GASTROENTEROLOGY

Effect of chronic dietary ethanol in the promotion of *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis in rats

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Abstract

Background: The pathogenetic correlation between chronic alcohol consumption and development of esophageal cancer is not clear. The role of alcohol abuse in the carcinogenic action of *N*-nitrosomethylbenzylamine, which induces tumors in the esophagus, has been evaluated.

Methods: Twenty male rats were fed liquid diets containing ethanol or carbohydrates for 30 weeks. *N*-nitrosomethylbenzylamine (0.1 mg/kg, twice a week) was injected i.p. from the 9th to 19th week. The pair feeding was stopped at 9.00 AM and *N*-nitrosomethylbenzylamine was administered at 10.00 AM. Ethanol was not detected in the blood at the time of injection. Liquid diets were provided again at 3 PM until 9 AM next day. The animals were killed at the end of the 30th week. The esophagi were collected and examined for visible tumors. The tissue sections were stained for histology and CYP2E1 expression.

Results: While 5–8 esophageal squamous polyps developed in all rats in the ethanol group, only one polyp each was formed in five out of the 10 rats in the control group. The size of the polyps was significantly larger in the ethanol group, when compared to the control group. Invasive squamous cell carcinoma was also observed in 50% of the animals in the ethanol group. Cytochrome P4502E1 (CYP2E1) staining demonstrated marked expression in the esophageal mucosa in the ethanol group, but not in the control group.

Conclusions: The increased expression of CYP2E1 induced by chronic ethanol consumption promotes the development of *N*-nitrosomethylbenzylamine-induced esophageal tumorigenesis. However, the molecular mechanism of the increased production of esophageal tumors during alternative administration of *N*-nitrosomethylbenzylamine and ethanol is not clear.

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INTRODUCTION

Chronic alcohol consumption is considered to be one of the major risk factors for the development of esophageal cancer in humans.[1–5] A positive association between chronic ethanol consumption and enhanced cancer risk has been demonstrated by epidemiological studies.[6,7] The major targets of alcohol-associated cancers are oral cavity, esophagus and liver.[8] Approximately 75% of the esophageal cancers in the USA are attributable to excessive alcohol intake.[9] The relative risk factor of esophageal cancer in Japan is 16-fold higher in alcoholic subjects.[10]

Ethanol is mostly metabolized to acetaldehyde by the enzyme alcohol dehydrogenase with the oxidative transfer of the hydrogen of the ethanol. Xanthine oxidase oxidizes the acetaldehyde formed by ethanol metabolism and generates free radicals.[11,12] Because oxygen free radicals possess an unpaired electron, they are highly reactive. It is well known that ethanol increases the

production of superoxide anions and hydroxyl radicals, which react rapidly with biological materials, causing oxidative damage to the biological system both in humans and in ethanol-treated experimental animals.

It is generally assumed that ethanol itself is not carcinogenic. Its carcinogenic effects may be attributable to the metabolic modification of other carcinogens into more active forms through its metabolic internediates. Many chemical entities are carcinogenic or toxic but require metabolic activation or modifications *in vivo* to form the ultimate carcinogens or toxins.[13] Conversion of procarcinogens into carcinogens occurs through the drug-metabolizing system of the microsomes, the cytochrome P450-mediated monooxygenase system.[14,15] It has been demonstrated that the liver drug-metabolizing enzyme system cytochrome P4502E1 (CYP2E1) is induced by long-term consumption of ethanol.[16–18] The induction of CYP2E1 by ethanol is considered to be one of the reasons for the high frequency of gastrointestinal cancers in alcoholic patients.[19]

Cytochrome P4502E1 plays a key role in the toxicity of drugs, solvents, and environmental procarcinogens through its extensive capacity to convert such compounds into reactive intermediates that can elicit organ damage or tumorogenesis.[14,20] Because CYP2E1 has low Km forms of *N*-nitrosodimethylamine demethylase[21] and *N*-nitrosomethylbenzylamine debenzylase,[22] CYP2E1 is responsible for the metabolism of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosomethylbenzylamine (NMBA) that are common environmental contaminants, present in tobacco products, some food and beverages and also formed *in vivo*. It has been demonstrated that CYP2E1 is present not only in the liver but also in the gastrointestinal tract, including the esophagus.[22] The induction of esophageal CYP2E1 during chronic ethanol consumption may promote the development of esophageal cancer. It has been well documented that NMBA induces tumors in the esophagus.[23,24] In the present investigation we have evaluated the role of chronic alcohol consumption in the promotion of the carcinogenic action of NMBA, which incites tumors in the esophagus.

