The Enhancing Effect of Ethanol on the Development of Glutathione S-Transferase Placental Form-Positive Foci Induced by Diethylnitrosamine in F344 Rat*

Kyung-Ja. Cho, Na-Hye Myong and Ja-June Jang

Department of Anatomical Pathology, Korea Cancer Center Hospital

The effects of ethyl alcohol and pig serum administration on the development of preneoplastic hepatic enzyme-altered foci were examined in an in vivo mid-term assay system. Rats were initially given a single dose (200mg/Kg) intraperitoneal injection of diethylnitrosamine (DEN). Two weeks later, treatment was started with 10% ethanol+10% sucrose solution, 10% sucrose solution, or tap water as drinking water for 6 weeks with or without intraperitoneal injection of porcine serum twice a week. All rats were subjected to a two-thirds partial hepatectomy at week 3. The modification potentials were evaluated by comparing the number and area per cm² of glutathione S-transferase placental form-positive (GST-P⁺) foci in the liver of each group. As a result, ethanol significantly enhanced the development of GST-P⁺ foci. Unfortunately, the porcine serum injection produced no hepatic fibrosis and no significant alteration in GST-P⁺ foci.

Key Words: Ethanol, Porcine serum, DEN, GST-P⁺ foci

INTRODUCTION

Alcohol is one of the most important toxins consumed regularly by humans. A number of epidemiologic studies have shown the associations between heavy alcohol drinking and cancers of various organs. However, effects of ethanol on experimental chemical carcinogenesis, especially hepatic carcinogenesis, have been reported as variable, and the actual action of alcohol on hepatic carcinogenesis is not precisely known (Seitz & Simanowski, 1988). The association of liver cirrhosis and hepatocellular carcinoma has been rather well-documented, and not only the initiating action of the hepatotropic viruses but also fibrosis itself is thought to play a role, because various forms of noninfectious cirrhosis can also predispose the occurrence of liver cell carcinoma in humans. Hepatic

fibrosis without hepatocytic regenerating activity could be experimentally produced by immunologic mechanism with repeated intraperitoneal injections of heterologous serum (Paronetto & Popper, 1966). In the present experiment, porcine serum was used to produce hepatic fibrosis, and the effects of ethanol ingestion and hepatic fibrosis on the development of DEN-induced GST-P⁺ foci in rat liver were examined.

MATERIALS AND METHODS

The experimental schedule for this study is shown in Figure 1. Seven-week-old F344 male rats (Korea Research Institute of Chemical Technology) were divided into six groups and initially given a single dose (200mg/Kg) of DEN (Tokyo Chemical Industry Co., Japan) intraperitoneally. After two weeks on a basal diet, 10% ethanol (Ferak, Germany)+10% sucrose solution, 10% sucrose solution, or tap water was supplied as drinking water to each two group, respectively, for 6 weeks. One of each two group was treated with regular intraperitoneal injections of 0.5ml of porcine serum (JR Scientific, Calif., USA) twice a week during

Address correspondence to: K.J. Cho, Department of Anatomical Pathology, Korea Cancer Center Hospital, Gongneung-dong 215-4, Nowon-ku, Seoul 139-240, Korea
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Fig. 1. Experimental Schedule

Animal: F344 male rats, 7-week-old (Total 67)

▽ : DEN 200mg/kg i.p.

▼ : Partial hepatectomy

▨ : 10% ethanol + 10% sucrose solution

▤ : 10% sucrose solution

↓ : Porcine serum 0.5ml/rat i.p.

() : Number of rats

the experimental period, except for one time after three injections due to the poor general condition and increasing mortality rate of the animals. The average consumption of the ethanol+sucrose solution was 264.1 ± 25.1 ml and 273.1 ± 22.9 ml per 100 g of body weight, respectively, which was equivalent to 20.86 ± 1.98 g and 21.57 ± 1.81 g of ethyl alcohol in Groups 1 and 3, respectively. All rats were subjected to a two-thirds partial hepatectomy at week 3 and sacrificed at week 8. All the rats that survived until week 8 were considered as effective number of rats. They were fed a standard pellet diet *ad libitum* and made to fast for 18 hours before being killed. Body weights were measured biweekly.

