Jpn. J. Cancer Res. **91**, 973–978, October 2000

Increase in 8-Hydroxyguanine and Its Repair Activity in the Esophagi of Rats Given Long-term Ethanol and Nutrition-deficient Diet

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Epidemiological studies have shown that an increased risk of esophageal cancer is associated with the chronic consumption of alcoholic beverages, although alcohol itself is not a carcinogen in animal models. Reactive oxygen species produced by the metabolism of ethanol or by chronic inflammation may play an important role in the carcinogenic process. In this study, we analyzed one type of oxidative DNA damage, 8-hydroxyguanine (8-OH-Gua), and its repair activity in the esophagus as indicators of cellular oxidative stress in rats given long-term ethanol and an autoclaved diet (nutrition-deficient diet). Three-week-old male Sprague-Dawley rats were fed an ethanol beverage whose concentration was increased from 12 to 70% over 20 weeks. When the concentration reached 50%, the diet of one group was changed from the regular diet to an autoclaved diet. At the feeding periods of 20, 25, 30, and 35 weeks, the rats were sacrificed and the 8-OH-Gua levels and repair activities within the esophagi were measured. After 30 weeks of ethanol- and autoclaved diet-feeding, significant increases of 8-OH-Gua and its repair activity were observed in the esophagi, but not in those of the ethanol- and normal diet-fed rats. This result indicates that the combined effects of long-term ethanol consumption and nutritional deficiency may be involved in inducing oxidative stress in the rat esophagus.

Key words: Oxidative stress — 8-Hydroxyguanine — DNA repair — Ethanol — Nutrition-deficient diet

A positive association between chronic ethanol consumption and an enhanced cancer risk has been shown by epidemiological studies.[1)] The main sites associated with this risk are the oral cavity, the esophagus and the liver.[2, 3)] Ethanol is normally metabolized to acetaldehyde by oxidative transfer of the hydrogen of the ethanol by alcohol dehydrogenase (ADH) to nicotinamide dinucleotide (NAD^+), which is reduced to NADH, and by the microsomal ethanol oxidation system (MEOS). Xanthine oxidase oxidizes the acetaldehyde formed by ethanol metabolism and generates free radicals.[4, 5)] Therefore, ethanol is capable of generating free radicals during its metabolism. Oxygen free radicals are highly reactive, because they possess an unpaired electron. It is well known that ethanol increases the production of superoxide anions and hydroxyl radicals, which react rapidly with biological materials, causing oxidative damage in the ethanol-treated rat.[6)]

It is common knowledge that long-term alcohol consumption is a major risk factor for liver disease in humans. The consumption of large amounts of ethanol over a long period can lead to liver cirrhosis and dysfunction. Liver dysfunction can also inhibit the detoxification of carcinogenic compounds that are ingested. The effects of ethanol on nutritional status and liver function may also decrease the immune response. By generating free radicals, ethanol may affect nutritional status, liver function,[4)] immune function,[3)] and other body functions, and may influence the risks for various types of cancer.[2)]

Oxygen radicals cause extensive damage to DNA. Among the various forms of DNA damage induced by oxygen radicals, 8-hydroxyguanine (8-OH-Gua) is a major form of oxidative DNA damage and a useful marker of cellular oxidative stress.[7)] Studies of the DNA damage induced by oxygen radicals should be helpful in elucidating the mechanisms of cancer induction by alcohol intake. In a previous preliminary study, we observed increases in the 8-OH-Gua level and its repair activity in the rat liver after long-term alcohol- and autoclaved diet-feeding.[8)] In a previous study, we made an animal model that simulated the drinking and eating patterns of heavy alcohol drinkers, who tend to eat unbalanced meals at irregular intervals.[9)] Additionally, most of the heavy drinkers had started drinking when they were less than 20 years old. Based on this information, we fed young (3-week-old) rats an initial concentration of 12% ethanol and increased the concentration up to 70% with an autoclaved diet or a normal diet.[10)] The reason why the ethanol concentration was increased by 3%/week is that rats need training to drink a high concentration of ethanol, otherwise they refuse to drink 70% ethanol.[10)] In this study, we focused on the induction of oxidative stress in the rat esophagus by a similar system.