METHODS

Chemicals

N-nitrosomethylbenzylamine was purchased from Nacalai tesque, Kyoto, Japan. High-grade absolute ethanol was procured from Wako Pure Chemical Industries, Osaka, Japan. Immunohistochemical kit (CSA system) was obtained from Dako, Carpinteria, USA. All other chemicals were of the highest purity available commercially. Milli-Q water (Nihon Millipore, Tokyo, Japan) was used for all experiments.

Animals and treatments

The animal experimental protocol was approved by the committee for the Care and Use of Laboratory Animals,

Kanazawa Medical University. Twenty albino male rats of the Wistar strain (156 ± 15 g bodyweight) were divided into two groups of 10 rats each. One group (ethanol group) was given an ethanol-containing liquid diet (36% of total calories, 5% ethanol v/v) and the other group (control group) was pair-fed with a control diet in which ethanol was replaced isocalorically with glucose for 8 weeks as described previously.[25] Subsequently, NMBA was injected i.p. into both the ethanol and control group animals at a concentration of 0.1 mg/kg bodyweight, twice a week for 10 consecutive weeks. The ingestion of ethanol-containing liquid diet was continued during the administration of NMBA. The animals were fed with liquid diet for another 12 weeks after the termination of NMBA injection. Figure 1 demonstrates the protocol for the administration of ethanol and NMBA to the experimental animals. During the administration of NMBA, the pair feeding with liquid diet was stopped at 9.00 AM and NMBA was injected at 10.00 AM. Prior to the injection of NMBA, blood sample was collected under anesthesia from the orbital sinus of the animal using a heparinized capillary tube and analyzed for the presence of ethanol by headspace capillary gas chromatography.[26] Ethanol was not detected in the blood at the time of NMBA administration. Liquid diet was again provided for the animals at 3.00 PM until 9.00 AM the next morning.

Procedures for histopathology

All the experimental animals were killed at the end of the 30th week after anesthesia. None of the animals died during the experimental period. The esophagi were carefully removed, cut opened longitudinally and spread on a cardboard with aluminum foil. The esophagi were instantly examined, confirmed by esophageal mucosa and counted for visible tumors. The esophagi were then temporarily placed in 5% phosphate-buffered formalin solution. The esophageal tumor surface and color were again examined macroscopically, confirmed by stereoscopy, and photographed. The size of the

Whole schedule
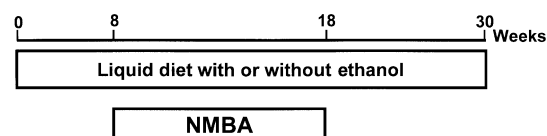
Daily schedule during NMBA injection
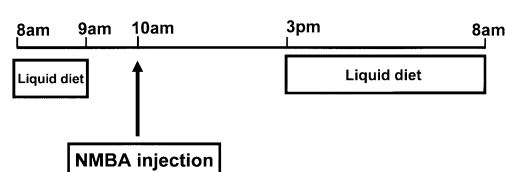
Figure 1 Experimental schedule for the administration of ethanol and *N*-nitrosomethylbenzylamine (NMBA) for the induction of esophageal carcinogenesis in rats.

visible tumors was also measured. Finally the esophagi were fixed in 10% phosphate-buffered formalin. The fixed esophageal tissue was processed in an automated tissue processor and embedded in paraffin blocks. Serial sections of 5 µm thickness were cut and stained with hematoxylin and eosin (HE). The stained sections were examined using an Olympus microscope with camera attached and then photographed.

## Immunohistochemical procedures for cytochrome P4502E1

The CYP2E1 antibody was raised and purified as described before.[27] The serial esophagus sections were deparaffinized using xylene and alcohol, hydrated to water followed by cold phosphate-buffered saline (PBS). The immunohistochemical staining for CYP2E1 was carried out using the catalyzed signal amplification system from Dako, which offers a sensitivity 20–200-fold greater than traditional avidin–biotin methods. The endogenous peroxidase activity present in the esophagus sections was first quenched by incubating the specimens with 3% hydrogen peroxide for 10 min. The specimens were washed with PBS and incubated with protein block for 30 min to reduce the non-specific protein binding. The sections were then incubated overnight at 4°C with CYP2E1 primary antibody raised in rabbit. It was then treated with a few drops of biotinylated antirabbit and antimouse immunoglobulins followed by horseradish peroxidase-labeled streptavidin and biotinyl tyramide. The color was developed using 3–3′diaminobenzidine (DAB) as substrate chromogen, which results in a brown-colored precipitate at the antigen site.

