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Enhancing Effect of Ethanol on Aflatoxin B_1-induced Hepatocarcinogenesis in Male ACI/N Rats

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The modifying effect of ethanol (EtOH) on aflatoxin B_1 (AFB_1)-induced hepatocarcinogenesis was examined in male ACI/N rats by chronic treatment at the post-initiation phase. Rats received an ip injection of AFB_1 (1.5 mg/kg) twice a week for 10 weeks (a total of 20 doses). Following a week of acclimation, they were given 10% EtOH as drinking water for 56 weeks. The effect of EtOH on the hepatocarcinogenesis was evaluated in terms of the incidence of altered hepatocellular foci and neoplasms at the end of the experiment. Exposure to AFB_1 alone induced a substantial number of altered foci (6.98 iron-excluding foci/cm^2) in rats. The number of altered liver cell foci in rats receiving AFB_1 followed by EtOH was significantly increased (26.39 iron-excluding foci/cm^2). In the rats given EtOH after AFB_1, the total area and mean diameter of both iron-excluding foci and altered foci identified in hematoxylin and eosin-stained sections were significantly higher than in the rats exposed to AFB_1 alone. The incidence of liver cell tumors of the group given AFB_1 and EtOH (3/15, 20%) was higher than that of the group treated with AFB_1 alone (0/14, 0%). Treatment with EtOH alone for 56 weeks did not induce either. These results indicate an enhancing effect of EtOH on AFB_1-induced hepatocarcinogenesis when it is given in the promotion phase.

Key words: Enhancing effect — Ethanol — Hepatocarcinogenesis — Aflatoxin B_1 — Rats

Epidemiological studies have suggested that chronic alcohol consumption is associated with an increased risk of cancer development in various organs such as the liver and upper digestive organs.[1-3] In experimental studies, no clear evidence that ethanol (EtOH) is carcinogenic has been obtained,[4-6] although there are some reports showing its co-carcinogenic or tumor-promoting action.[2,5] The mechanism of the modifying effect of EtOH on the tumor development is still not clear. In liver carcinogenesis, conflicting results on the effect of EtOH on tumor development have been reported.[7-15] Schwarz *et al.*[11] suggested that the effect of EtOH on liver carcinogenesis depends on the time schedule of administration. They reported that the incidence of preneoplastic hepatocellular lesions increased when EtOH was given during carcinogen treatment and it decreased when EtOH was given after cessation of carcinogen exposure. However, Takada *et al.*[14] found that EtOH had a promoting action in diethylnitrosamine (DEN)-induced hepatocarcinogenesis.

To determine the modifying effects of EtOH on tumorigenesis by possible human carcinogens is clearly important. In this study, the effect of EtOH on aflatoxin B_1 (AFB_1)-induced liver carcinogenesis was examined in rats. AFB_1 is a potent liver carcinogen in various experimental animals,[16] and has recently been classified as a human carcinogen on the basis of epidemiological studies in exposed human populations as well as experimental data.[17]

MATERIALS AND METHODS

Male inbred ACI/N rats, which have been maintained as an inbred strain in our laboratory, were used. At 6 weeks of age, these rats were transferred to the holding room and randomized into experimental and control groups. Rats were housed 3 or 4 to a wire cage. The holding room was maintained at 23±2°C, 50±10% humidity, and a 12-h light/12-h dark cycle.

A total of 54 rats were divided into four groups as shown in the table. The protocol in this study was as shown in Fig. 1. Fourteen rats in group 1 and 15 rats in group 2 were given AFB_1 (Aldrich Chemical Co., Milwaukee, WI) freshly dissolved in dimethyl sulfoxide (DMSO, Aldrich Chemical Co.) by ip injection twice a week for 10 weeks as a single dose of 1.5 mg/kg body weight (total dose: 30 mg/kg body weight). Each rat received a volume of 0.2 ml of DMSO per dose. The injections were done between 10:00 and 11:00 am. Rats in group 2 were given EtOH (10% in the drinking water) for 56 weeks. Rats in group 3 (15 rats) were given DMSO by ip injection and administered EtOH for 56 weeks. Animals in group 4 (10 rats) were given the DMSO vehicle alone according to the same schedule as

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for group 1 and served as controls. Rats in groups 1 and 4 were given tap water throughout the experiment. All animals were maintained on a basel diet, CE-2 (CLEA Japan Inc., Tokyo).

