

## RESEARCH COMMUNICATION

# Effects of Cessation of Alcohol Exposure on Rat Hepatocarcinogenesis

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### Abstract

Cessation of long-term alcohol exposure is reported to enhance rat hepatocarcinogenesis. The purpose of the present study was to assess this possibility using glutathione-S transferase placental form (GST-P) positive foci as end point lesions. All rats were treated with a single i.p. injection of diethylnitrosamine (DEN) (200 mg/kg body weight) and then given a MF pellet diet for 2 weeks. Thereafter, the animals were maintained on: alcohol liquid diet in which 36% of total calories were provided by alcohol (5% Al diet) for 6 weeks (group 1); control liquid diet (C diet) for 6 weeks (group 2); 5% Al diet for 6 weeks and subsequently C diet for 4 weeks (group 3); 5% Al diet for 10 weeks (group 4); or C diet for 10 weeks (group 5). All rats were subjected to two thirds partial hepatectomy at 3 weeks after DEN injection. The number and area of GST-P positive foci per cm<sup>2</sup> of liver tissue were slightly increased in group 1 compared to the group 2 and significantly elevated in the group 4 compared to group 5. However, numbers in group 3 were significantly lower in group 4 and similar to the group 5 values. PCNA positive cells in the GST-P positive foci in the group 1 and group 4 were significantly increased as compared with respective controls (groups 2 and 5, respectively), while indices in the group 3 were again similar to values for group 5. Cessation of short-term alcohol administration thus had no promoting effects on development of GST-P foci, suggesting that the duration of alcohol treatment may be important. The results also imply the existence of a cumulative exposure time or dose threshold for alcohol if promoting effects of cessation are to be seen on rat hepatocarcinogenesis.

**Key Words:** Alcohol - GST-P positive foci - hepatocarcinogenesis

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### Introduction

Recent epidemiological studies have shown that the excessive and chronic alcohol consumption contributes to cancer development in liver, oral cavity, esophagus, pharynx, larynx colorectum and breast (Poschl and Seitz, 2004; Tatsuta et al., 1997). It is nowadays believed that with regard to liver cancer in humans, the risk for hepatocellular carcinoma (HCC) development increases approximately 5-fold with chronic alcoholic beverage consumption greater than 80 g/day for more than 10 years (Oshima et al., 1984; Morgan et al., 2004). Furthermore, alcohol per se has recently been shown to act as a cocarcinogen or tumor promoter in rodents. For example, alcohol promoted hepatocarcinogenesis in male rats initiated with 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), a food-related heterocyclic amine present in cooked meat and fish (Kushida et al., 2005). However, lack of the carcinogenic activity of alcohol even after lifelong exposure in experimental and human studies

has been reported (Holmberg and Ekstrom, 1995).

The results concerning alcohol influence are still contradictory and the reasons for its promoting influence have yet to be unequivocally clarified. Alcohol has been found to enhance hepatocarcinogenesis in rats, playing an important role in the initiation phase (Yamagiwa et al., 1994). Clear cocarcinogenic effects have been previously demonstrated by Tsutsumi et al (1993) when applied before and/or during carcinogen administration, but others have shown inhibition rather than adverse influence (Habs and Schmähl, 1981; Mandl et al., 1989). Regarding alcohol influence on postinitiation stage of hepatocarcinogenesis, modifying effects were recognized as enhancement (Porta et al 1985; Takada et al., 1986; Mufti and Sipes, 1991), or inhibition (Tanaka et al., 1989). Recently, we have shown that incidences of hepatocellular adenoma and total tumors increased dose-dependently in F344/DuCrj rats initiated with 200 ppm MeIQx in the diet for 8 weeks and thereafter receiving alcohol at doses of 10 and 20% in drinking water

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ad libitum for 16 weeks (Kushida et al., 2005). The incidence of HCC was also observed to be significantly elevated at a dose of 20%.