Statistical analysis

Arithmetic mean and standard deviation were calculated for the data. The results are expressed as mean $\pm$ SD. The control mean values were compared with the ethanol-treated mean values either using Student's t-test or χ^2 test. $P < 0.05$ was considered as statistically significant.

RESULTS

In order to study the effect of ethanol on the bodyweight of the experimental animals, the bodyweight was measured every week during the experiment. Figure 2 demonstrates the bodyweight changes of the experimental animals during the entire course of the study. No significant alteration was observed in the bodyweight between ethanol and control groups during the treatment period. The average daily intake of liquid diet was 80 mL/day in both alcohol and control group by pair feeding. Because the liquid diet contained 5% alcohol (v/v), the average daily intake of alcohol in the alcohol-fed group was 4.0 mL.
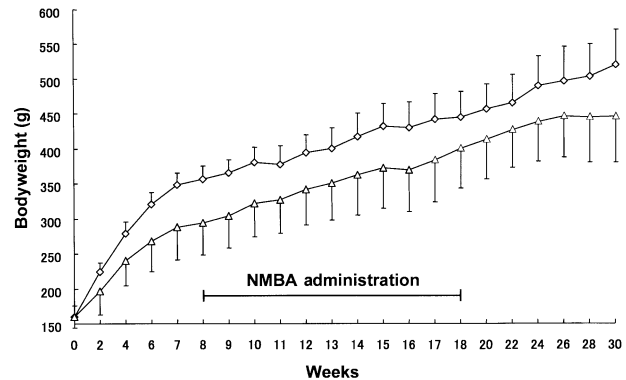

Figure 2 Bodyweight changes of the experimental animals during the course of alcohol and N-nitrosomethylbenzylamine (NMBA) administration. ($\diamond$) Control; ($\triangle$) alcohol.

Figure 3 demonstrates macroscopic and stereoscopic examination of esophagus after ethanol and NMBA administration in rats. In the control group, one visible esophageal tumor $(5.0 \pm 0.7$ mm in width) was observed in five out of the 10 rats studied (Fig. 3a). Flat small white nodules (2–3 mm in width) were observed mainly in the middle portion of the esophagus using a stereoscopic microscope (Fig. 3b). In the ethanol group, 5–8 visible tumors of 5–15 mm width $(7.3 \pm 3.6$ mm) with irregular surface and/or reddish in colour were observed in the whole esophagus in all of the rats studied (Fig. 3c,d). Many small white nodules were also observed in the whole esophagus. The total number of visible esophageal tumors that developed in the ethanol group was 58. The frequency and size of esophageal tumors in the ethanol group was significantly higher than that in the control group (Table 1).

On histopathological examination it was found that the visible esophageal tumors were squamous polyps with hyperkeratosis, acanthosis, and a prominent fibrovascular core (Fig. 4). There were no atypical changes in most of the polyps, but a few of them demonstrated mild to moderate dysplasia. The flat mucosal lesions between the polyps demonstrated areas ranging from mild to moderate dysplasia in both the ethanol and control groups. The incidence of dysplasia per rat in the ethanol group was significantly higher than that in the control group (Table 2). In the ethanol group, invasive squamous cell carcinoma was present in many areas, but there was no such lesion in the control group (Fig. 5; Table 2).