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MATERIALS AND METHODS

Chemicals The DNA Extractor WB Kit was purchased from Wako Biochemicals (Osaka). Nuclease P_1 (from *Penicillium citrinum*) was from Yamasa Co. (Chiba). The acid phosphatase (type XA, P-1435) was from Sigma Chemical Co. (St. Louis, MO). The protein assay kit was purchased from Bio-Rad (Hercules, CA). The [γ-^{32}P]adenosine 5′-triphosphate (ATP) (specific activity, >5000 Ci/mmol) was from Amersham (Buckinghamshire, UK). T4 polynucleotide kinase was from TaKaRa Shuzo Co. (Otsu). Other chemicals were of the highest purity commercially available. Milli-Q water was used for all experiments.

Protocol for animal experiments Three-week-old Sprague-Dawley male rats were purchased from Seiwa Experimental Animal Ltd. (Fukuoka). They were provided with commercial rat chow (CE-2: Clea Japan Inc.) or a nutrient-depleted diet (CE-2 autoclaved for 30 min at 120°C) (Table I). They were divided into 4 groups: 1) 32 rats fed aqueous ethanol (12–70%) *ad libitum* and autoclaved diet, 2) 32 rats fed aqueous ethanol *ad libitum* and normal diet, 3) 32 rats fed tap water *ad libitum* and autoclaved diet, 4) 32 rats fed tap water *ad libitum* and normal diet. For groups 1 and 2, the ethanol concentration was increased from 12 to 70% over a 20-week period. Once the concentration reached 50%, the normal diet (CE-2) was maintained for one group, and for the other group, the normal diet and the autoclaved diet were given every other week. The rats were killed at time points of 20, 25, 30, and 35 weeks, and the thoracic and abdominal cavities of each rat were opened under light ether anesthesia. The esophageal tissue was quickly removed and opened along the longitudinal axis, and the mucosa layer was scraped off with slide glass. The 8-OH-Gua level and repair activity in the esophageal mucosa were measured (Fig. 1).

Table I. Nutritional Value of the Diet before and after Autoclaving

	Before (%)	After (%)
Vitamin B_1	1.73 mg (100)	0.35 mg (20)
Vitamin C	19.00 mg (100)	9.00 mg (47)
Retinol	0.36 mg (100)	0.19 mg (53)
Vitamin B_{12}	4.90 μg (100)	2.60 μg (53)
Inositol	1.63 mg (100)	1.09 mg (67)
Pantothenic acid	2.85 mg (100)	2.21 mg (78)
Vitamin B_6	1.26 mg (100)	0.99 mg (79)
Niacin	15.90 mg (100)	14.00 mg (88)
Vitamin B_2	1.37 mg (100)	1.21 mg (88)
Vitamin E	11.00 mg (100)	10.00 mg (91)
Folic acid	0.15 mg (100)	0.15 mg (100)
Biotin	47.80 μg (100)	48.10 μg (101)
Choline	250.00 mg (100)	260.00 mg (104)

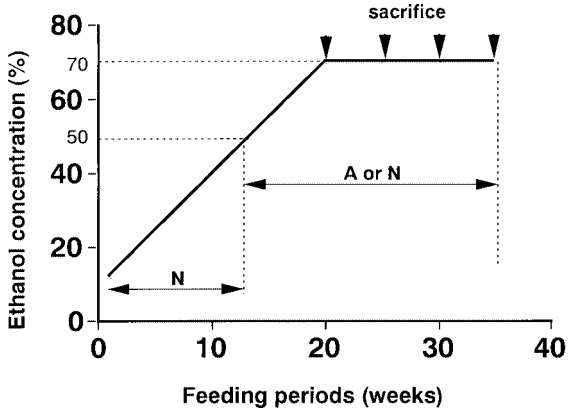

Fig. 1. Experimental protocol. The ethanol concentration was increased from 12 to 70% over a 20-week period. Once the concentration reached 50%, the normal diet and the autoclaved diet were given every other week. A, alternating diets every other week (autoclaved and normal diets); N, normal diet.