The mechanism of enhancement of hepatocarcinogenicity by alcohol is not clear. Alcohol is normally metabolized to acetaldehyde due to oxidative transfer of hydrogen by alcohol dehydrogenase to nicotinamide dinucleotide by the microsomal ethanol oxidation system. Acetaldehyde is then oxidized by xanthine oxidase and free radical species (ROS) are generated (Oei et al., 1982; Li, 1983). ROS, which highly reactive with cellular macromolecules such as DNA or proteins, have been found to cause oxidative damage in the alcohol-treated rats (Nordmann et al., 1992). DNA is a potential target of free radical damage, and the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a marker of oxidative DNA damage in the rat liver, on administration of ethanol after MeIQx initiation has been recently reported (Karim et al., 2003).

It has long been believed that alcohol-associated liver cancers do not increase after stopping alcohol intake. However, recent studies have reported that interruption of long-term alcohol administration enhances development of hepatocellular carcinoma in patients with alcoholic cirrhosis (Nishiuchi and Shinji, 1990; Tsukuma et al., 1993; Tanaka et al., 2000). Therefore it is of much interest to investigate the influence of transient alcohol exposure on hepatocarcinogenesis. Furthermore, the effect of stopping of short-term alcohol administration has not been yet clarified. Therefore, in the present study we focused on the effects of short-term alcohol administration in male F344/DuCrj rats using a medium-term rat liver bioassay with GST-P positive foci as end point preneoplastic lesions. To assess mechanisms, we also assessed alterations in gene expression and cellular proliferation. The results indicated that formation of GST-P positive foci of liver tissue was slightly increased after 4 weeks on an alcohol rich diet following

DEN initiation and significantly elevated after 10 weeks. However, rat liver carcinogenesis was not enhanced after termination of short-term treatment with alcohol and the results suggested that this effect might be closely related to the recovery of regulation of cell proliferation.

## Materials and Methods

### Animals

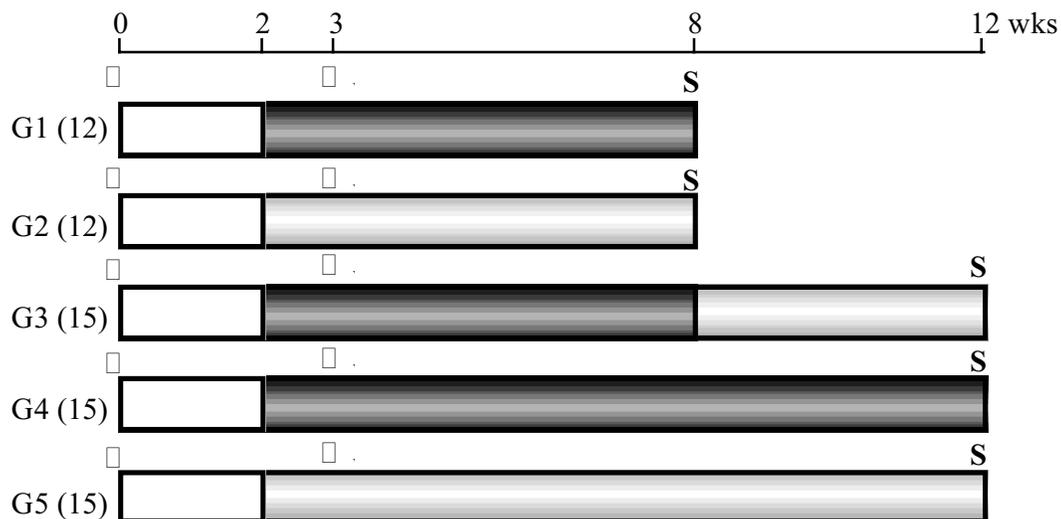
Male 6-week-old Fisher 344 rats (Charles River, Japan, Hino, Shiga, Japan) were quarantined for 1 week before the start of the experiment. They were housed in an animal facility maintained on a 12 h (7:00 -19:00) light/dark cycle, at a constant temperature of  $23 \pm 1^\circ\text{C}$  and relative humidity of  $44 \pm 5\%$ , and were given free access to tap water and food (Oriental MF pellet diet, Oriental Yeast Co., Tokyo, Japan).

### Chemicals and diet

Diethylnitrosamine (DEN) was obtained from Tokyo Chemical Industry Co., Tokyo. The 5% alcohol diet (A1 diet) and control diet (C diet) were purchased from Oriental Yeast Co., Ltd. Tokyo. Anti-rat GST-P primary (IgG,  $100 \mu\text{g/ml}$ ) antibodies were from MBL Co., Nagoya.