Immunohistochemical staining clearly demonstrated the presence of CYP2E1 antigen in the esophageal mucosa of the ethanol group, indicating increased expression of the enzyme in the ethanol-treated rats (Fig. 6). But the staining was very feeble or absent in the control group. Quantitative evaluation of the staining using imagepro software (Media Cybernetics, Silver Spring, MD, USA) revealed a total number of 85 positive stained cells in the picture (Fig. 6b). None of the cells was positive in the control group (Fig. 6a). The papilloma obtained from the ethanol group rats also stained strongly for CYP2E1 (Fig. 7), which indicates increased expression of the enzyme. Color computation
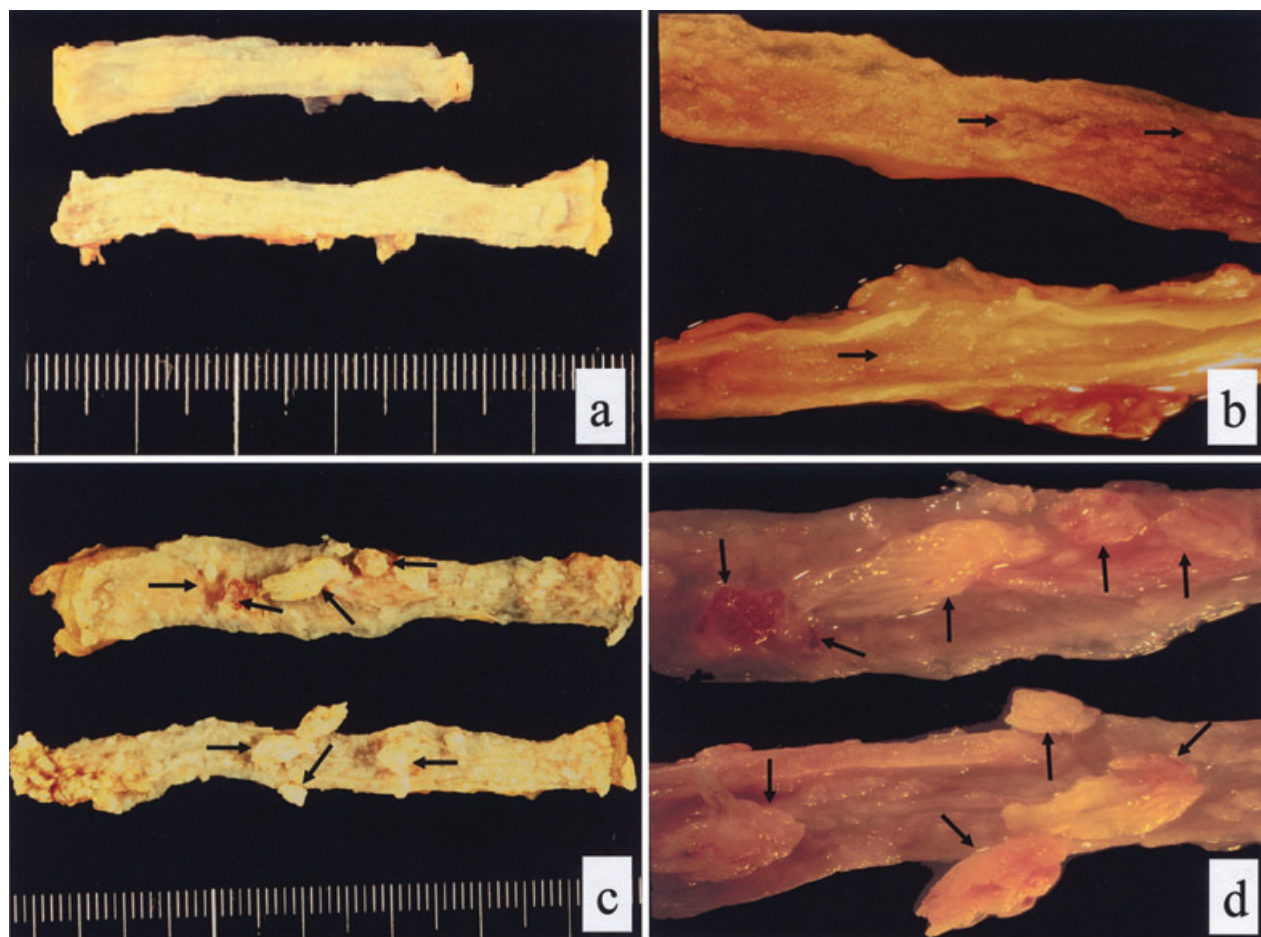
Figure 3 Macroscopic and stereoscopic examination of esophagus after ethanol and *N*-nitrosomethylbenzylamine administration in rats. (a,b) Control group. (a) Esophageal tumor was not observed by macroscopy; (b) flat small white nodules were present in middle esophagus by stereoscopy. (c,d) Ethanol group. Tumors with irregular surface and/or a reddish colour were observed in the whole esophagus by both (c) macroscopic and (d) stereoscopic examination.

