

RESEARCH COMMUNICATION

Time Course of Change in Glutathione S-Transferase Positive Foci and Ornithine Decarboxylase Activity after Cessation of Long-term Alcohol Administration in Rats

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Abstract

In our previous experiments, we showed that cessation of long-term alcohol administration enhances hepatocarcinogenesis in the rat. In the present study, we examined the time course of hepatocarcinogenesis induced by diethylnitrosamine (DEN) after cessation of alcohol using numbers and areas of glutathione S transferase placental form (GST-P)-positive foci and the activity of ornithine decarboxylase (ODC) in males of the Wistar strain. Fifty six rats were given a single i.p. injection of DEN (200 mg/kg body weight), maintained on basal solid diet for two weeks, then maintained on liquid diet in which 36% of total calories were provided by ethanol (Al diet) for 12 weeks, and then eight rats were killed. The remaining rats were divided into 6 groups. Three alcohol cessation groups were maintained on control liquid diet (C diet) instead of Al diet for 3, 6 and 12 weeks, respectively. The others, as reference groups were maintained on the Al diet continuously for the same periods, respectively. The numbers of GST-P-positive foci per unit area of the liver were not markedly changed after cessation of alcohol. However, their areas were increased with time, so that values in the alcohol cessation groups at 3 and 12 weeks were significantly higher than those in the reference groups at the same points, respectively. Furthermore, ODC activity was significantly elevated in the alcohol cessation groups at 3 and 6 weeks compared to reference groups, but not at 12 weeks when reduction was rather observed. These results suggest that cessation of long-term alcohol administration enhances hepatocarcinogenesis and this effect may be closely related to activation of cell proliferation due to the interruption of alcohol insult.

Key words: alcohol - hepatocarcinogenesis - glutathione S transferase placental form - ornithine decarboxylase - cessation of alcohol administration

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Introduction

In Japan, preventive measures for hepatocellular carcinoma are urgently required, because the prevalence of this neoplasm and associated mortality have been increasing (Takada et al., 1994). It is clear that most non-viral cases of hepatocellular carcinoma arise with a background of alcoholic liver cirrhosis and that the incidence of hepatocellular carcinoma is higher in heavy drinkers than in non-drinkers, even in those with viral liver cirrhosis

(Tuyns, 1979; Lieber et al., 1979; Yu et al., 1983; Tsukuma et al., 1990; Tsutsumi et al., 1996). Thus it has been thought that alcohol drinking is positively linked with hepatocarcinogenesis. On the other hand, there has been a report of acceleration due to abstinence from alcohol drinking (Nishiuchi et al., 1990). Recently, in a previous report, we demonstrated that cessation of long-term alcohol administration enhances hepatocarcinogenesis in rats (Tanaka et al., 2000).

In the present study, we examined the time course of

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hepatocarcinogenesis after cessation of alcohol using the same experimental design and indicators as previously employed. The experimental model used is a modified Ito's medium-term bioassay system for hepatocarcinogenesis in rats (Ito et al., 1988). Attention was concentrated on the time course of change in numbers and areas of cell foci positive for the glutathione S transferase placental form (GST-P), early indicators for hepatocarcinogenesis, and ornithine decarboxylase (ODC) activity, as a marker for cell proliferation.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) was obtained from Tokyo Chemical Industry Co., Ltd., Tokyo. [1-¹⁴C]Ornithine was obtained from Moravек Biochemicals, Inc., Brea, CA, USA.

Animals and treatment

The protocol of the experiment was approved by the Animal Research Committee, Osaka City University, and care of the animals was according to the standards of this institution (Guide for Animal Experimentation, Osaka City University).

Fifty six male Wistar rats (Clea Japan, Inc., Tokyo) purchased at 6 weeks of age were housed in an air-conditioned room with a 12-h light, 12-h dark cycle, and given a pellet diet (Oriental Yeast Co., Tokyo) and tap water ad libitum.

The experimental design is shown in Figure 1. Fifty six rats were given a single i.p. injection of DEN (200 mg/kg body weight) and maintained on pellet diet for two weeks, and then administered a liquid diet in which 36% of total calories were provided by ethanol (A1 diet) for 12 weeks. The rats of group 1 (N = 8) were then killed. The remaining animals were divided into 6 groups. Three alcohol cessation groups, namely, groups 3, 5 and 7 were maintained on control liquid diet (C diet) instead of the A1 diet for 3, 6 and 12

weeks, respectively. Continued administration groups 2, 4 and 6, as references for groups 3, 5 and 7, respectively, were maintained on the A1 diet continuously for the same periods. All the liquid diets were purchased from Oriental Yeast Co., Tokyo. All rats were subjected to partial hepatectomy under light ether anesthesia 3 weeks after DEN injection using the method of Higgins and Anderson (1931).

