Short Communication

Ethanol Promotes Intestinal Tumorigenesis in the MIN Mouse[1]

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Abstract

Epidemiological studies suggest that alcohol consumption increases the risk of developing colorectal cancer; however, these data are confounded by numerous cosegregating variables. Previous experimental reports with the rodent carcinogen model have also yielded discordant results. To clarify the alcohol-colon cancer relationship, we used the MIN (multiple intestinal neoplasia) mouse, a genetic model of intestinal tumorigenesis. Twenty-four MIN mice were randomized to ethanol supplementation in the drinking water (15% alternating with 20% on a daily basis) or control. Mice were sacrificed after 10 weeks, and the intestinal tumors were scored under magnification. Tissue sections were assessed for apoptosis and cell proliferation rates, along with the presence of the malondialdehyde-acetaldehyde (MAA) adduct, a mutagenic adduct associated with ethanol consumption. Ethanol supplementation resulted in a significant increase in tumor number (135 ± 35%; $P = 0.027$ *versus* control). The induction of tumorigenesis by ethanol was most dramatic in the distal small bowel (167 ± 56%; $P = 0.01$). In the uninvolved intestinal mucosa, there was no difference in proliferative or apoptotic indices. Cytoplasmic and nuclear MAA adducts were detected in both ethanol-treated and control mice. We demonstrated that ethanol ingestion increased intestinal tumorigenesis in the MIN mouse model. Furthermore, whereas mechanisms remain incompletely elucidated, our data implicate formation of MAA adducts. This report provides further support that ethanol consumption is a risk factor for colorectal cancer.

Introduction

In the United States this year, it is estimated that there will be 148,300 new cases of colorectal cancer resulting in 56,600 deaths (1). The pathogenesis of this common malignancy is complex, involving interactions between genetic substrates and environmental factors such as diet, obesity, sedentary lifestyle, and tobacco use (2). Alcohol is a well-established risk factor for a number of malignancies. With regard to colorectal cancer, a majority of the epidemiological studies suggest a 1.5–3-fold increase in risk by alcohol consumption (3–5). However, the relationship between alcohol and colorectal cancer remains enigmatic, confounded by beverage type, gender, regional distribution of the colonic tumors, and other variables associated with alcohol intake (3–5).

Therefore, experimental animal studies are critical in clarifying the role of alcohol in colon carcinogenesis. There are two major experimental models for colorectal cancer: (*a*) the carcinogen (generally azoxymethane or its precursor, 1,2-dimethylhydrazine) rodent model; and (*b*) the MIN mouse model (6). Ethanol studies with the azoxymethane rat model have yielded discordant data, potentially from altered carcinogen activation as a result of chronic ethanol ingestion (7, 8). This confounding factor is avoided in the MIN mouse, in which a germ-line mutation in the *APC*[3] tumor suppressor gene leads to spontaneous development of intestinal adenomas (9). *APC* mutations are the initiating event in most sporadic human colon cancers, underscoring the biological relevance of the MIN mouse model (10). No previous studies have assessed the effect of ethanol supplementation on a genetic model of colon carcinogenesis. We therefore investigated the effect of ethanol on intestinal tumorigenesis in the MIN mouse. To explore potential mechanisms, we evaluated apoptosis, cell proliferation, and presence of mutagenic MAA adducts.

Materials and Methods

Approval was obtained from the Institutional Animal Care Utilization Committee of the Omaha Veterans Administration Medical Center. Twenty-four 7–8-week-old male C57/B6$^{APC\ min}$ mice (Jackson Laboratory, Bar Harbor, ME) were randomized to an AIN93a diet with or without ethanol supplementation in the drinking water (15% alternating with 20% every other day). The mice were sacrificed after 10 weeks, and intestines were removed, rinsed, and divided into proximal small bowel, distal small intestine, and colon as described previously (11). Tumors were scored under magnification by two independent observers blinded to treatment group.