Table 1 Tumors in NMBA-induced esophageal carcinogenesis in rats

Group	Incidence *n* (%)	Size (mm) (Mean ± SD)	Total no. visible tumors	Tumor incidence/rat (Mean ± SD)
Control	5/10 (50)	5.0 ± 0.7	5	0.5 ± 0.5
Ethanol	10/10 (100)★	7.3 ± 3.6★	58	6.1 ± 1.0★

NMBA, *N*-nitrosomethylbenzylamine.
★$P < 0.001$ *versus* control group.

Table 2 Dysplasia and squamous cell carcinoma during NMBA-induced esophageal carcinogenesis in rats

Group	Dysplasia		Incidence of squamous cell carcinoma *n* (%)
	Incidence *n* (%)	Incidence/rat (Mean ± SD)	
Control	5/10 (50)	0.5 ± 0.5	0/10 (0)
Ethanol	10/10 (100)★	2.3 ± 0.5★	5/10 (50)★

NMBA, *N*-nitrosomethylbenzylamine.
★$P < 0.001$ *versus* control group.
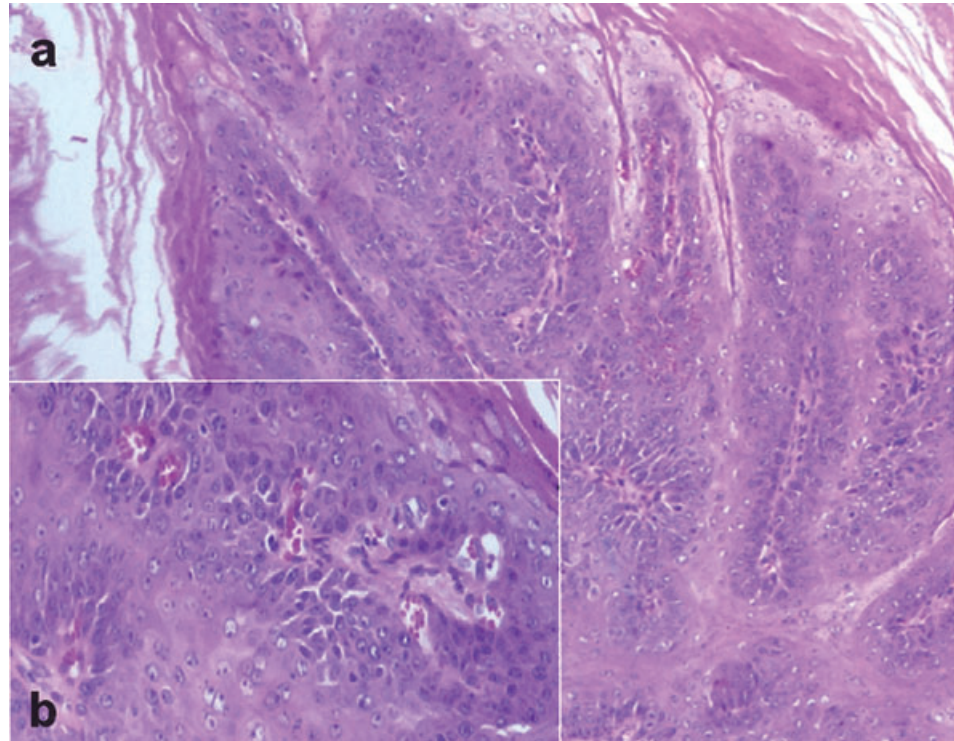
Figure 4 Histopathological examination of esophageal tumors in rat treated with ethanol (HE). Histopathology showed that the visible tumors were squamous polyps with hyperkeratosis, acanthosis, and prominent fibrovascular core.

of the staining using imagepro showed a total of 37 positive stained cells in the Fig. 7(b) area.

DISCUSSION

Chronic alcohol consumption is one of the major risk factors for the development of esophageal cancer in humans.[1–5] The probability of developing esophageal cancer is much higher in alcohol abusers who are also heavy smokers.[28,29] It has been reported that most macronutrients, vitamins and minerals are inversely associated with the etiology of squamous cell carcinoma of the esophagus.[30] However, the exact mechanism of the development of esophageal cancer during chronic alcohol consumption is not clear. In people who drink and smoke heavily, the nitrosamines present in tobacco smoke condensate could be metabolically transformed into more toxic carcinogenic compounds through increased expression of the cytochrome P450 monooxygenase system and incite esophageal carcinogenesis. In the present investigation we have demonstrated the increased expression of CYP2E1 during chronic ethanol consumption and simultaneous promotion of esophageal carcinogenesis by the chemical carcinogen NMBA in rats.