### Experimental design

Before the start of the experiment, a total of 56 male F344 rats were divided into 5 groups (Fig. 1). All animals underwent a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis and maintained on pellet diet (Oriental MF pellet diet, Oriental Yeast Co., Tokyo, Japan) and tap water for 2 weeks. Thereafter, group 1 was maintained on 5% alcohol liquid diet in which 36% of total calories were provided by ethanol (5% A1 diet) for 6 weeks, group 2 was administered control liquid diet (C diet) for 6 weeks, group 3 was maintained on 5% alcohol liquid diet for 6 weeks and subsequently on control liquid diet for 4 weeks, and groups 4 and 5 were



**Figure 1. Experimental Protocol.** □, MF pellet diet; ■, alcohol liquid diet; □, control liquid diet (all Oriental Yeast Co., Ltd.); i, DEN i.p. injection, 200 mg/kg b.w.; 2/3 partial hepatectomy; S, sacrifice

administered 5% alcohol and control liquid diets, respectively, for 10 weeks. All animals were subjected to two third partial hepatectomy at 3 weeks after DEN initiation treatment. Survivors in groups 1 and 2 were killed at the end of week 8, and in groups 3, 4, and 5 at week 12. Animals were anesthetized by diethyl ether, and livers were immediately excised for immunohistochemical demonstration of GST-P positive foci. Serum was collected for examination of insulin-like growth factor-1 (IGF-1).

#### Liver tissue processing

The excised livers were cut into 2-3 mm slices with razor blades. Three slices, one from the right posterior, anterior, and caudate lobes each, were fixed in 10% buffered formalin for GST-P positive foci and PCNA immunohistochemical examination. The remaining liver tissue was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for molecular assessment.

#### Immunohistochemistry for GST-P positive foci

Immunohistochemical assessment of GST-P was performed with the avidin-biotin complex method as described by Hsu et al (1981) using an anti-rat GST-P primary (1:2000) (IgG, 100  $\mu\text{g}/\text{ml}$ ) antibody, and diaminobenzidine tetrahydrochloride (DAB) as the substrate to demonstrate sites of peroxidase binding. Quantitation of GST-P positive foci was performed using two-dimensional evaluation (Imaida et al., 1989). The numbers and areas of foci greater than 0.2 mm in diameter, and total areas of liver sections, were measured using a color image processor (IPAP; Sumica Technos Osaka, Japan) to give values per  $\text{cm}^2$  of liver section.

#### Double staining for GST-P and PCNA

For double staining of GST-P and PCNA, rat liver sections were treated sequentially with the polyclonal rabbit anti-rat GST-P antibody at 1:2000 dilution and an anti-PCNA mouse monoclonal (PC-10, IgG2a; DAKO, Kyoto, Japan; 1:500) antibody. Immunohistochemical detection of GST-P was accomplished according to the protocol described above. The sites of peroxidase binding were demonstrated with alkaline phosphatase (Vectastain ABC-AP kit, Vector Red (SK-5100)) solution. Thereafter sections were sequentially treated with 0.2 M glycine, pH 2.2, for 2 h to remove immune complexes. Immunohistochemistry for PCNA was essentially performed as described previously (Kinoshita et al., 2002) with color development by DAB. GST-P positive foci were stained red, while brown/black staining of nuclei showed a positive immunoreaction for the monoclonal primary antibody with PCNA. The PCNA indices were estimated for GST-P positive areas and in background liver parenchyma as numbers of positive nuclei per 100 cells.

#### Determination of serum IGF-1 levels

Blood was collected in serum separator tubes, following centrifugation at 1000 g for 15 min, decantation, after which serum was frozen at  $-80^{\circ}\text{C}$  until analysis. Serum IGF-1 concentrations were determined using a radioimmunoassay

(RIA, Nichols Institute Diagnostics, San Juan Capistrano, CA) (Marc et al., 1999).

