, the GSH-PO was mainly localized in portal zones of the hepatic lobules (Fig. 1). In the livers from 4-APP-administered rats, the intensity of GSH-PO staining was weaker than that of control (Fig. 2). Especially, this tendency was predominantly observed in portal zones of the hepatic lobules.

Table 1 Effect of 4-APP administration on the activity of GSH-PO and the TBA value in rat liver homogenates

	Control	4-APP
GSH-PO activity (unit/g liver)	103.8 ± 8.4	68.0 ± 5.2
TBA value (nmol/g liver)	126.0 ± 11.0*	401.0 ± 26.5**

TBA: Thiobarbituric acid
Values are mean ± SD.
*$P < 0.05$, **$P < 0.01$, significant difference from control
4-APP: 4-aminopyrazolopyrimidine

Biochemical studies

Lipid peroxide value (TBA value) and GSH-PO activity in the liver homogenates are sown in Table 1. Lipid peroxide levels as TBA value of liver homogenates from rats given 4-APP administration were significantly higher than that of controls. However, the GSH-PO activity in the same homogenates was much less, being only 40 % of the control value.

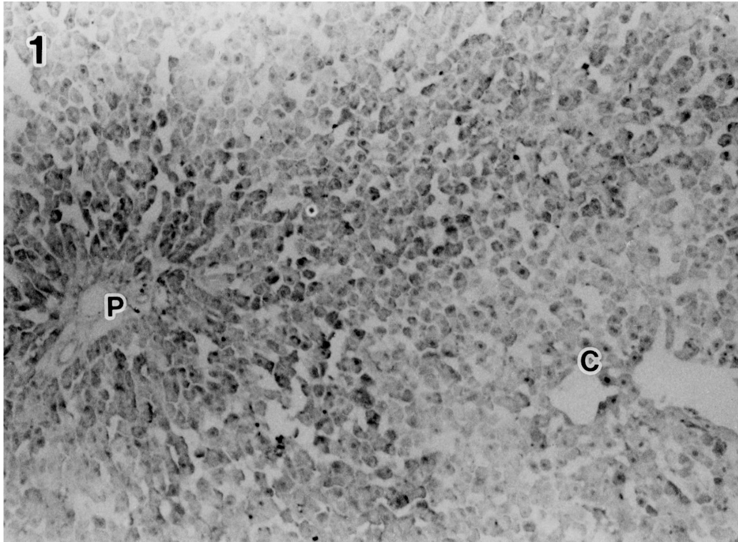

Fig. 1 Immunohistochemical localization of GSH-PO in the liver of a control rat. GSH-PO is manly observed in the hepatocytes of portal zones (P) of the hepatic lobules. C: Central zone. Peroxidase-labeled antibody method, × 150.

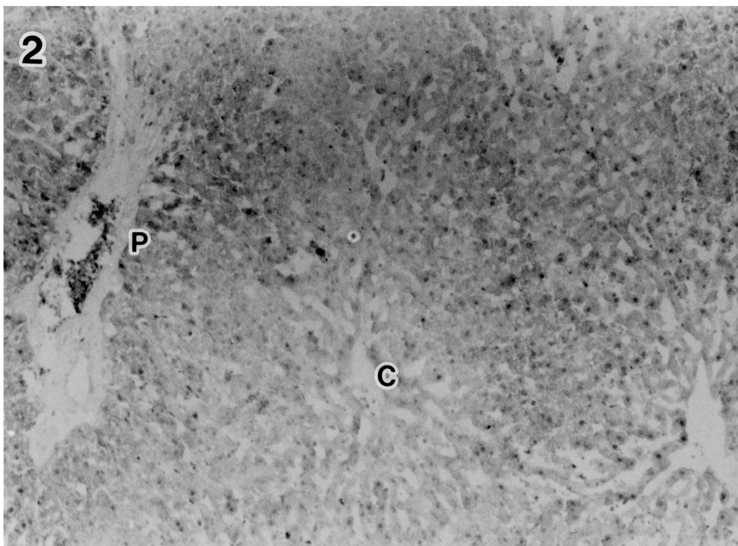

Fig. 2 Immunohistochemical localization of GSH-PO in the liver of a 4-APP-tretaed rat. The intensity of GSH-PO staining in portal zones (P) of the hepatic lobules are weaker than that of control. C: Central zone. Peroxidase-labeled antibody method, × 150.

DISCUSSION

Four-APP, an adenine analog, is well known to inhibit hepatic release of lipoprotein including triglycerides and to decrease serum cholesterol levels markedly [1, 7, 12]. Henderson reported that 4-APP inhibits the secretion of triglycerides from the liver and he speculated that the changes in serum and liver lipids produced by 4-APP were not the result of altered protein synthesis because he found no reaction in plasma protein synthesis after administration of 4-APP to mice [7]. Furthermore, Schiff *et al*. speculated that accumulation of lipid in the liver and decrease in serum lipoprotein concentrations resulting from administration of 4-APP might be due to inhibition of the synthesis of lipoprotein or to decreased transport, or both [12]. This class of fatty liver is well known to be induced in the rat by the administration of CCl_4, ethionine, phosphorus, choline deficiency or orotic acid.

In our previous report, pathogenesis of 4-APP-induced fatty livers was demonstrated by electron microscopic investigation [9]. As a result, we proposed that 4-APP induced-fatty livers might show defective, protein synthesis, at least in part, and that imperfect lipoprotein formation might result in lipid accumulation [9].

In the present study, we found that lipid peroxide values (TBA value) of liver homogenates were markedly increased following 4-APP administration. These biochemical data strongly suggest that lipid peroxidation may be actively involved in the genesis of fatty livers due to the administration of 4-APP. In fact, electron microscopical examination of liver sections from 4-APP-administered rats has been observed disorganization and detachment of ribosomes of the endoplasmic reticulum but no equivalent damage to the mitochondria [9]. Dasai *et al*. [4] have shown that intracellular particle bounded by a double-layered membrane such as mitochondria are far less susceptible to disorganization induced by free radicals than are systems bounded by a unit membrane such as endoplasmic reticulum. In addition, Arstile *et al*. [2] showed by electron microscopy that increased peroxidation caused detachment of ribosomes from membrane followed by dissociation of the ribosomal particles and that the structure of the microsomes then changed from round to less regular vesicles and functionally to a dense, amorphous precipitate that contained membrane debris.

On the other hand, 4-APP-induced fatty changes have been predominantly observed in the portal zones of the hepatic lobules. In the rat liver, GSH-PO, which effectively reduced lipid peroxides, has been localized mainly in the portal zones of the hepatic lobules as previously documented [14]. In the present study, immunohistochemical localization of GSH-PO in 4-APP-treated rat liver was weaker than that of control, mainly of the portal zones of the hepatic lobules. In addition, GSH-PO activity of liver homogenates from 4-APP-treated rats was markedly decreased. Many studies have demonstrated a protective role of GSH-PO against lipid peroxidation induced by liver injury [3, 14], in some processes for steroidogenesis [10], and also prostaglandin synthesis [10].

Based on our data and these facts, we strongly suggest that pathogenesis of 4-APP-induced fatty liver, at least in part, may involve uncontrolled free radical formation and peroxidation of lipids, which then causes an associated lipid accumulation in the liver.

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