Consumption of Low-Dose of Ethanol Suppresses Colon Tumorigenesis in 1,2-Dimethylhydrazine-Treated Rats

Yongshou YANG1, Kan TAKAHARA1, Thanutchaporn KUMBUNGSEE1, Akiko KIMOTO2, Fumio SHIMAMOTO3,*** and Norihisa KATO1,***

1 Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima 739–8528, Japan
2 Faculty of Human Ecology, Yasuda Women’s University, Asaminami-ku, Hiroshima 731–0153, Japan
3 Faculty of Health Sciences, Hiroshima Shudo University, Asaminami-ku, Hiroshima 731–3195, Japan

(Received April 17, 2019)

Summary The effect of low-dose of ethanol consumption on the development of colon cancer is unclear. This study aimed to investigate the effects of low-dose ethanol (0.5%, 1%, and 2% [v/v] ethanol in drinking water) for 28 wk on colon tumor incidence in rats injected with 1,2-dimethylhydrazine. Body weight, fluid and food consumption, and the total numbers of colon adenomas (mild-, moderate-, and severe-grade dysplasia) per rat were unaffected by ethanol consumption. However, the numbers of severe-grade dysplasia were significantly reduced by 1% ethanol compared with the control (0% ethanol; 93%) but not by 0.5% and 2% ethanol. Although the numbers of total adenocarcinomas were unaffected, those of total adenomas and adenocarcinomas together were significantly reduced by 0.5% and 1% ethanol (−39% and −41%, respectively). Intriguingly, real-time PCR assay indicated the abundance of cecal Clostridium leptum (a putative immunosuppressor) was least in rats received 1% ethanol. Furthermore, 1% ethanol markedly increased colonic mRNA of IL-6, a putative suppressor of regulatory T-cells and cytoprotector. This study provides the first evidence for the potential of 1% ethanol, but not 2% ethanol, to prevent colon tumorigenesis in rats, supporting the J-curve hypothesis of the effect of low-dose alcohol on health. Further, the modulation of C. leptum and expression of IL-6, potentially linking to carcinogenesis, by 1% ethanol may provide an insight into the underlying mechanisms of the anti-colon tumor effect.

Key Words ethanol, colon cancer, Clostridium leptum, gene expression, IL-6, rat

A high dose of alcohol has harmful effects on human health, and oxidative stress and acetaldehyde are involved in the toxicity of alcohol (1). Conversely, several epidemiological studies have indicated that mortality risk of some diseases, including coronary heart disease, cerebrovascular disease, and colon cancer, is lower in individuals consuming alcohol in moderation than in non- and heavy consumers (2–6). These study results appear to be consistent with the J-curve hypothesis for the effect of alcohol consumption on human health, as proposed by Marmot and Brunner (2). However, other epidemiological studies have not supported the preventive effect of low-dose alcohol against total cancer and colon cancer risk (7, 8). A study in a Mediterranean population suggested that consuming alcohol in moderation exerts a protective effect against colorectal cancer, possibly related to the effects of red wine (9). Thus, the relationship between low-dose alcohol consumption and colorectal cancer risk remains controversial.

In epidemiological studies, it is difficult to identify general factors and lifestyle choices (such as race, type of alcoholic beverage, drinking habits, social status, diet, and personality type) that may validate the beneficial effects of moderate or low dose of alcohol itself. Animal experiments are useful for examining the direct effects of alcohol; however, most experimental animal models have focused on high toxicological doses with forced and excessive ingestion involving intragastric ethanol infusion and liquid diets (10, 11), and animal studies involving low alcohol consumption are limited. A study by Pérez-Holanda et al. (12) indicated no significant effect of ethanol consumption (1.23 g/kg body
weight/d) on the development of colon carcinogenesis in rats treated with the colon carcinogen 1,2-dimethylhydrazine (DMH) for 18 wk. However, they investigated the effect of only one dose of alcohol in rats. Further study is necessary to understand the effect of graded low-dose ethanol consumption on the colon tumorigenesis in rats administered with DMH.

Recently, we reported that consumption of 1% (v/v) ethanol in drinking water improved the liver function in rats fed a high-fat diet (1,3) and delayed the aging process in senescence-accelerated mice (SAMP8), whereas 2% (v/v) ethanol exhibited a minimal effect on the liver function and aging (14). These studies exhibit a clear beneficial effect of 1% ethanol but less so for 2% ethanol, supporting the J-curve hypothesis of alcohol consumption. Thus, we aimed to investigate if long-term ethanol consumption at 0.5%, 1%, and 2% in drinking water prevents colon tumorigenesis in DMH-treated rats. We also assessed intestinal microflora and gene expression of cancer-causing risk factors because growing evidence has suggested the involvement of intestinal microflora in the occurrence of colon cancer (1-5).