Immediately upon killing, the liver was resected and weighed. Three 2-3mm thick slices were taken from the right posterior, anterior and caudate lobes, respec-

tively, and fixed in ice-cold acetone. They were processed for embedding in paraffin, sectioning, and Hematoxylin-Eosin, van-Gieson and immuno-histochemical staining for GST-P⁺ foci by the ABC method. Rabbit anti-GST-P antibody was kindly provided by Professor Kiyomi Sato, Second Department of Biochemistry, Hirosaki University School of Medicine. The numbers and areas of GST-P⁺ foci of more than 0.2mm diameter were measured with an image analyzer (VIDAS, Kontron Ltd., Germany) and expressed as number/cm² or area (mm²)/cm². Statistical analyses were performed by *t*-test employing the Cochran and Cox method. For electron microscopy, three representative blocks of the livers and kidneys in each group were subjected to fixation in 2.5% glutaraldehyde and subsequent processings.

RESULTS

The survival rates of rats in each group ranged from 66.7 to 100%. The survival of animals given 10% ethanol and/or porcine serum tended to be lower.

The mean body weights of the rats at the beginning and end of the experiment showed no significant variation among the groups. The mean relative (% body weight) liver weights were lower in Groups 2 and 4 (Table 1).

On histologic examination, suspicious preneoplastic foci composed of clear or eosinophilic cells were occasionally observed in all groups. Peculiarly, the livers of rats that were given intraperitoneal porcine serum injections 11 times revealed no demonstrable fibrosis on H-E and van-Gieson stained sections. Instead, two cases in Group 1 showed marked bile duct proliferation and severe portal inflammation.

Quantitative values of GST-P⁺ foci are shown in Table 2. The number of foci per unit area was greater in Group 1 than in Groups 2 and 5, and in Group 3 than in Group 4, showing a stimulatory effect of

Table 1. Mean Body and Liver Weights (g)

Group	Effective No. of Rats	Body Weight		Liver Weight	L.W./100g B.W.
		Initial	Final		
1	10	132.41 ± 12.15	231.93 ± 28.92	6.50 ± 1.48	2.83 ± 0.69
2	14	136.25 ± 12.88	253.49 ± 23.84	5.89 ± 1.42	$2.30 \pm 0.41^*$
3	10	138.92 ± 12.05	253.87 ± 23.06	7.08 ± 0.87	2.80 ± 0.36
4	10	140.40 ± 14.27	236.74 ± 19.63	5.27 ± 1.15	$2.13 \pm 0.40^{**}$
5	12	141.53 ± 15.23	235.50 ± 28.39	5.87 ± 1.23	2.48 ± 0.35
6	11	133.19 ± 13.41	237.10 ± 26.69	6.11 ± 1.25	2.57 ± 0.45

* Significantly different from Group 1 at $p < 0.05$

** Significantly different from Groups 3 & 5 at $p < 0.001$ & $p < 0.05$

Table 2. Quantitative Values of GST-P⁺ Foci

Group	Effective No. of Rats	Number/cm ²	Area (mm ²)/cm ²
1	10	9.80 ± 4.65 ^a	0.40 ± 0.18 ^b
2	14	6.24 ± 3.29 ^c	0.29 ± 0.18
3	10	6.78 ± 3.51 ^d	0.30 ± 0.14 ^e
4	10	3.41 ± 1.83	0.17 ± 0.09
5	12	3.79 ± 1.69	0.24 ± 0.10
6	11	4.41 ± 1.91	0.22 ± 0.10

^a Significantly different from Groups 2 & 5 at $p < 0.05$ & $p < 0.001$

^b Significantly different from Group 5 at $p < 0.02$

^c Significantly different from Group 4 at $p < 0.05$

^d Significantly different from Group 4 at $p < 0.02$

^e Significantly different from Group 4 at $p < 0.05$

ethanol. The increasing tendency of the number of foci in the porcine serum-treated groups was significant only between Groups 2 and 4. The area of foci in Groups 1 and 3 was significantly larger than in Groups 5 and 4, respectively, suggesting an enhancement by ethanol, but the difference between Groups 1 and 2 was insignificant. Sucrose treatment revealed no significant effect.

An ultrastructural study revealed no immunoglobulin deposit or other evidence of immune reaction in the kidney and liver.