#### Ornithine decarboxylase activity assays

Liver was homogenized in 4 volume of  $\text{mmol}/\text{L}$  Tris-HCl (PH 7.4) containing 1  $\text{mmol}/\text{L}$  EDTA, 1  $\text{mmol}/\text{L}$  dithiothreitol and 50  $\mu\text{mol}/\text{L}$  pyridoxal phosphate. The homogenate was centrifuged at 30,000 g for 30 min, and the supernatant was used for assay of ODC activity by measurement of the  $^{14}\text{C}$  putrescine formed from 5- $^{14}\text{C}$  ornithine as described previously (Otani et al., 1984).

#### Statistical analysis

Statistical analysis of the obtained results was performed using the Student's t-test.

## Results

#### General observations

The daily intake of alcohol in the liquid diet ranged from 2.5 to 3.0 ml per day per rat throughout the experiment. There were no physical signs of toxicity although slight loss of body weight after switching to the liquid diet was observed in both the control- and the alcohol-fed rats (data not shown). Loss of body weight also occurred after partial hepatectomy. Animals in the alcohol-fed groups demonstrated lower body weights compared to the controls despite isocaloric content. The rats in group 3 gained body weight rapidly after withdrawal of alcohol diet.

#### Evaluation of GST-P positive foci

Data for GST-P positive foci are summarized in Table 1. The trend for increase of number and area of GST-P positive foci per  $\text{cm}^2$  was found after 4 weeks on A1 diet following DEN initiation (group 1) as compared to the initiation control group 2. Furthermore, values for both parameters in the group 4 (given 5% alcohol liquid diet for 10 weeks) were significantly elevated over the control level in group 5. When alcohol diet administration was terminated after 6 weeks followed by control diet administration (group 3), numbers of GST-P positive foci were significantly lower than in group 4 given the A1 diet for 12 weeks.

#### Alteration in cell proliferation

Results obtained by double immunohistochemistry for

**Table 1. Numbers and Areas of GST-P Positive Foci**

Group	Treatment	GST-P positive foci	
		Number (/cm <sup>2</sup> )	Area (mm <sup>2</sup> /cm <sup>2</sup> )
G1	DEN + A1 diet	7.8 $\pm$ 3.5*	0.69 $\pm$ 0.33
G2	DEN + C diet	6.3 $\pm$ 1.8	0.56 $\pm$ 0.24
G3	DEN + A1 diet +C diet	5.7 $\pm$ 1.6 <sup>a</sup>	0.64 $\pm$ 0.2
G4	DEN + A1 diet	8.3 $\pm$ 3.1 <sup>b</sup>	0.90 $\pm$ 0.39 <sup>c</sup>
G5	DEN + C diet	3.8 $\pm$ 1.5	0.59 $\pm$ 0.39

A1 diet, 5% alcohol diet; C diet, Control diet; G1 and 2, 8 weeks and 10 animals; G 3-5, 12 weeks and 11 animals; \*Mean  $\pm$ SD

<sup>a</sup>P<0.05 vs G4 <sup>b</sup>P<0.0001 vs G5 <sup>c</sup>P<0.05 vs G5

**Table 2. PCNA labeling indices**

Group	Treatment	PCNA (No./100 cells)	
		Surrounding area	GST-P foci
G1	DEN + Al diet	1.6 ± 0.8*	14.5 ± 5.9 <sup>a</sup>
G2	DEN + C diet	2.4 ± 0.6	6.7 ± 4.1
G3	DEN + Al diet + C diet	2.8 ± 0.4	9.5 ± 2.6 <sup>b</sup>
G4	DEN + Al diet	2.7 ± 0.8	18.6 ± 2.8 <sup>c</sup>
G5	DEN + C diet	3.0 ± 0.7	9.9 ± 4.04

Al diet, 5% alcohol diet; C diet, Control diet; \*Mean ±SD  
<sup>a</sup>P<0.05 vs G2 <sup>b</sup>P<0.0001 vs G4 <sup>c</sup>P<0.05 vs G5

