Inhibition by Dietary Ethanol of Experimental Colonic Carcinogenesis Induced by High-Dose Azoxy methane in F344 Rats

Stanley R. Hamilton,2 Ock Soon Sohn, and Emerich S. Fiala

ABSTRACT

Epidemiological studies have shown an association between consumption of alcoholic beverages and increased occurrence of large bowel carcinoma; but studies in experimental models of colonic carcinogenesis have produced conflicting results. We assessed the effects of chronic dietary ethanol consumption during the preinduction and induction phase (period of acclimatization and carcinogen administration) in a high-dose azoxymethane-treated rat model (14 mg/kg/wk for 10 wk). Ten-wk-old male Fischer 344 rats were given 33% of calories as ethanol or no ethanol (controls). Pair-feeding with Lieber-DeCarli-type liquid diets provided comparable total carbohydrates, proteins, fats, and calories. After 3 wk of dietary acclimatization, injections of azoxymethane (AOM) were given s.c. to all rats in Wk 1 to 10. At necropsy in Wk 25, dramatic suppression of gastrointestinal tumorigenesis was evident in the ethanol-fed group: the prevalence of colonic tumors was 5% as compared with 91% in controls; and the prevalence of small bowel tumors was 0% versus 74% (P < 0.0001). In an analogous study of [14C]AOM metabolism, exhaled 14CO2 was decreased in the ethanol-fed rats, indicating suppression of AOM metabolism. Similarly, in the ethanol-fed rats the levels of the DNA adducts O6-methylguanine and 7-methylguanine 24 h after AOM injection were reduced in the colonic mucosa to 14 ± 7% and 61 ± 11% of controls and in the liver to 80 ± 9% and 86 ± 6% of controls. By contrast, rats changed from the ethanol diet to no-ethanol diet for 12 h prior to the dose of [14C]AOM metabolized the carcinogen at a faster rate than controls, indicating loss of suppression with cessation of ethanol intake along with induction of metabolizing enzymes; DNA adduct levels were reduced in the colonic mucosa to 90 ± 13% and 76 ± 9% of controls and in the liver to 81 ± 6% and 85 ± 3% of controls. Our findings indicate that dietary ethanol during the preinduction and induction phase of the AOM model dramatically inhibits tumorigenesis, even with high dosage of carcinogen, and suggest that: (a) inhibition of tumorigenesis may result from suppression of metabolic activation of AOM and the consequent reduced formation of DNA adducts during the induction (initiation) phase of the model; (b) these antiinitiation effects of ethanol are unrelated to the epidemiological association between consumption of alcoholic beverages and large bowel cancer; and (c) mechanisms of action of agents found to modulate carcinogenesis in experimental models should be determined before the results can be generalized to human beings.

INTRODUCTION

Colorectal carcinoma is the second most common cause of cancer mortality in the United States (1). Epidemiological studies have shown the importance of dietary factors, particularly high dietary fat and low dietary fiber, in the pathogenesis of colorectal carcinoma (2, 3). In addition, some epidemiological studies using a variety of data sources and statistical methods have found an association between consumption of alcoholic beverages and cancer of the large bowel (e.g., 4, 5). Studies of chronic dietary alcohol consumption using experimental models with colonic carcinogens have produced conflicting results (6–12); some authors have found enhanced large bowel tumorigenesis in ethanol-fed rats, providing experimental support for the epidemiological association (6, 7, 12); while others have not (7–11).

In our previous study of the mechanisms of ethanol effects in the AOM1-treated rat model of experimental colonic carcinogenesis (11), we found that dietary ethanol inhibited tumorigenesis when given during the preinduction and induction phase (before and during carcinogen administration) but had no effect on tumorigenesis when given during the postinduction phase (after carcinogen administration). We also found that inhibition was dependent on the quantity of ethanol consumed: marked suppression of tumorigenesis occurred with high levels of dietary ethanol (33% of calories, about 70 g of ethanol/kg of body weight/wk), but there was a threshold effect with no inhibition of tumorigenesis at low levels of ethanol consumption (11% of calories, about 20 g/kg/wk). Our in vivo and in vitro studies of carcinogen metabolism using [14C]AOM showed that ethanol inhibited the metabolism of AOM (11, 13).