Analysis of 8-OH-Gua formation Approximately 50 mg of esophageal mucosa was homogenized in lysis buffer (1% (w/v) Triton X-100, 0.32 *M* saccharose, 5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 7.5) with a Potter-type homogenizer, and the nuclear DNA in the homogenate was extracted using the DNA Extractor WB Kit. One microliter of 2 *M* sodium acetate (pH 4.5), 4 μl of nuclease P_1 (0.8 units), and 2 μl of acid phosphatase (1 unit) were added to the extracted DNA solution. After incubation at 37°C for 30 min, the mixture was treated with ion exchange resin, Muromac (1×8) (Muromachi Kagaku Kogyo, Tokyo), to remove the I^-, and was centrifuged at 15 000 rpm for 5 min. The supernatant was transferred to an Ultrafree-Probind Filter (Millipore, Bedford, MA) and was centrifuged at 10 000 rpm for 1 min. The filtrate was injected onto an HPLC column (Beckman Ultrasphere-ODS, 5 mm, 4.6×250 mm; elution, 10 m*M* NaH_2PO_4 containing 8% methanol) equipped with an ECD (Coulochem II, ESA Inc., Chelmsford, MA), as described previously.[11] As standard samples, 20 μl each of deoxyguanosine (0.5 mg/ml) and 8-hydroxydeoxyguanosine (5 ng/ml) solutions were injected. The 8-OH-Gua value was calculated as the number per 10^5 guanine residues.

Endonuclease nicking assay Approximately 100 mg of esophagus were homogenized in 50 m*M* Tris-HCl buffer (pH 7.5) containing protease inhibitors (5 μg/ml each of pepstatin, leupeptin, antipain, and chymostatin) by a Potter-type homogenizer. The cellular debris was removed by centrifugation at 12 000 rpm for 30 min, and the supernatant was aliquoted. The total protein concentration was determined with a protein assay kit (Bio-Rad) and adjusted to 5 mg/ml. A 22-mer double-stranded synthetic oligonucleotide containing 8-OH-Gua (5′-GGTGGCCTGACG*

CATTCCCCAA-3′; G*, 8-OH-Gua) was ^{32}P end-labeled. The crude extract (50 μg total protein) was incubated with 0.05 pmol of the double-stranded DNA substrate at 25°C for 1 h. The samples were applied to a 20% denaturing polyacrylamide gel for electrophoresis. Details of the methods used have been described.[12)]

Statistical analysis The data are presented as means±SD. The significance of the differences in the results was evaluated by applying the Wilcoxon rank sum test. All analyses were carried out using the Stat View 4.01 program (Berkeley, CA).

RESULTS

The mean body weights throughout the experiments are shown in Fig. 2. From the 9th week to the last week, the body weights of ethanol- and autoclaved diet-fed rats were lower than those of the other groups. During the experiment, eight rats died in both the ethanol- and autoclaved diet-fed group and the ethanol- and normal diet-fed group. Fig. 3 shows a comparison of the esophageal 8-OH-Gua repair activities (nicking assay) of rats fed an ethanol and autoclaved diet for 30 weeks with those fed a normal diet (no ethanol). After 30 weeks, significant increases of 8-OH-Gua and its repair activity were observed in the esophagi of the ethanol- and autoclaved diet-fed rats (Fig. 4A). After 30 weeks, a significant increase in 8-OH-Gua was also observed in the esophagi of the tap water and autoclaved diet group, while the 8-OH-Gua repair activity was not significantly changed (Fig. 4B). Neither the 8-OH-Gua level or its repair activity significantly changed in the esophagi of the ethanol- and normal diet-fed rats (Fig. 4C). Fig. 4D shows the 8-OH-Gua levels in the esophagi of rats fed a normal diet (no ethanol) over 35 weeks.