MATERIALS AND METHODS

Animals and diet. Five-week-old male F344 rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) were kept under controlled conditions (ambient temperature, 22±2˚C; 12-h light/dark cycle). The animals were cared for in compliance with the principles and guidelines of the Ethical Committee for Animal Care of the Prefectural University of Hiroshima (Hiroshima, Japan) and the Prefectural University of Hiroshima Animal Ethics Committee in accordance with the Japanese National Law on Animal Care and Use. The Ethical Committee for Animal Care of the Prefectural University of Hiroshima (Hiroshima, Japan) approved the experiments undertaken (approval number: 13HA005). Animals were allowed to acclimatize for 1 wk before commencing the experiments and were given free access to diet and tap water ad libitum. The rats were randomly divided into four groups, namely, control (0% ethanol group), 0.5% ethanol, 1% ethanol, and 2% ethanol groups. After an acclimation period of 1 wk, the rats received either drinking deionized water with 0.5% (v/v), 1% (v/v), or 2% (v/v) ethanol (experimental groups, n=15 each) or deionized water alone (control group, n=15) and had free access to commercial stock diet (MF: Oriental Yeast Co., Ltd., Tokyo, Japan) for 28 wk. DMH (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in 0.9% sodium chloride, and the pH value was adjusted to 6.5 with NaHCO3. Upon reaching the age of 6 wk, all rats were subcutaneously injected with DMH (20 mg/kg body weight) once a week for 8 consecutive weeks to induce colon carcinogenesis. All rats were sacrificed at 28 wk after the first DMH injection and dissected to obtain various samples. At the end of the feeding period, the rats were sacrificed by decapitation after administering anesthesia (45 mg/kg body weight of sodium pentobarbital). Colon tissue samples were immediately obtained for histological examination of tumor-bearing areas from 10 rats per group. Cecal content samples for the analysis of microflora and colon tissue samples for the analysis of gene expression were immediately excised from the remaining five rats per group and stored at −80˚C until further analysis.

Visualization and histological examination. At the termination of the studies, the colon was removed, slit open longitudinally from cecum to anus, placed on a paper towel and fixed in neutral formalin for 24 h. Sections (4 μm thick) of formalin-fixed, paraffin-embedded, colon tissues were prepared and stained with hematoxylin and eosin (HE) and examined under a light microscope (Olympus, Tokyo, Japan). The tumors in the colon were classified into two types, i.e., adenomas and adenocarcinomas. Adenomas were tumors with no evidence of malignancy, and further classified into three grade of dysplasia: mild-grade dysplasia, moderate-grade dysplasia, and severe-grade dysplasia. Adenocarcinomas were tumors with evidence of malignancy (well differentiated tubular adenocarcinoma, moderately differentiated tubular adenocarcinoma, and poorly differentiated adenocarcinoma).

Abundance of cecal microflora. For the analysis of cecal microflora, bacterial genomic DNA was isolated from the cecum digesta using the UltraClean™ Fecal DNA extraction kit (MO BIO Laboratories, CA, USA) according to the manufacturer’s instructions. Real-time quantitative polymerase chain reaction (qPCR) was performed to investigate the variation in the total numbers of bacteria. The 16S rRNA primers for the bacteria were used for qPCR have been described in Table S1 (Supplemental Online Material) and our recent papers (16, 17). The total volume of reagent mixture for each PCR was 20 μL [4.4 μL distilled water, 10 μL Master mix (Takara Bio Inc., Shiga, Japan), 2 μL Plus solution (Takara Bio Inc.), 0.8 μL of each forward and reverse primers and 2 μL DNA sample]. The reaction conditions were as follows: 95˚C for 30 s, followed by 40 cycles at 95˚C for 5 s, 55˚C for 15 s and 72˚C for 30 s. The fluorescent products were detected at the last step of each cycle. Melting curve analysis was performed after amplification to distinguish the targeted PCR product from the non-targeted PCR product. Data were analysed using the second derivative maximum method of the StepOne™ Real-time PCR Software version 2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The plasmid copy numbers/μL were determined for the standard plasmid solution ([μg standard plasmid mixture ng/μL]×(molecules bp/1,0×109 ng)×(1/660 DNA length bp/plasmid)=plasmid copies/μL]. Real-time qPCR products were run on five 10-fold serial dilutions of each standard mixture to compare the threshold cycle number with the copy number of the target sequence and to generate standard curves for the quantification of unknown samples. Typically, standard curves were linear across five orders of magnitude (R²>0.98).