In the study reported here, we assessed dietary ethanol effects on colonic tumorigenesis from a strong carcinogenic stimulus in a high-dose AOM model (14 mg/kg/wk for 10 wk as compared with 7 mg/kg/wk in our previous study). We also studied the mechanism of ethanol effects on initiation phase events in the model. During the initiation phase, AOM undergoes metabolic activation, ultimately to form a reactive species which methylates DNA (14, 15). The formation of DNA adducts is a key step in carcinogenesis (16, 17), and its extent is determined by the in vivo activities of the activation versus detoxification reactions in carcinogen metabolism. Therefore, we assessed ethanol effects on the in vivo metabolism of [14C]AOM and on the levels of the DNA adducts O6-methylguanine and 7-methylguanine as markers for initiation phase events in this model. We discuss our findings in relation to the epidemiological association between consumption of alcoholic beverages and large bowel cancer.

MATERIALS AND METHODS

Animals and Housing. Guidelines for care and use of laboratory animals promulgated by the Division of Comparative Medicine of The Johns Hopkins Medical Institutions were followed (18). Ten-wk-old male Fischer 344 rats weighing 210 to 260 g were obtained from Harlan-Sprague Dawley, Indianapolis, IN. The rats were housed singly in suspended wire-bottomed metabolic cages as in our previous study (11).

Diet. Experimental Groups, and Pair-Feeding Technique. Two isocaloric Lieber-DeCarli-type liquid diets (19) were formulated by BioServ, Frenchtown, NJ, to provide 33% of total calories as ethanol and a no-ethanol control diet. The diets had equivalent percentages of calories from protein, carbohydrates, and fats. The detailed compositions are shown in our previous study (11).

The ethanol-fed group was given the ethanol diet continuously during the 3 wk of acclimatization (preinduction phase, Wk A1 to A3) and...
during the 10 wk of AOM injection (induction phase, Wk 1 to 10). This group was then given the no-ethanol diet until sacrifice (postinduction phase, Wk 11 to 25). The control group was given the no-ethanol diet throughout the study. Pair-feeding with graduated feeding tubes (BioServ) was used to control diet consumption, as in our previous studies (7, 11).

Carcinogen and Administration Procedures. All carcinogen-handling procedures were carried out in accordance with regulations promulgated by the Office of Safety and Environmental Health of The Johns Hopkins Medical Institutions (20). A single batch of AOM (CAS:25843-45-2; Ash Stevens, Detroit, MI) was used as in our previous studies (7, 11). After 3 wk of dietary acclimatization, all rats were given 10 weekly injections of AOM s.c. at a dosage of 14 mg/kg. Injections were given in the mid-morning.

Necropsy Procedures. The rats were killed 16 wk after the last injection of AOM (Wk 26). Necropsy and pathological examinations were done as in our previous studies (7, 11).

Studies of Azoxymethane Metabolism. The procedures were analogous to those for the first carcinogen dose in the studies of tumor outcome, except that the metabolic study included a third experimental group which was switched from ethanol diet to no-ethanol diet 12 h before [14C]AOM injection (11). On the morning of the 22nd day of feeding, 4 rats (Charles River, Kingston, NY) from each group received an injection s.c. of [14C]AOM (dimethyl-14C; specific activity, 3.99 mCi/ mmol; New England Nuclear, Boston, MA), 14 mg/kg of body weight (about 30 μCi). The rats were immediately placed in Delmar-Roth-type metabolism cages for the determination of exhaled 14CO2 by methods described previously (11, 14). Exhaled air was collected continuously, and samples for analysis of 14CO2 were obtained hourly for the first 7 h and at 24 h. At the end of 24 h, the rats were killed. Throughout the experimental period, the rats had their respective liquid diets available ad libitum.

Studies of DNA Adduct Levels. The procedures were those for the first carcinogen dose in the studies of tumor outcome, except that a third experimental group switched from ethanol diet to no-ethanol diet was also included, as in the studies of carcinogen metabolism. Six rats from each experimental group were killed by decapitation 24 h after the dose of AOM. The colon and liver were snap frozen in liquid nitrogen and stored at −80°C until DNA analysis.