At autopsy, livers were excised and slices 2-3 mm thick were cut with a razor blade. These were fixed in ice cold acetone and routinely processed for immunohistochemical examination of putative preneoplastic lesions, GST-P-positive foci. For measurement of ODC activity, samples of rat livers were frozen in liquid nitrogen immediately after resection.

Immunohistochemical staining of GST-P

The avidin-biotin-peroxidase complex (ABC) method described by Hsu et al. (1981) was used to demonstrate GST-P-positive foci. After deparaffinization, liver sections were treated sequentially with normal goat serum, anti-rabbit GST-P antibody, biotin-labeled goat anti-rabbit IgG and ABC (Elite Vectastain, Vector Labs, Burlingame, CA). The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. The numbers and the areas of GST-P-positive foci >0.1 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (Olympus-IKEGAMI VIP-21CH, Tokyo).

Measurement of ODC activity

ODC activity was measured by the method of Otani et al. (1985). Frozen samples of liver were suspended in 0.5 ml of 50 mM Tris (pH 7.5) containing 0.25 M sucrose and disrupted by homogenization for a few minutes. The homogenized suspensions were centrifuged at 100,000g for 30 min, and the supernatants assayed for ODC activity by measurement of the amount of radioactive CO₂ produced from [1-¹⁴C] ornithine.

Statistical analysis

Data were expressed as means ± SD, and statistical analysis was performed using the Student's t test.

Results

There were no significant differences in final body weights and relative liver weights between alcohol cessation and reference groups (i.e. between groups 2 and 3, groups 4 and 5, and groups 6 and 7).

On immunohistochemical staining, GST-P-positive foci were stained dark brown. With hematoxylin staining, the regions in serial sections had no obvious structural changes, in terms of nuclear density or atypia, and differentiation of foci from background parenchyma was difficult. Fibrosis was not apparent in any group, but fatty change was remarkable in all groups equivalently.

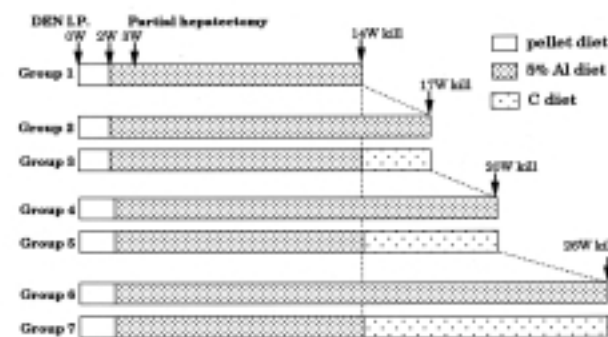


Figure 1. Schematic Diagram of the Experimental Protocol for Examining the Effects of Cessation of Alcohol Administration. Details are Described in the "Materials and Methods." N=8 for Each Group.

Numbers of GST-P-positive Foci

The numbers of GST-P-positive foci per unit area of the liver were not markedly changed after cessation of alcohol (Fig. 2). The slight differences between alcohol cessation and reference groups (i.e. between groups 2 and 3, groups 4 and 5, and groups 6 and 7) were not statistically significant.

Areas of GST-P-positive Foci

The areas of GST-P-positive foci per unit area of the liver were increased with time after cessation of alcohol (Fig. 3). The values in group 3 and 7 were significantly higher than those in groups 2 and 6 ($p < 0.05$, $p < 0.01$, respectively). That for group 5, also, showed a tendency for increase as compared to group 4 ($p < 0.1$).

Liver ODC Activity

ODC activity in the liver was significantly elevated at 3 and 6 weeks after cessation of alcohol administration. Thus values for groups 3 and 5 were significantly higher than those for groups 2 and 4 ($p < 0.05$, $p < 0.01$, respectively, Fig. 4). However, at the 12 week time point, it was reduced to the base level.

Discussion

It has been said that most non-viral cases of hepatocellular carcinoma arise with a background of alcoholic liver cirrhosis and that the incidence of hepatocellular carcinoma is higher in heavy drinkers than in non-drinkers, even in those with viral liver cirrhosis (Tuyns, 1979; Lieber et al., 1979; Yu et al., 1983; Tsukuma et al., 1990; Tsutsumi et al., 1996). Thus it generally considered that alcohol drinking is positively linked with hepatocarcinogenesis. On the other hand, there has been a report of acceleration due to abstinence from alcohol drinking (Nishiuchi et al., 1990). Recently, in our previous report, we described cessation of long-term alcohol administration to enhances hepatocarcinogenesis in