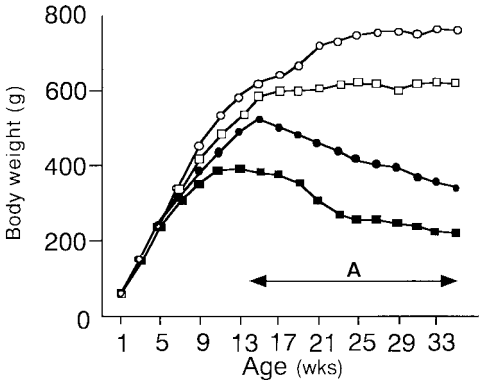

Fig. 2. Changes in body weight (g) with respect to diet over a 35-week period. ○ autoclaved diet(–) ethanol(–), □ autoclaved diet(+) ethanol(–), ● autoclaved diet(–) ethanol(+), ■ autoclaved diet(+) ethanol(+).

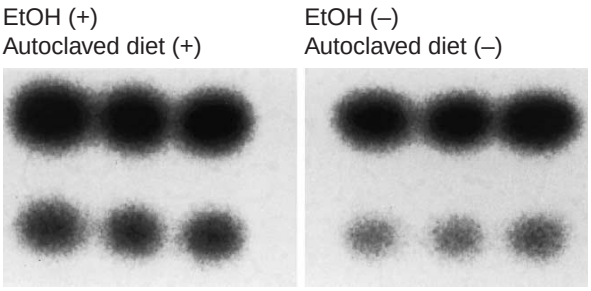

Fig. 3. A comparison of esophageal 8-OH-Gua repair activity in rats fed ethanol and autoclaved diet for 30 weeks with those fed a normal diet (not autoclaved and no ethanol). The gel position of the cleaved fragment, generated as a consequence of the excision repair activity, was confirmed by comparison with the hot piperidine-treated oligonucleotide as a fragment marker (data not shown). Upper band, substrate DNA; lower band, excised fragment.

DISCUSSION

Epidemiological studies have indicated an increased risk of esophageal cancer in alcoholics and there have been many reports concerning the mechanisms of alcohol-induced esophageal cancer.[13, 14)] Since several somatic mutations of tumor suppressor genes, such as *p53*[15–17)] and *Fhit*,[18)] have been observed in esophageal cancer patients, it is believed that these mutations are responsible for the cancer development. However, the factors that induce these mutations have not been clarified. Direct ethanol- and/or acetaldehyde-mediated toxic effects on the esophageal epithelium, and esophagitis induced by the direct effect of ethanol are possibly involved in esophageal carcinogenesis.[19)] There have been many explanations for the enhanced cancer risk among heavy drinkers, e.g., alcoholic beverages contain several substances, such as nitrosamine, that may initiate the carcinogenic process,[20)] alcohol intake causes nutritional deficiency,[4)] which increases cellular exposure to oxidants, and the cellular depletion of antioxidants induced by alcohol intake may lead to liver cirrhosis and defective liver function.[21, 22)]

However, the exact mechanisms of carcinogenesis induced by ethanol are still unclear. Most previous studies were concerned with the enhancing effects of ethanol on esophageal carcinogenesis by smoking[23)] and on liver carcinogenesis due to chronic hepatitis.[14)] These studies suggested that ethanol enhances the effects of carcinogens by altering their solubility or penetration efficiency into cells, or their metabolism, or by other unknown mechanisms. Hence, more direct evidence of the effect of ethanol on carcinogenesis is needed.

Recently, interest has been focused on the effect of ethanol on genomic DNA, to clarify the exact role of ethanol