DNA was isolated from coded colonic mucosal scrapings and liver homogenates by the method of Margison and Kleihues (21). The DNA was subjected to acid hydrolysis and analyzed for O6-MeG and 7-MeG content by the high-pressure liquid chromatographic-fluorometric detector method of Herrón and Shank (22). Results were expressed as ng of methylated guanine/μg of guanine.

Statistical Analysis. In the study of tumor outcome, 10 rats died before time of scheduled sacrifice, representing 5 rats in each experimental group. Seven rats dying before Wk 16 of causes unrelated to tumors (pneumonia in 3 ethanol-fed rats, gastrointestinal hemorrhage due to acute hemorrhagic gastritis in one ethanol-fed rat and 2 control rats, and no anatomical cause of death in one ethanol-fed rat) were excluded from analysis. Three control rats dying in Wk 21 or 23 as a result of tumors were included.

Prevalences of tumors in the various segments of the gastrointestinal tract were compared by the Fisher exact test. Differences between means were assessed by calculation of 2-tailed t tests for paired or unpaired data, and the Mann-Whitney U test or Wilcoxon signed-rank test.

RESULTS

Effects of Chronic Dietary Ethanol Consumption on Tumorigenesis. Mean weekly caloric intakes (kcal/kg of body weight/wk) are shown in Fig. 1. The intakes rose during the acclimatization period (Wk A1 to A3) but then declined during the course of carcinogen administration (Wk 1 to 10). This decline was attributed to the combined toxicities of ethanol and AOM. Despite pair-feeding, mean weekly caloric intake per kg of body weight was higher in the ethanol diet group, since their body weights were lower (see Fig. 3 below). With cessation of AOM and ethanol, intake rose abruptly but then fell during the remainder of the study.

Mean ethanol consumption (g/kg of body weight/wk) is shown in Fig. 2 and reflected the intake of liquid diet and the ethanol content.

Mean body weights. The body weights of the ethanol-fed group lagged behind the control despite pair-feeding, possibly due to energy wastage by ethanol (EtOH) metabolism (19), but later surpassed control.

Mean body weights are shown in Fig. 3. Despite pair-feeding, the body weight curve of the ethanol-fed group lagged behind the controls during the period of ethanol consumption. Such a lag has been attributed in previous studies to energy wastage associated with ethanol metabolism (19). During the latter
weeks of the study, the mean body weights of the ethanol-fed group, which was found ultimately to have dramatically reduced tumorigenesis, exceeded those of the controls.

Tumor outcome is summarized in Table 1, and the anatomical sites of the colonic tumors are shown in Fig. 4. The strong carcinogenic stimulus of the high-dose AOM protocol was reflected in the high prevalence, frequency, multiplicity, and size of tumors in the carcinogen-treated control group. Tumorigenesis in the small and large bowel was reduced dramatically in the ethanol-fed group.

Table 1 Summary of tumor results

<table>
<thead>
<tr>
<th>Diet group</th>
<th>33% of calories as ethanol</th>
<th>Control (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumors of all sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Frequency* (mean ± SE)</td>
<td>0.05 ± 0.05</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Multiplicity* (mean ± SE)</td>
<td>1</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Colonic tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>1</td>
<td>57</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>5</td>
<td>91</td>
</tr>
<tr>
<td>Frequency (mean ± SE)</td>
<td>0.05 ± 0.05</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Multiplicity (mean ± SE)</td>
<td>1</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Diameter (mean mm ± SE)</td>
<td>2.5</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Small bowel tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td>Frequency (mean ± SE)</td>
<td>0</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Multiplicity (mean ± SE)</td>
<td>NA*</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Diameter (mean mm ± SE)</td>
<td>NA</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>Ear canal tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

* Mean number of tumors per rat including all rats in diet group.
* Mean number of tumors per rat excluding rats without a tumor.
* NA, not applicable.

The rats switched from ethanol diet to no-ethanol diet 12 h before [14C]AOM administration metabolized the carcinogen at a faster rate than controls for 3 h after injection (P = 0.05). These findings suggest that the enzymes involved in AOM metabolism were induced by chronic ethanol consumption and that increased levels of these enzymes were still present 12 h after ethanol consumption ceased.