Colonic gene expression. For the analysis of gene expression in the descending and ascending colon, the areas of flat mucosa with no visible tumors were used. The analysis of colonic gene expression was done using
Table 1. Effects of ethanol consumption on body weight, food intake and fluid intake.

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>0% (Control)</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>375±3</td>
<td>384±4</td>
<td>383±5</td>
<td>384±4</td>
</tr>
<tr>
<td>Total food intake (g/28 wk)</td>
<td>2,568±20ab</td>
<td>2,587±20a</td>
<td>2,568±20ab</td>
<td>2,508±20b</td>
</tr>
<tr>
<td>Total fluid intake (g/28 wk)</td>
<td>3,744±78ab</td>
<td>3,763±59ab</td>
<td>3,783±59b</td>
<td>3,567±59a</td>
</tr>
<tr>
<td>Total ethanol intake (g/28 wk)</td>
<td>0</td>
<td>14.8±0.2a</td>
<td>29.8±0.3b</td>
<td>56.3±0.9c</td>
</tr>
</tbody>
</table>

Values are means±SE (n=13–15). Values with different superscript are significantly different by Tukey-Kramer HSD test (p<0.05).

RESULTS

Food consumption, fluid consumption, and body weight

The final body weight, and total food and fluid consumption were unaffected by ethanol compared with control (0% ethanol), and was not significantly different among the four groups (Table 1). From the calculation based on the body weight and ethanol consumption, rats in the 0.5%, 1% and 2% ethanol groups on the 14th week ingested approximately 0.25, 0.5 and 1 g ethanol/kg body weight/d, respectively; those on the 28th week ingested approximately 0.2, 0.4 and 0.8 g ethanol/kg body weight/d, respectively.

Histological analysis in tumor tissues

The numbers of mild and moderate dysplasia (Fig. S1A and B, Supplemental Online Material) per rat were unaffected by the consumption of 0.5%, 1%, and 2% ethanol compared with control (p>0.05, Fig. 1A and B). The numbers of severe-grade dysplasia (Fig. S1C) were markedly lower in the 1% ethanol group than in the control group (−93%, p<0.05, Fig. 1C), whereas those in the 0.5% and 2% ethanol groups were not significantly different from those in the control group (p>0.05). The total numbers of adenomas (mild-grade, moderate-grade, and severe-grade dysplasia; Supplemental Fig. 1A, B and C) (Fig. 1D) and those of adenocarcinomas (well differentiated tubular adenocarcinoma, moderately differentiated tubular adenocarcinoma, and poorly differentiated adenocarcinoma; Supplemental Fig. 1D, E and F) (Fig. 1E) were unaffected by the consumption of ethanol. The numbers of well differentiated tubular adenocarcinoma, those of moderately differentiated tubular adenocarcinoma, and those of poorly differentiated adenocarcinoma were also unaffected (data not shown).

The total numbers of adenomas and adenocarcinomas together were significantly lower in the 0.5% and 1% ethanol groups than in the control group (−39% and
The total numbers of adenomas and adenocarcinomas together were not different between the 2% ethanol and control groups. Table 2 indicates the numbers of tumor-bearing rats and the percentage of the tumor-bearing rats. The percentage of the numbers of severe dysplasia-bearing rats was significantly lower in the 1% ethanol group than in the control and 2% ethanol groups (p<0.05). The percentage of the rats bearing both adenomas and adenocarcinomas was unaffected by ethanol consumption.

Cecal microflora

The numbers of the total numbers of all bacterial species (Fig. 2A) and some bacteria such as Bifidobacterium (Fig. 2B), Lactobacillus (Fig. 2C), Enterobacteriaceae (Fig. 2D), and Clostridium cocoides (Fig. 2E) per gram of cecal contents were unaffected by ethanol consumption. Among the four groups, the numbers of Clostridium leptum bacteria were the lowest in the 1% ethanol group (Fig. 2F), with a significant difference between the 1% and 2% ethanol groups (p<0.05).