N-nitrosomethylbenzylamine has been demonstrated to be an esophageal-specific carcinogen in the rat,[31–33] inducing squamous cell carcinoma, which is histologically similar to human esophageal carcinoma.[33] Moreover, environmental exposure to NMBA has been reported to be associated with the increased incidence of esophageal cancer in China.[34,35] Because CYP2E1 is an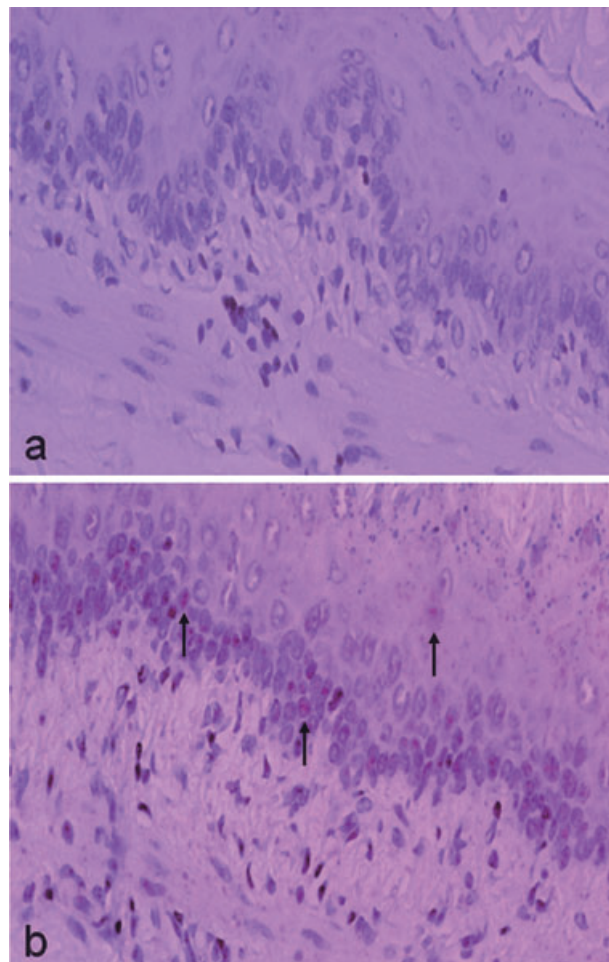 ethanol inducible enzyme,[36–38] the induction of CYP2E1 during chronic alcohol consumption may play a major role in the development of cancer in the gastrointestinal tract organs of alcoholic individuals. Although there are various reports associated with chronic alcohol abuse and gastrointestinal cancers, experimental evidence to confirm the pathogenetic correlation between alcohol abuse and development of cancer is lacking. This could be attributed to the microsomal procarcinogen activation during ethanol metabolism and the consequent induction of the cytochrome P450 monooxygenase system. It has not been demonstrated as to which class of cytochrome P450 enzyme is responsible for the metabolism of NMBA in the esophagus. Gopalakrishnan *et al.* demonstrated the presence of cytochrome P4502A3 protein and mRNA in the esophagus tissue of rats, but concluded that the amounts are low and may not be sufficient to activate NMBA.[39] It was reported that NMBA could be metabolized by human CYP2E1 expressed in mammalian cell lines.[40] In the present study, in order to induce CYP2E1 prior to NMBA administration, first the rats were treated with ethanol for 8 weeks. Second, to avoid the competition of ethanol and NMBA metabolism through CYP2E1, NMBA was administered to rats 1 h after stopping the liquid diet containing ethanol. Ethanol was not detected in the blood 30 min after terminating the liquid diet with ethanol. Furthermore, in the present investigation we used only 0.1 mg NMBA/kg bodyweight, which is a very low dose to incite esophageal carcinogenesis. Therefore the induction and increased expression of CYP2E1 by ethanol played an important role in the development of esophageal tumors in the present study.
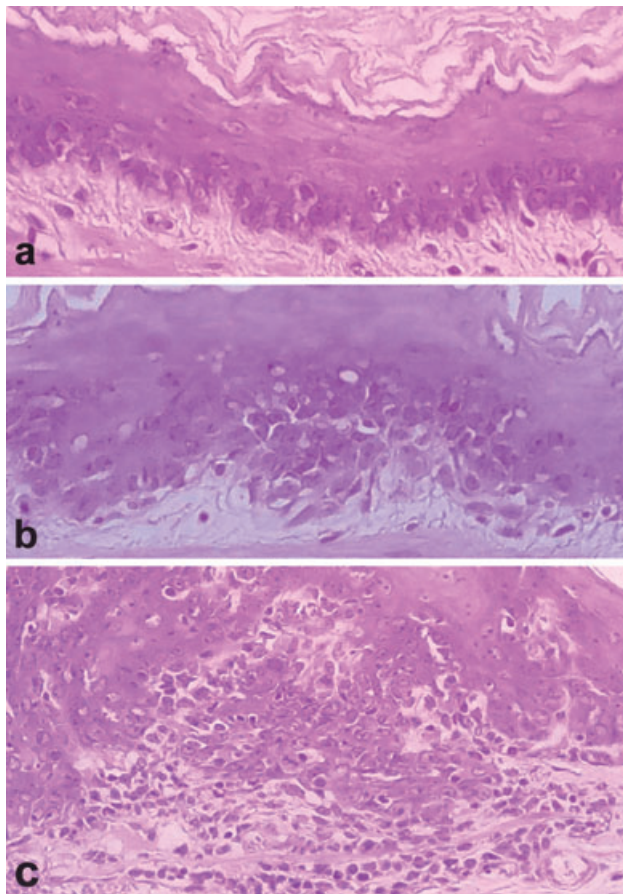
Figure 5 Histopathological examination of esophageal mucosa in rat treated with ethanol (HE). (a) normal, (b) dysplasia, (c) squamous cell carcinoma.