The animals were carefully observed and killed by decapitation at 67 weeks after the start of the experiment in order to evaluate the incidence of preneoplastic and neoplastic lesions of the liver. For the demonstration of iron-excluding liver lesions, all rats were iron-loaded by sc injections of 12.5 mg elemental iron/100 g body weight (Ferrimicrodex brand, 100 mg elemental iron/ml) in the inguinal regions, alternating sides three times a week for 2 weeks prior to killing.[18] Complete necropsies were performed on all animals. At necropsy, the livers were removed and weighed, and slices (2 mm thick) were taken from each sublobe. All organs were fixed in 10% buffered formalin. All tissues and gross lesions were processed for histology by the conventional methods and stained with hematoxylin and eosin (H & E). Two serial liver sections were cut and one was stained with H & E while the other was reacted for iron by the Prussian blue technique. Liver cell foci and neoplasms were quantified by using the histologic criteria of the Institute of Laboratory Animal Resources.[19] The incidence of altered hepatocellular foci was quantified on the H & E- and Prussian blue-stained sections using a microscope and expressed as number of foci/cm^2. For morphometric analysis of altered hepatic cell foci (>0.15 mm diameter), a color image analyzer (CA-102, Olympus Optical Ind. Co., Tokyo) was used. The average focal area was expressed as (mm^2)/cm^2. Differences between measurements made in the groups were tested for significance by using Student's t test. The incidence of liver cell tumors (neoplastic nodules and carcinomas) was compared using Fisher's exact probability test.

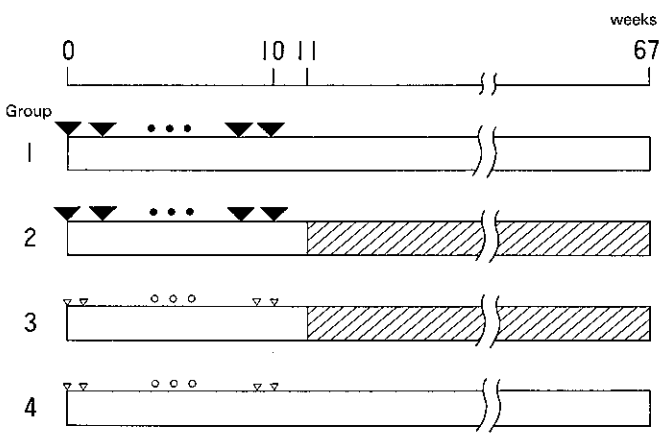

Fig. 1. Experimental protocol. ▼: AFB_1, ip injection (1.5 mg/kg body weight) twice a week for 10 weeks. ▽: DMSO (0.2 ml), ip injection. ▨:10% ethanol as drinking water.

RESULTS

The mean daily and cumulative intakes of EtOH were 9.20±1.80 ml/rat and 3543±684 ml/rat in group 2 and 9.73±2.46 ml/rat and 3692±891 ml/rat in group 3. The intake of EtOH tended to be lower in rats of group 2 but no clear differences were seen between groups 2 and 3. The average body weight gain and relative liver weight in each experimental group at the end of the experiment are shown in Table I. Body weight gain curves for all groups were similar. Administration of AFB_1 in group 1 slightly increased liver weights compared to the controls in group 4. The liver weights and relative liver weights in groups 2 and 3 were slightly decreased when compared to groups 1 and 4, respectively.

The ip administration of AFB_1 induced a substantial number of altered liver cell foci detectable by iron-exclusion and H & E staining (Table II). At the end of the study, an average of 6.98 iron-excluding foci/cm^2 was present in group 1, but there were no liver cell neoplasms. In group 2 treated with AFB_1 and EtOH, the number of iron-excluding foci (28.44 foci/cm^2) was significantly increased when compared to group 1 treated with AFB_1

Table I. Body and Liver Weights of ACI/N Rats Treated with AFB_1 and Ethanol (EtOH) at the End of the Experiment

Group no.	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AFB_1	14	270±42[a]	9.6±1.2	3.66±0.72
2	AFB_1/EtOH	15	268±48	9.2±1.9	3.51±0.82
3	DMSO/EtOH	15	265±42	8.2±1.7	3.11±0.47
4	DMSO	10	263±24	8.7±1.3	3.38±0.61

a) Mean±SD.