Fig. 4. Diagram of anatomical sites of large bowel tumors. The hepatic and splenic flexures are indicated by arrows. The marked reduction in tumorigenesis in the ethanol-fed group is evident.
range of values in the other 3 rats was 0.010 to 0.019 ng of O6-MeG/μg of guanine. 7-MeG was reduced to 61 ± 11% of controls (difference of borderline statistical significance with P < 0.02 by paired t test and P < 0.10 by Wilcoxon signed-rank test). Because of the limits of detectability for low levels of O6-MeG, no conclusions could be drawn with regard to O6-MeG/7-MeG ratios. In the liver, both O6-MeG and 7-MeG were reduced (to 80 ± 9% and 86 ± 6% of controls, respectively; P = 0.05 by Wilcoxon signed-rank test), but the magnitude of the reductions was not as striking as in the colon mucosa.

The rats switched from ethanol diet to no-ethanol diet 12 h before carcinogen injection (36 h before necropsy) also showed reduced O6-MeG and 7-MeG in the colon mucosa (to 90 ± 13% and 76 ± 9% of controls, respectively; P < 0.05 for 7-MeG by paired t test). The magnitude of the reductions was much less than in the rats fed ethanol continuously, particularly for O6-MeG levels. By contrast, the liver showed reduced levels of both adducts (to 81 ± 6% of controls for O6-MeG and 85 ± 3% for 7-MeG), and these levels were similar to those in the rats fed ethanol continuously.

**DISCUSSION**

We found in a high-dose AOM model that chronic dietary ethanol consumption during the preinduction and induction phase (before and during the course of carcinogen administration) dramatically inhibited small bowel and colonic tumorigenesis. Small bowel tumorigenesis is characteristic of high-dose regimens of AOM (23), and the high prevalence of small bowel as well as colonic tumors in our control group attests to the strong carcinogenic stimulus which was blocked by ethanol. The marked inhibitory effect of ethanol given during the induction phase focused our attention on the relationship between ethanol and metabolic activation of AOM with resultant DNA adduct formation.

According to current views (Fig. 7), AOM is metabolically activated by a series of reactions (14, 15, 24). AOM is hydroxylated in the liver to MAM by the cytochrome P-450-dependent MFO of the endoplasmic reticulum (Fig. 7, Step a). MAM may be further metabolized by alcohol dehydrogenase (25) in both liver and colonic mucosa (Steps b and c, respectively) or by MFO in the liver (Step b) to the highly unstable methylazoxymethanol formaldehyde which spontaneously breaks down to yield the highly reactive methylazonium ion and formic acid (24). The methylazonium ion can methylate cellular nucleophiles, including DNA, to form adducts, or it can react with water to form methanol. DNA adduct formation is considered to be the determining step in initiation events which may result ultimately in tumorigenesis (17, 26). Of particular note, the enzymes involved in the metabolic activation of AOM (microsomal oxidase in liver, alcohol dehydrogenase in liver and colonic mucosa) also metabolize ethanol to acetaldehyde (27). Thus, the level of DNA adduct formation and, ultimately, of tumorigenesis itself appears to depend on the activities of these enzymes and the competition between AOM and ethanol for the active sites (13).

Our in vivo studies with [14C]AOM showed that dietary ethanol consumption inhibited the metabolism of [14C]AOM to [14CO2] (Figs. 5 and 7). These findings are in agreement with our previous observations in vitro which showed that ethanol competes with the metabolism of [14C]AOM to [14C]MAM (13). Our previous in vivo and in vitro studies showed that the next step, i.e., metabolism of MAM, is also inhibited by ethanol (28). This paper provides direct evidence that metabolic activation of AOM to the DNA-alkylating species is inhibited by dietary ethanol, as indicated by the lower levels of methylated guanines in the colonic mucosal and hepatic DNAs of ethanol-fed rats. Taken together, our findings suggest that the inhibition of tumorigenesis by dietary ethanol...
consumption during the preinduction and induction phase of the AOM model is due in large part to a markedly reduced effective dose of carcinogen in the colonic mucosa.