Sections (5 μm) were made from the paraffin-embedded intestinal rolls. Apoptosis was analyzed using a modified terminal deoxynucleotidyl transferase-mediated nick end labeling assay kit (Oncogene Science, Cambridge, MA) as per the manufacturer's directions (11). Immunohistochemical analysis for PCNA and MAA adduct was performed using

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[3] The abbreviations used are: *APC*, adenomatous polyposis coli; MAA, malondialdehyde-acetaldehyde; PCNA, proliferating cell nuclear antigen.

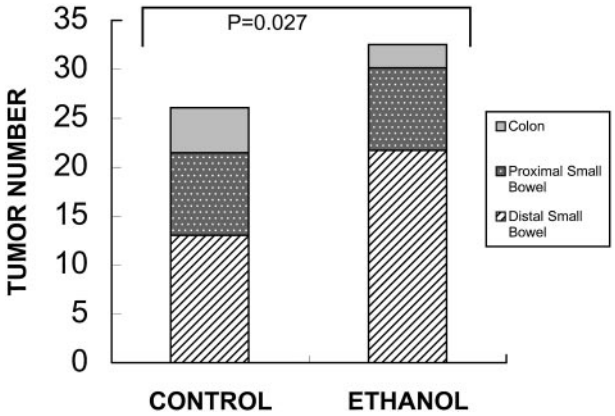

Fig. 1. Dietary ethanol supplementation induced MIN mice tumorigenesis. Ethanol supplementation resulted in a 35% increase in intestinal tumor multiplicity ($p = 0.027$). The increase in tumor number with ethanol was most dramatic in the distal small bowel, which is where most of the tumors were located in both the control and ethanol groups.

the Vectastain A,B,C kit (Vector Laboratories, Burlingame CA). A monoclonal PCNA antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal MAA adduct antibody (1:200 dilution produced as described previously; Ref. 12) was incubated for 1.5 h at 37°C. All slides were scored by a gastrointestinal pathologist (J. M. G.) blinded to treatment group.

Results

Ethanol Was Well Tolerated. There was no evidence of toxicity from the ethanol supplementation as indicated by animal behavior or necropsy examination. Body weights were monitored weekly, and there were no significant differences between the ethanol and control groups at any time point.

Tumorigenesis. Tumor multiplicity, the classic end point for MIN mice studies, was increased from 26.8 ± 8.9 tumors in the control group to 36.9 ± 10.1 in the ethanol-supplemented mice ($P < 0.05$; Fig. 1). The most remarkable augmentation was found in the distal small bowel, where the tumor count increased by 67% ($P = 0.01$). Whereas tumor size was not formally assessed, tumors in the ethanol-treated group appeared to be somewhat larger.

Apoptosis/Cell Proliferation. Based on our tumor data, we focused on the distal small bowel for the apoptosis and proliferation analysis. A modified terminal deoxynucleotidyl transferase-mediated nick end labeling assay (which labels free 3′ ends of degraded DNA) failed to demonstrate any significant alterations in apoptotic indices with ethanol treatment (101.9 ± 4.3% of control). Furthermore, these negative results were duplicated using another apoptosis assay based on the immunohistochemical detection of the M30 neo-epitope (Boehringer Manheim, Indianapolis, IN; Ref. 11).

Proliferation was assessed through immunohistochemical detection of PCNA, whose expression is increased early during S phase (13). Epithelial PCNA staining was not significantly altered by ethanol treatment (92.7 ± 9.7% of control). Western blot analysis of intestinal epithelial scrapings also failed to demonstrate any significant increase of PCNA expression in the ethanol group (data not shown).

MAA Adducts. Immunohistochemical detection of the MAA adduct was performed using a polyclonal antibody (12). Strong cytoplasmic and nuclear staining was evident in the distal small bowel epithelium (Fig. 2). However, we detected MAA adducts in both the ethanol-supplemented and the control mice, consonant with previous reports on acetaldehyde in the rodent carcinogen model. To assure antibody specificity, we confirmed abrogation of immunoreactivity through preincubation of the antibody with a 10-fold excess of the immunogenic molecule hexyl-MAA (data not shown).