Table II. Effect of Ethanol (EtOH) on Hepatocarcinogenesis Induced by AFB_1

Group no.	Treatment	No. of rats	Preneoplastic liver cell lesions: Iron-excluding: No. of foci (/cm²)	Iron-excluding: Mean diameter (mm)	Iron-excluding: Total area (mm²/cm²)	H&E-stained section: No. of foci (/cm²)	H&E-stained section: Mean diameter (mm)	H&E-stained section: Total area (mm²/cm²)	No. of rats with liver cell tumors: NN[a]	No. of rats with liver cell tumors: HCC
1	AFB_1	14	6.98[b] ±0.91	0.24 ±0.07	0.32 ±0.10	7.63 ±0.80	0.25 ±0.05	0.38 ±0.13	0	0
2	AFB_1/EtOH	15	26.39[c] ±10.33	0.34[c] ±0.13	2.38[c] ±0.41	28.44[c] ±15.29	0.37[c] ±0.12	2.60[c] ±0.39	2 (3)[d]	1 (1)
3	DMSO/EtOH	15	0.11 ±0.21	0.10 ±0.04	0.01 ±0.01	0.10 ±0.11	0.11 ±0.04	0.01 ±0.02	0	0
4	DMSO	10	0.09 ±0.11	0.10 ±0.05	0.01 ±0.01	0.08 ±0.10	0.10 ±0.04	0.01 ±0.01	0	0

a) NN=neoplastic nodules; HCC=hepatocellular carcinomas.
b) Mean±SD.
c) Significantly different from group 1 by Student's *t* test ($P<0.001$).
d) Numbers in parentheses are numbers of tumors.

alone ($P<0.001$). There were a few iron-excluding foci in groups 3 and 4. In H & E-stained sections, similar incidences of foci were obtained in each group (Table II). Similarly, in group 2, given EtOH after AFB_1, the total area and mean diameter of both iron-excluding foci and altered foci identified in H & E-stained sections were significantly increased in comparison with group 4 (Table II). The incidence of hepatocellular neoplasms (neoplastic nodules and carcinomas) is also shown in Table II. In group 2, liver cell neoplasms had developed in rats with a low incidence (3/15, 20%). There were no hepatocellular neoplasms in rats of groups 1, 3 and 4.

Histologically, altered liver cell foci were mostly eosinophilic or of clear cell type. Almost all foci and liver cell neoplasms were iron-excluding. A hepatocellular carcinoma in a rat of group 2 was of trabecular type. In other organs, a renal cell carcinoma was found in a rat of group 1.

DISCUSSION

In the present study, the incidence of iron-excluding liver cell foci, which are considered to be precursors of liver cell neoplasms,[20,21] in the liver of rats given AFB_1 followed by EtOH was significantly higher than that of animals that received AFB_1 alone. However, the number of hepatocellular foci detected by H & E staining and the incidence of liver cell neoplasms in rats of group 2 were larger than those of group 1, though the differences were not statistically significant. These results indicate that EtOH has a weak enhancing effect on the hepatocarcinogenesis induced by AFB_1.