Two points about our proposed mechanism for ethanol inhibition of tumorogenesis deserve further comment. (a) We assessed the levels of methylated DNA guanines at 24 h after carcinogen injection. Because the levels of DNA alkylation are determined by the rate of removal of DNA adducts as well as by their formation (16), it is theoretically possible that the lower levels in the ethanol-fed rats resulted from more rapid removal. (b) There is notable disparity between the transient, modest inhibition of carcinogen metabolism by ethanol as contrasted with the dramatic inhibition of tumorogenesis. Although we studied AOM metabolism and DNA adduct levels after only the first of ten doses of carcinogen, it seems likely that similar inhibitory effects of ethanol would occur with the other nine doses of carcinogen. As a result, the cumulative effects of ethanol inhibition of DNA adduct formation would be substantial. In addition, the colonic mucosa appears to be particularly “protected” by ethanol since the reduction in DNA adduct levels was much greater than in the liver. These effects on AOM metabolism and DNA alkylation probably account for the dramatic inhibition of colonic and small bowel tumorogenesis in the ethanol-fed rats.

Our studies of AOM metabolism and DNA alkylation in rats with chronic dietary ethanol consumption which was discontinued 12 h before harvesting were 2 to 3 times more active in AOM metabolism than microsomes from control rats (13). In rats fed ethanol continuously, the stimulation of AOM metabolism due to enzyme induction appears to be overshadowed in vivo by the presence of consumed ethanol, which acts as a competitive inhibitor. Similar interactions between acute and chronic effects of ethanol on hepatic metabolism of other carcinogens have been reported previously (29, 30).

The effects of interrupted dietary ethanol on AOM metabolism and DNA alkylation in vivo appear contradictory at first. Since we found that ethanol inhibition of AOM metabolism resulted in decreased DNA adduct levels, the more rapid metabolism of AOM in rats fed the interrupted ethanol diet might be expected to increase DNA adduct levels. Rather, we found that DNA adduct levels were decreased in the colonic mucosa and especially in the liver (Fig. 6). These results may be explained by the finding of others that the total amount of carcinogen metabolized, rather than its rate of metabolism, determines the level of DNA alkylation when the amount of carcinogen administered is the limiting factor (31). Our study of supplemental ethanol administration showed that the group with interrupted ethanol diet metabolized AOM at a faster rate than controls during the first 6 h, but did not show increased cumulative amount of metabolized AOM by 24 h (Fig. 5). Thus, DNA alkylation in the interrupted ethanol group is likely to occur earlier due to faster AOM metabolism, but would not occur to any greater extent than in controls. If the rate of removal of alkylated species is similar in the interrupted ethanol and control groups, we suggest that the level of methylated guanines at 24 h is lower in the interrupted ethanol group due to the longer period during which removal could occur. Determination of the time course of DNA alkylation in the interrupted ethanol and control groups would be necessary to evaluate this explanation, but is beyond the scope of the present work.

Clearly, our results in the AOM-treated rat model do not provide supporting evidence for the epidemiological association between the consumption of alcoholic beverages and increased occurrence of large bowel cancer. However, since our tumorogenesis studies were accompanied by determinations of AOM metabolism, we are able to attribute this apparent discrepancy between animal and human data to the inhibitory effects of ethanol on the metabolic activation of the particular carcinogen used. Although the carcinogen(s) involved in the etiology of human large bowel cancer is thus far unidentified (32), it is extremely improbable that AOM is in any way involved. The antinitiation effects of ethanol we found emphasize the importance of taking into account the mechanisms of action of modulating agents in experimental models before generalizing the results to human beings (33). Agents such as ethanol which suppress the metabolic activation of the systemic chemical carcinogens used in animal models of colonic carcinogenesis may have completely different effects in human beings. Thus, additional studies of modulation by alcoholic beverages in more appropriate models of large bowel carcinogenesis appear warranted for comparison with epidemiological results.

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D. Enhancement of acetoxymethylmethylnitrosamine (AMMN)-induced colorectal tumors following chronic ethanol consumption in rats (abstract).


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