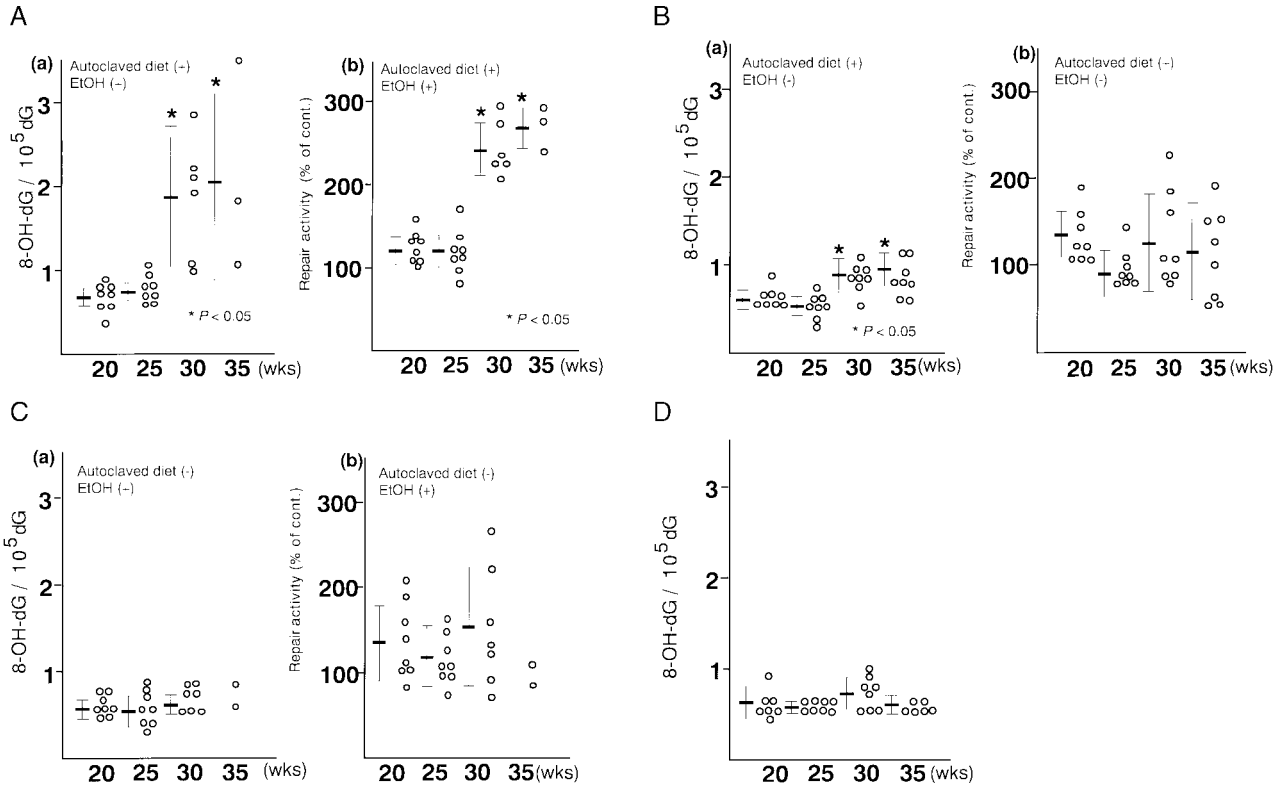

Fig. 4. Changes in the levels of 8-OH-Gua (A-a) and its repair activity (A-b) in esophagi of rats fed ethanol and an autoclaved diet (20 wks, *n*=8; 25 wks, *n*=8; 30 wks, *n*=6; 35 wks, *n*=3). Changes in the levels of 8-OH-Gua (B-a) and its repair activity (B-b) in esophagi of rats fed an autoclaved diet with no ethanol (20 wks, *n*=8; 25 wks, *n*=8; 30 wks, *n*=8; 35 wks, *n*=8). Changes in the levels of 8-OH-Gua (C-a) and its repair activity (C-b) in esophagi of rats fed a normal diet (not autoclaved) with ethanol (20 wks, *n*=8; 25 wks, *n*=8; 30 wks, *n*=5; 35 wks, *n*=2). (D) 8-OH-Gua levels in the esophagi of rats fed a normal diet (not autoclaved and no ethanol) over 35 weeks (20 wks, *n*=8; 25 wks, *n*=8; 30 wks, *n*=8; 35 wks, *n*=8).

in carcinogenesis. Acetaldehyde, which is one of the major metabolites of ethanol, induces cross-links in isolated DNA.[24] DNA alkylation is also increased by ethanol, which might involve the inhibition of the O^6-methylguanine transferase repair activity.[25] However, information regarding ethanol-induced oxidative DNA damage in the esophagus has not been available thus far.