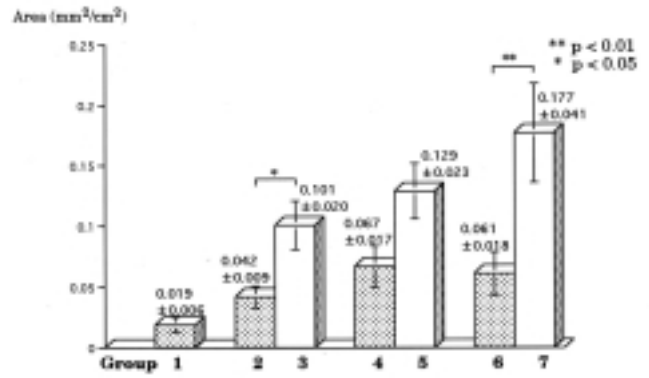


Figure 3. Areas of Glutathione S Transferase-P (GST)-Positive Foci per cm². Groups 1-7; See also Fig.1 for Further Details . Values are Means±SD.

rats (Tanaka et al., 2000). In the present study, we examined the time course after cessation of alcohol in order to clarify the underlying mechanism using Ito's medium-term model of hepatocarcinogenesis (Ito et al., 1988) in rats.

The process of chemical carcinogenesis can be divided into initiation, promotion and progression phases. In Ito's model, a carcinogenic substance (DEN) is administered once for initiation; from 2 weeks after, a test substance is then administered for 6 weeks; at the 3rd week, partial hepatectomy is performed as a stimulation to proliferation; and after 8 weeks, the animals are killed and carcinogenic activity is assessed. To determine the effect of alcohol on hepatocarcinogenesis, longer-term exposure was considered necessary and therefore we prolonged the period to 12 weeks or more.

While the numbers of GST-P-positive foci per unit area of the liver were not markedly changed after cessation of alcohol (Fig. 2), the areas were clearly increased with time after cessation of alcohol (Fig. 3). In fact the values in

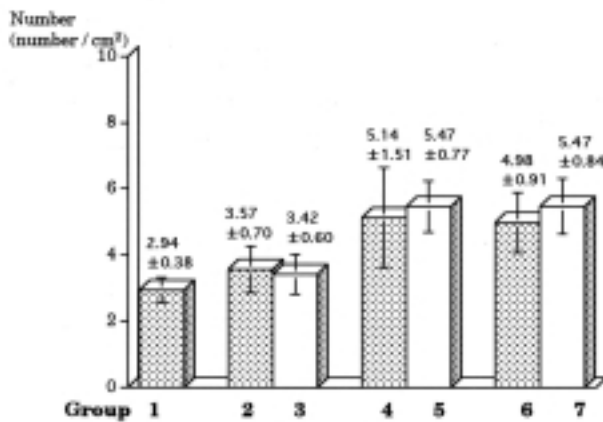


Figure 2. Numbers of Glutathione S Transferase-P (GST)-Positive Foci per cm². Groups 1-7; See also Fig.1 for Further Details. Values are Means±SD.

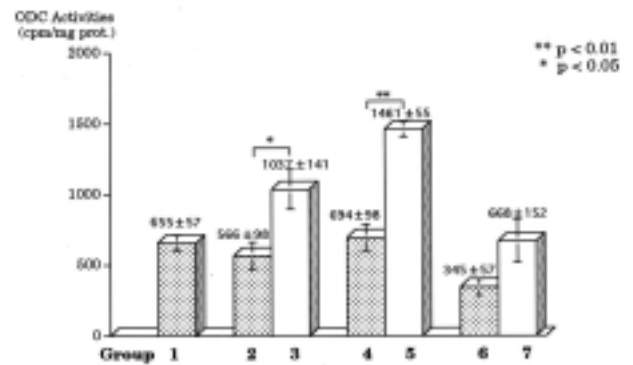


Figure 4. Hepatic Ornithine Decarboxylase (ODC) Activity at Week 26. Groups 1-7; See also Fig.1 for Further Details. Values are Means±SD.

cessation groups were significantly higher than those in reference groups. With regard to this apparent discrepancy between numbers and areas, we speculate as follows. Cell proliferation in whole liver was activated for a while after cessation of alcohol and this caused growth, perhaps preferentially, in foci. This speculation is supported by the results for ODC activity, with significant elevation found at 3 and 6 weeks after cessation of alcohol.

In conclusion, the results suggest that cessation of long-term alcohol administration enhances hepatocarcinogenesis and this effect may be closely related to the activation of cell proliferation due to alcohol interruption. Of course, we can not simply extrapolate to the human situation. However, clinically, hepatocarcinogenesis may be accelerated by abstinence from alcohol drinking in patients with liver cirrhosis (Nishiuchi et al., 1990). It has also been reported that α -fetoprotein increases temporarily when patients consuming alcohol abstain from drinking. Further studies are needed as to the changes in foci with observations over a longer period of time after interruption of alcohol and differences in hepatic function and morphology in comparison with the continuous drinking state.

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