Gene expressions in descending and ascending colon

As shown in Fig. 3, the consumption of 1% ethanol markedly increased interleukin (IL)-6 (IL-6) mRNA expression in the descending colon (4-fold, p<0.05)
compared with the control, whereas the consumption of 0.5% and 2% ethanol did not affect IL-6 expression. The expressions of other interleukins such as IL-2, IL-8, IL-10, IL-12, IL-15, IL-17, IL-21, IL-23 and IL-17 were unaffected by ethanol intake in our preliminary study (unpublished data, Yang et al.). Consumption of 0.5% and 2% ethanol significantly increased c-myc mRNA levels in the descending colon than the control (p<0.05, Fig. 3), whereas the consumption of 1% ethanol did not cause any significant change. There was no influence of ethanol intake on the expression of several genes relating to inflammation (interferon-γ [IFN-γ], tumor necro-
sis factor-α [TNF-α], inducible nitric oxide synthase [iNOS] and cyclooxygenase-2 [COX-2]), cell proliferation (vascular endothelial growth factor [VEGF], Kras, epidermal growth factor receptor [EGFR] and proliferating cell nuclear antigen [PCNA]), and tumor suppressor adenomatous polyposis coli [APC]) (p < 0.05). Similarly, in the ascending colon, consumption of 1% ethanol caused a 3.3-fold increase in IL-6 mRNA expression compared with the control (p < 0.05), whereas 0.5% and 2% ethanol had no effect. The expression of other genes such as TNF-α, iNOS and COX-2 in the ascending colon was unaffected by ethanol consumption.

Several alcohol-metabolizing enzymes, such as alcohol dehydrogenase (Adh), aldehyde dehydrogenase (Aldh) and cytochrome P450 2E1 (Cyp2e1), have been implicated in colon carcinogenesis and in the metabolic activation of the procarcinogen, DMH (1, 22–24). Therefore, gene expression of these enzymes was also examined. Table S2 (Supplemental Online Material) shows that ethanol consumption had no effect on the gene expression of the alcohol-metabolizing enzymes (Adh-1, Adh-2, Adh-3, Adh-4, Aldh-1, Aldh-2 and Cyp2e1), although 1% ethanol tended to increase the gene expression of Aldh-1 (1.94-fold, p = 0.052). Because Toll-like receptors (TLRs) are associated with cancer (25), expressions of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8 and TLR9 expression were also examined, and the results indicated no significant effect of ethanol consumption on the gene expression of TLRs (Table S2).

Cecal IgA, ammonia, ethanol and bile acids

As shown in Table S3 (Supplemental Online Material), the levels of IgA, ammonia (a carcinogen) and ethanol were unaffected by ethanol consumption. The levels of bile acids such as cholic acid, deoxycholic acid (a carcinogen) and hyodeoxycholic acid were also unaffected. Lithocholic acid (a carcinogen) was not detectable.

**DISCUSSION**

The most important novel finding of the present study was that consumption of 0.5–1% ethanol for 28 wk suppressed colon tumorigenesis in DMH-treated rats, whereas that of 2% ethanol did not. These results were, in large part, consistent with our preliminary study (26): male F344 rats (five-wk-old) were fed 0%, 1%, 2% or 5% (v/v) ethanol in drinking water (10 rats per group) for 28 wk. They were treated with injections of DMH (20 mg/kg body weight) once a week for initial 8 wk. As a result, 1% ethanol group, compared with other groups, showed statistically lower numbers of adenoma and adenocarcinoma together in the colon. In the current study, the dose of 0.5–1% ethanol consumption in drinking water is approximately 0.2–0.5 g ethanol/kg of body weight/d in the 14th and 28th week. The dose of 2% ethanol consumption is approximately 0.8–1 g ethanol/kg body weight/d. Pérez-Holanda et al. also indicated no significant effect of ethanol consumption (1.23 g/kg body weight/d) on the development of colon carcinogenesis in DMH-treated rats for 18 wk (12). Taken together, 0.2–0.5 g ethanol/kg of body weight/d is likely to be effective for the anti-colon tumor effect, while more than 1 g/kg body weight/d is not likely.

As a 0.5–1% ethanol intake had a preventive effect on colon tumorigenesis in the current study, it is of interest to extrapolate the results to humans. The average body weight of the rats on the 28th week in the 0.5–1% ethanol groups was ~380 g with a daily ethanol intake of 0.2–0.4 g. Rats are known to metabolize ethanol 3 times faster than humans (300 mg/kg body weight/h in rats versus 100 mg/kg body weight/h in humans) (26, 27). Therefore, the daily dose of ethanol intake in rats is approximately equivalent to 11–21 g ethanol/d in a standard human male weighing 60 kg. These levels appear to be close to those of 2.5–15 g ethanol/d, associated with the lowest total mortality in human males (3). However, this extrapolation method may be based on the overly simplified assumption that rats metabolize ethanol 3 times faster than humans (27, 28). Therefore, further information comparing the effects of alcohol dose in humans and rats is necessary.