Figure 6 Immunohistochemical staining for cytochrome P4502E1 in the esophageal mucosa. (a) Control rat; (b) ethanol rat.

Induction of CYP2E1 has been previously demonstrated in the rat esophagus after chronic ethanol administration.[22] In the present investigation also we have observed prominent staining of CYP2E1 in the esophageal mucosa in the ethanol group, indicating increased expression of the enzyme. Moreover, CYP2E1 staining was markedly enhanced in the squamous cells of the visible papilloma in the ethanol group. Because CYP2E1 can metabolize NMBA,[40,41] these results suggest that NMBA might be metabolized more actively in the esophageal mucosa of ethanol-treated rats. In all ethanol group rats, 5–8 visible squamous papillomas with a size of 5–10 mm developed in the esophagi. Furthermore, 2–3 mucosal lesions including moderate dysplasia to invasive squamous cell carcinoma were present. However, in the control group, only one papilloma was developed in five out of the 10 rats studied and squamous cell carcinoma was not recognized in the esophageal mucosa. These results suggest that chronic ethanol consumption may promote esophageal cancer through enhanced CYP2E1 expression induced by ethanol.

It was reported that the incidence of esophageal nodules and tumors induced by NMBA decreased during simultaneous ethanol administration, but was promoted when ethanol was administered after initiation.[42] It was also reported that simultaneous ethanol and NMBA administration have weak enhancing effects on NMBA-induced esophageal tumorigenesis in rats, while there is are promoting effects in the post-initiation phase with continuous ethanol administration.[43] These could be explained by the competition of ethanol and NMBA metabolism through CYP2E1 induced by ethanol, which is avoided in the present investigation by removing the ethanol-containing liquid diet 1 h prior to the NMBA injection (Fig. 1). There is also a chance of a similar induction of other CYP subfamily enzymes such as CYP2A6 and CYP2B1/2, which have been recently demonstrated in the esophagus tissue of rats and which are capable of metabolizing NMBA.[44,45] However, there is no report on the induction of these enzymes by chronic ethanol consumption either in the liver or esophagus.