Similar results have been obtained by Takada *et al.*[14] showing that 20% EtOH increased the number of enzyme-altered foci in male Wistar rats pretreated by partial hepatectomy and DEN administration. However, other reports on the effect of EtOH on the carcinogen-induced preneoplastic and neoplastic liver lesions induced by various carcinogens have provided conflicting results as shown in Table III. Misslbeck *et al.*[13] found no significant effect of EtOH on the formation of γ-glutamyltranspeptidase (GGT)-positive foci induced by AFB_1 in male Sprague-Dawley rats fed a low- or high-fat diet when EtOH was given in the post-initiation phase. However, rats fed EtOH in a high-fat diet had an increased number and size of GGT-positive foci compared to rats on EtOH in a low-fat diet. They postulated that a positive correlation between the development of GGT-positive foci and high fat intake may be due to the effect of peroxidases. The differences between the results in the present study and those reported by Misslbeck *et al.*[13] may be due to the different experimental schedule, diets used, species difference, dose, etc.[7-15]

The mechanism of the enhancing effect of EtOH on hepatocarcinogenesis as proved here is not clear. Nevertheless, several possible mechanisms for such an enhancing effect of EtOH have been proposed[1-3,22-26]: (a) liver cell injury by EtOH; (b) activation of chemical carcinogens by EtOH, altering the metabolism and/or distribution of carcinogens; (c) interference of EtOH with the repair of carcinogen-mediated DNA alkylation; (d) interference with the immune response; (e) exacerbation of dietary deficiency; (f) the "preneoplasia" hypothesis involving EtOH in Mallory body pathogenesis, which may

Table III. Summary of Studies Showing Modifying Effect of Ethanol (EtOH) on Carcinogen-induced Liver Carcinogenesis

Carcinogen	Species	Time of EtOH treatment[a]	Results	Reference
Vinyl chloride	Rat	Before, during and after	(↑)[b]	Radike *et al.* (7)
DMN[c]	Mouse	During	(−)	Griciute *et al.* (8)
DEN	Rat	After	(↓)	Habs & Schmähl (9)
Vinyl chloride	Rat	Before, during and after	(↑)	Radike *et al.* (10)
DEN	Rat	During or after	(↑) or (−)	Schwarz *et al.* (11)
DMN	Rat	Before	(−)	Teschke *et al.* (12)
AFB_1	Rat	After	(−)	Misslbeck *et al.* (13)
DEN	Rat	After	(↑)	Takada *et al.* (14)
DEN	Rat	After	(↓)	Ikawa *et al.* (15)
AFB_1	Rat	After	(↑)	Present study

a) Time of EtOH treatment: before, during or after the carcinogen exposure.
b) (↑), enhancing effect; (↓), inhibiting effect; (−), no effect.
c) DMN, dimethylnitrosamine; DEN, diethylnitrosamine; AFB_1, aflatoxin B_1.

reflect genetic control of hepatotumorigenesis. In the present study, Mallory bodies were not observed in the lesions indicating that the enhancing effect of EtOH on AFB_1-induced hepatotumorigenesis is probably not related to Mallory bodies. Furthermore, the body weight of rats given 10% EtOH and DMSO was almost the same as that of rats given DMSO alone. This appears to show no negative nutritional impact of EtOH at this dose. EtOH did not induce distinctive toxic changes such as fatty metamorphosis of hepatocytes or cirrhosis in the livers of rats in group 3. Recent reports have demonstrated that EtOH enhances the metabolism of AFB_1[27,28] and AFB_1-induced hepatotoxicity.[29] It has also been proved that EtOH acts as an inducer of the microsomal cytochrome P-450-dependent biotransformation system.[30,31] AFB_1 is known to require metabolic activation by cytochrome P-450s, which are efficient catalysts of AFB_1 8,9-epoxidation to elicit its toxic and carcinogenic effects.[32] It seems likely that the enhancing effect of EtOH as shown in the present study is due in some part to a change in the balance between enzymatic activation and detoxification of the carcinogen, since the interval of 7 days between AFB_1 exposure and commencement of administration of EtOH was relatively short in order to permit remission of AFB_1-induced molecular damage. However, the precise mechanism of the enhancing effect of EtOH on AFB_1-induced liver carcinogenesis needs to be further investigated.

EtOH is reported to be weakly mutagenic or non-mutagenic to bacteria.[33] In eukaryotic (including mammarian) cells, it shows clastogenic activity under conditions where its biotransformation to acetaldehyde occurs.[33] In the present study, exposure to EtOH alone produced a low incidence of preneoplastic liver lesions without development of hepatocellular neoplasms. Accordingly, it appears that EtOH may act as a weak promoter of hepatocarcinogenesis under the conditions employed in this study.

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