DISCUSSION

This study was initially planned to examine the effects of ethyl alcohol and/or hepatic fibrosis inducible by heterologous serum on rat hepatic carcinogenesis. Experimentally, regular intraperitoneal injection of swine serum for more than 16 times was reported to be successful in producing collagenous septa linking the central-central or central-portal area without hepatocytic change in rats (Rubin et al., 1968) and mice (Paronneto & Popper, 1966), with a co-product of mesangial immunoglobulin deposits in the renal glomeruli (Kitamura et al., 1984). We used porcine serum for this purpose with the same method of application, but failed to develop hepatic fibrosis or an immune reaction. For a reason of this result, the total dose of porcine serum must have been insufficient for the development of fibrosis. Although the histologic changes in the liver of porcine serum-treated rats proved to be insignificant, the general condition of these rats rapidly deteriorated, especially with ethanol administration, requiring a transient cessation of the treatment. This might have been the result of partial hepatectomy, poor oral intake or dehydration. Another

problem in the experiment was the method of ethanol administration. We tried mixing a 10% ethanol and 10% sucrose solution for better drinking but could not guarantee adequate ethanol intake, sufficient hydration and nutritional support. The liquid diet feeding technique would be more ideal, because the total amount of ethanol intake and nutritional status of the animals might influence the effect of the ethanol.

In this study, ethanol significantly increased the number of GST-P⁺ foci, but an increase in the area was inconsistent among the groups. The number of preneoplastic lesions possibly indicates the potency of the initiating activity of chemicals, while the area or size of the lesion seems more related to the intensity of the promoting activity of chemicals in liver carcinogenesis. Thus the present effect of ethanol might be suspected of potentiating the initiating activity of DEN. The previously reported effects of alcohol on hepatic carcinogenesis were variable, which were thought by some workers to be related to the time of alcohol administration (Seitz & Simanowski, 1988). When alcohol was applied prior to the carcinogen, carcinogenesis was not affected or inhibited (Teschke et al., 1983), or stimulated (Radike et al., 1977). Simultaneous ethanol treatment has been reported to inhibit (Griciute et al., 1981 & 1986; Habs & Schmahl, 1981), stimulate (Schwarz et al., 1983), or not affect hepatocarcinogenesis by nitrosamines (Weisburger et al., 1964). The effects were diverse as well when ethanol was given during promotion (Driver & McLean, 1986; Ikawa et al., 1986; Misslbeck et al., 1984; Porta et al., 1985; Takada et al., 1986; Yanagi et al., 1989). So it cannot be concluded that there is any consistent relationship between the effects and the time of application of alcohol.

The possible mechanisms by which ethanol enhances carcinogenesis include activation of procarcinogen by microsomal enzyme induction, direct hepatocellular injury, inhibition of the repair of carcinogen-induced DNA alkylation, stimulation of cel-

lular regeneration, and exacerbation of dietary deficiencies. Among them the microsomal enzyme induction demonstrates the most important and complicated role of alcohol in carcinogenesis. A variety of chemical procarcinogens is increasingly activated following chronic ethanol ingestion with the appearance of a distinct form of cytochrome P-450 (Yang et al., 1985), but detoxifying enzymes of the same chemicals are also inducible by ethanol. Ethanol even competitively inhibits procarcinogen activation in liver, and alters the organ specificity, resulting in the concomitant stimulation of extrahepatic tumor development. In vitro biochemical studies revealed both enhancement (Garro et al., 1981; Gellert et al., 1980; Maling et al., 1975; Sato et al., 1986; Schwarz et al., 1980) and competitive inhibition (Peng et al., 1982) of microsomal metabolism of nitrosamines after pretreatment with ethanol. Thus, what is most important in the relationship between the enzyme activity induced by ethanol and carcinogenesis is the actual level of the activated carcinogen.

It is not considered that hepatic carcinogenesis is directly related to the enzyme induction, because high-dose phenobarbital, a potent inducer of P-450b-e which also activates various nitrosamines, is weaker in its tumor-promoting effect than ethanol (Driver & McLean, 1986). Phenobarbital is considered to act as a pure promoter being negative in all mutagenicity tests, but it is not clear whether alcohol exhibits its stimulatory effects as a promoter or co-carcinogen (Schwarz et al., 1983). Alcoholic hepatocellular injury, especially cirrhosis, might play an important role in human liver carcinogenesis. However, rat is known to be unsusceptible to ethanol-induced cirrhosis, and even fatty metamorphosis could hardly be observed in the present study. Inhibition of hepatocytic regeneration by acute and chronic ethanol administration has been described (Wands et al., 1979), and this action might influence carcinogenesis in a bidirectional way in the sense of interference of repair or proliferation.

In summary, the action of ethanol on chemical carcinogenesis is very complex, and our results permit us to state that ethanol enhanced the development of GST-P⁺ foci induced by DEN in rats. A further exploration is required to document the exact role of alcohol on the carcinogenesis of various organs.

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