Recently, the role of acetaldehyde in the etiology of esophageal cancer has been suggested by several authors, especially polymorphism concerned with alcohol dehydrogenase (ADH) and aldehyde
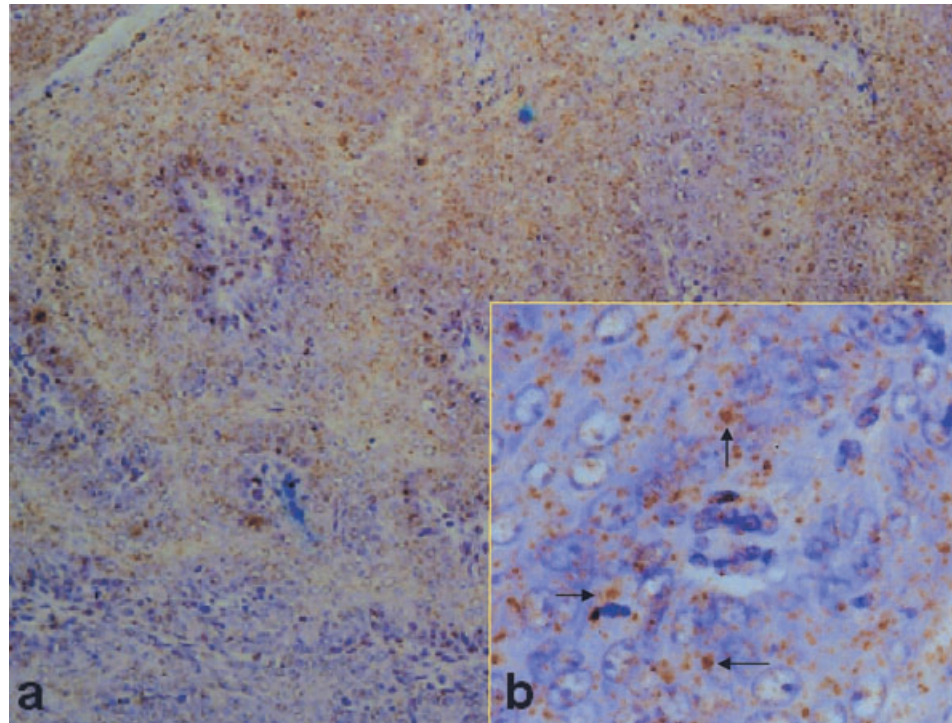
Figure 7 Immunohistochemical staining for cytochrome P4502E1 in the esophageal papilloma obtained from ethanol-treated rat.

dehydrogenase (ALDH) genes.[46–50] Ethanol is oxidized to acetaldehyde and then to acetate by ADH and ALDH, respectively, both of which have genetic polymorphisms. Inactive ALDH2, encoded by ALDH2★1/2★2, dramatically increases blood acetaldehyde levels after alcohol intake than the active form of ALDH2 encoded by ALDH2★1/2★1. Heavy and moderate alcoholic consumers with ALDH2★1/2★2 have a very high risk of esophageal cancer than those consumers with active ALDH2 encoded by ALDH2★1/2★1.[46] However, the high incidence of esophageal cancer observed in Caucasian alcoholics cannot be explained by the ALDH2 polymorphisms, because the frequency of ALDH2★1/2★2 genotype is very rare in Caucasians.[51] There are also several reports concerning the relationship between ADH polymorphisms and esophageal cancer. The ADH2★1/2★1 genotype, coding the less active form of ADH compared with the more active ADH2★1/2★2, is associated with an increased risk of esophageal cancer in Japanese alcoholics.[48] In those having ADH2★1/2★1 combined with ALDH2★1/2★2, the esophageal cancer risk is enhanced in a multiplicative fashion.[48] In contrast, the ADH3★1/3★1 genotype, which codes for the most active form of ADH3, produces a greater risk of laryngeal and oropharyngeal cancer.[50] Although these studies suggested that the associations between ALDH2, ADH2 and ADH3 polymorphisms and esophageal cancer may be corelated and that acetaldehyde may play an important role in the development of esophageal cancer, measurable levels of acetaldehyde in both blood and esophageal mucosa have not been reported.

In conclusion, the present investigation has demonstrated that the increased expression of CYP2E1 induced by chronic ethanol consumption promotes the development of NMBA-induced esophageal tumorigenesis in rats. However, the exact mechanism of the promotion and increased production of tumors in the esophagus during alternative administration of NMBA and ethanol is not clear. Other CYP subfamily enzymes such as CYP2A6 and CYP2B1/2 reported in the esophagus may also play a role. The present rat model is an excellent one in which to evaluate the effects of chronic ethanol consumption on the development of tumors induced by chemical carcinogens in various gastrointestinal organs.

ACKNOWLEDGMENTS

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