**Table 3. Data for IGF Levels and ODC Activity**

Group	Treatment	IGF-1 level	ODC activity
		(ng/ml)	(cpm/mg protein)
G1	DEN + Al diet	2.9 ± 1.2*	50.8 ± 32.2
G2	DEN + C diet	3.7 ± 1.3	37.1 ± 22.7
G3	DEN + Al diet + C diet	3.3 ± 1.2	101.7 ± 37.1
G4	DEN + Al diet	2.6 ± 0.8	120.2 ± 37.3 <sup>a</sup>
G5	DEN + C diet	3.2 ± 1.4	81.8 ± 16.5

Al diet, 5% alcohol diet; C diet, Control diet; \*Mean ±SD  
<sup>a</sup>P<0.05 vs G5

GST-P and PCNA are summarized in Table 2. Cell proliferation indices were expressed as number of PCNA-positive cells per 100 hepatocytes. Double staining of GST-P and PCNA revealed a significant increase in number of PCNA-positively stained nuclei within the area of GST-P positive foci in the group given alcohol continuously, as compared to control rats. After withdrawal of alcohol after 6 weeks of administration in group 3, the PCNA index significantly dropped within the area of GST-P positive foci as compared to that observed with rats given 5% alcohol diet continuously (group 4).

#### Serum IGF-1 levels

The level of serum IGF-1 was lower in groups 1 and 4, administered Al diet continuously for 6 and 10 weeks, than in groups 2 and 5, respectively, given the C diet. In group 3, however, values were similar to those in group 5 animals (Table 3).

#### Ornithine decarboxylase activity

ODC activity in the livers of rats administered 5% Al diet for 10 weeks (group 4) was significantly elevated over the control level (group 5). However, this was not the case 4 weeks after stopping Al diet administration at experimental week 12 (group 3) (Table 3).

## Discussion

In the relation of alcohol and hepatocarcinogenesis, it has been reported that interruption of long-term alcohol administration enhances hepatocarcinogenesis and this effect may be closely related to the activation of cell proliferation due to this interruption (Tanaka et al., 2000). In the present study, we applied a medium-term rat liver bioassay (Ito test) for the investigation of the short-term alcohol administration

in male F344 rats, but could not find any promoting effects after short-term alcohol treatment on hepatocarcinogenesis. While numbers and areas of GST-P positive foci in rats given 5% alcohol liquid diet continuously were higher than in the livers of control animals, they returned towards normal after stopping short-term alcohol administration.

Cellular proliferation has been shown to be significantly induced in the rat liver due to the alcohol application after initiation of hepatocarcinogenesis with MeIQx (Karim et al., 2003; Kushida et al., 2005). In line with our previous results, in the present study, PCNA indices within the areas of GST-P foci were significantly increased in the group given alcohol continuously for 10 weeks. Again, however, values after return to the normal diet were similar to those in controls.

ODC activity is high in carcinoma tissues induced experimentally by chemical carcinogens and also in tumors (Lamuraaglia et al., 1986; Furihata et al., 1987; Mori and Yamaguchi, 1989; Berdinskikh et al., 1991). In this study, ODC activity in the liver of rats given alcohol continuously was significantly elevated over the control level. However, as with PCNA labeling, the nucleotide synthesis enzyme activity in the rats after interrupted short-term alcohol administration did not differ from control 4 weeks after the removal of alcohol liquid diet. Our data concerning the increase of PCNA-positive cells by alcohol administration are in line with the elevation of ODC activity and again show no promoting effects of cessation of alcohol exposure.

IGF-1 is an anabolic hormone that exerts a strong mitogenic effect and has been shown to be effective for therapy of trauma-induced hypermetabolic response by improving wound healing, and gut and immune functions (Strock et al., 1990; Huang et al., 1993; Clemmons, 1994; Martin, 1997). In this study, serum IGF-1 level in the group given 5% alcohol liquid diet continuously for 10 weeks was reduced as compared with control group. In contrast, when the short-term alcohol administration was interrupted following by control diet for 4 weeks, IGF-1 concentrations were similar to control values.

In conclusion, the finding that cessation of short-term alcohol administration had no promoting effects on development of GST-P foci formation, suggests that effects of cessation of alcohol on rat hepatocarcinogenesis may be dependent on the duration of alcohol treatment. The results also imply the existence of a cumulative exposure time or dose threshold for alcohol if promoting effects of cessation are to be exerted on rat hepatocarcinogenesis.

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