Regarding the mechanism of the carcinogenic action of ethanol, we postulate that the oxygen radicals produced by chronic inflammation due to excessive alcohol intake play a major role, because epidemiological studies have shown that the consumption of strong alcoholic beverages is closely related to the induction of esophageal cancer.[14] Animal studies have shown that ethanol metabolism may affect the activities of superoxide dismutase,[22] catalase,[22] and glutathione peroxidase[26] in the liver. Other experiments suggested that antioxidant supplements may be able to reduce the severity of alcohol-induced tissue damage.[27] Oxygen radicals are known to cause various alterations in genomic DNA. 8-OH-Gua is one of the major forms of oxidative DNA damage, and causes GC-to-TA transversions, which occur frequently in the *p53* gene in esophageal cancer.[15, 17] Therefore, it is interesting to note the role of oxidative stresses in the mechanism of ethanol-related esophageal carcinogenesis. Since it is well known that inflammation itself produces oxidative stresses, it is important to investigate whether the accumulation of oxidative DNA damage is derived from the direct effect of ethanol or the inflammation induced by ethanol.

We measured the levels of 8-OH-Gua and its repair activity in esophageal tissue as indicators of the oxidative stress induced by ethanol intake. To our knowledge, this is the first study to examine the relationship between ethanol intake and oxidative DNA damage in rat esophagi. Our results indicate that the combination of long-term ethanol consumption and nutritional deficiency may play a critical role in producing oxidative DNA damage in the rat esophagus. Since 8-OH-Gua is known to cause mainly GC-to-

TA transversions, 8-OH-Gua may be a promutagen and a useful marker for the assessment of carcinogenesis.[28–31] Therefore, it is reasonable to conclude that an increase in 8-OH-Gua may be one mechanism by which ethanol stimulates esophageal carcinogenesis.

Based on all our observations, we speculate that oxidative DNA damage increases because the equilibrium between the attack and defense factors breaks down. Namely, ethanol induces an increase of an attack factor, oxygen free radicals, and a nutrient imbalance reduces defense abilities. This breakdown of the equilibrium might increase the 8-OH-Gua level. As shown in Fig. 4, increases of 8-OH-Gua and its repair activity were observed at 30 and 35 weeks in comparison with the levels at 20 weeks. It can be speculated that prolonged administration of ethanol might lead to changes in the defense system against oxidative stresses. The accumulation of 8-OH-Gua may also reflect the increase in oxidative stresses.

Several reports have suggested that ethanol is a tumor promoter.[20, 32, 33] A low dose of alcohol (7%) promoted esophageal carcinogenesis in rats initiated with N-nitrosomethylbenzylamine.[33] Although the administration of ethanol alone did not increase the accumulation of 8-OH-Gua in DNA in our experiment, 8-OH-Gua was increased in the esophageal tissue of rats fed a high concentration of ethanol (70%) and an autoclaved diet, suggesting that our proposed mechanisms may be restricted to tissues exposed to high concentrations of ethanol.

Another important point is the fact that a nutritional imbalance itself increases the level of oxidative DNA damage in the esophagus (Fig. 4B) and excessive alcohol intake enhances the increase (Fig. 4A). In general, heavy alcohol drinkers tend to have an irregular diet. Our results do not reveal the exact mechanism of the effect of ethanol, although they suggest that those who drink excessive amounts of alcohol should pay attention to the nutritional balance in their diet, otherwise the risk of cancer may increase.

In conclusion, the combined effects of long-term ethanol consumption and nutritional deficiency induce oxidative stress in the rat esophagus, which may be involved in esophageal carcinogenesis.

ACKNOWLEDGMENTS

This work was supported by a Grant from the Ministry of Education, Science, Sport and Culture of Japan.

(Received May 10, 2000/Revised July 17, 2000/Accepted July 24, 2000)

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