It is of great interest that the abundance of *C. leptum* appeared to be lowest in the 1% ethanol group among the four groups. Recent studies have suggested *C. leptum* exerts immunosuppression effect by promoting the activity of regulatory T-cells (Treg) (29, 30). Infiltration of a large number of Treg cells into tumor tissues is often associated with poor prognosis (31, 32). Accumulating evidence suggests Treg cells can suppress anticancer immunity, thereby hindering protective immunosurveillance of neoplasia and hampering effective anti-tumor immune responses in tumor-bearing hosts, thus promoting tumor development and progression (33). Accordingly, it might be possible that lower abundance of *C. leptum* by 1% ethanol partially relates to the anti-colon tumor effect. Further study is necessary to test this possibility.

Interestingly, the consumption of 1% ethanol caused a marked increase in colonic IL-6 expression. Expression of other genes related to inflammation and cancer examined were not affected. IL-6 has been suggested to play an important role in the development of cancer and inflammatory bowel disease but may exert antitumor activity under certain conditions (34–36). Accumulating evidence suggests that IL-6 signaling is condition-dependent, with dual aspects in the tumor microenvironment activity, tumor cell proliferation, survival, metastasis, and angiogenesis as well as immune suppression (36). Recent studies suggest IL-6 suppresses regulatory T-cells (Tregs) development, and lowers the balance of helper T (Th)-17/Tregs (37). Thus, there may be a possibility that the anti-tumor effect of 1% ethanol is partially mediated by the suppression of development of Tregs caused by higher IL-6 expression and lower abundance of *C. leptum*. To test this possibility, further study is necessary.

Recent study suggests that low ethanol consumption protects against cerebral ischemia/reperfusion injury in rats by suppressing post-ischemic inflammation (38). Meanwhile, in the current study, the expressions of genes of inflammatory mediators such as IL-6, TNF-α,
iNOS and COX-2 were not suppressed by 1% ethanol. Accordingly, it is unlikely that 1% ethanol reduces colon tumorigenesis by suppressing inflammation.

On the other hand, a previous study has indicated that ethanol treatment can induce the IL-6 protein expression in liver, thereby elevating IL-6 levels that act as endogenous protective cytokines against ethanol toxicity (39). IL-6 functions in a cytoprotective manner, partly by suppressing Bax translocation and by dimerization through PI3K/Akt-mediated Bax phosphorylation (39, 40). Thus, the increased IL-6 expression by consuming 1% ethanol may exhibit a protective effect against DMH-induced colon damage, relating to cancer risk. Further study remains to investigate the possibility of the involvement of increased IL-6 in the underlying mechanisms of the anti-colon tumor effect of 1% ethanol.

This study further indicated no effects of ethanol consumption on several factors relating to colon tumorigenesis, including IgA, ammonia and bile acids. In addition, the gene expression of the mediators relating to inflammation and cell proliferation, ethanol metabolizing enzymes, and TLRs were unaffected. Consequently, these data did not support the potential of these factors to play an important role in the anti-colon tumor effect of low-dose ethanol.

The current study has limitations. First, the anti-tumor effect of 0.5–1% ethanol was unremarkable, and the development of adenocarcinoma was not enough in our experimental condition. Therefore, feeding experiment with longer period than 28 wk is necessary together with increased numbers of animals per one group. Second, in the current study, the expression of genes responsible for alcohol-metabolizing enzymes responsible for the activation of procarcinogen, DMH were unaffected by ethanol consumption. However, since the activities of such enzymes were not investigated, the possibility of the modulation of the metabolism and activation of DMH by ethanol consumption still remains. Additionally, the effects of low-dose ethanol on expression of the enzymes for the activation of DMH in the liver should be also investigated because liver is primarily responsible for the activation of procarcinogen. DMH was the lowest in the 1% ethanol group. Furthermore, 1% ethanol profoundly increased the IL-6 mRNA. Thus, it is necessary to investigate if the modulation of C. leptum and IL-6 expression in response to ingestion of 1% ethanol causes a beneficial effect on the colon health of DMH-treated rats.

Disclosure of state of COI
The authors declare that they have no conflicts of interest.

Acknowledgments
This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant from the Brewers Association of Japan. The authors would like to thank Enago (www.enago.jp) for English language review.

Supporting information
Supplemental online material is available on J-STAGE.

REFERENCES