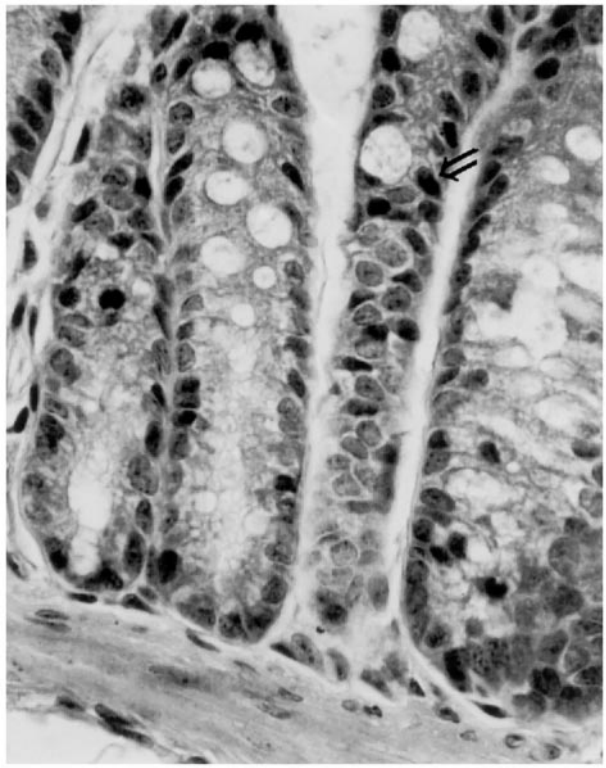

Fig. 2. MAA adducts were detected in the uninvolved intestinal epithelium of the MIN mouse. These were demonstrated in both the nucleus (*arrow*) as well as the cytoplasmic localization.

Discussion

Epidemiological studies on the role of alcohol in colon carcinogenesis have been limited by multiple confounding effects, underscoring the importance of data from experimental models. This report, the first to demonstrate that ethanol increased tumors in the MIN mouse, is of paramount importance given the discordant results obtained with the carcinogen rat model. For instance, Hamilton *et al.* (14) reported that if ethanol (36% calories from ethanol in Lieber-Decarli diet) was present in the initiation phase of the experiment, there was a decrease in hepatic azoxymethane activation, with a concomitant reduction in tumor number. On the other hand, Seitz and co-workers (8, 15) have demonstrated that ethanol potentiated colon tumorigenesis induced by both a procarcinogen (1,2-dimethylhydrazine) and a direct-acting carcinogen, acetoxymethyl-methylnitrosamine. In these latter reports, the magnitude and distribution (predominantly distal) of ethanol's promotion of colon neoplasms were consistent with our findings in the MIN mouse.

The strength of our report is that the MIN mouse recapitulates the genetic initiation of human colon carcinogene-

sis while avoiding confounding factors related to carcinogen metabolism. However, this model is somewhat limited in that the tumors are predominantly small bowel adenomas rather than colon carcinomas. Nevertheless, the assessment of adenoma multiplicity in the MIN mouse is an extraordinarily well-validated experimental model of human colon carcinogenesis (6, 9). Furthermore, the epidemiological data indicate that alcohol consumption is a risk factor for adenomatous polyp formation. For instance, heavy drinking increased the odds ratio of developing large (≥1 cm in diameter) adenomas to 1.8 (compared with nondrinkers; Ref. 16). Moreover, consuming ≥7 alcoholic beverages/week elevated the relative risk for the occurrence of metachronous adenomas to 2.04 (17). However, the relationship between alcohol and adenomas appears more striking for large rather than small polyps (18). Thus, our data with the MIN mouse model provide strong support for the epidemiological association of alcohol consumption and both colorectal adenomas and carcinomas. Whereas the reason for the distal predilection of the ethanol effect was not assessed, our previous work has suggested that there is a regional gradient in expression of a variety of proto-oncogenes including cyclooxygenase 2 (19).

To explore potential mechanisms of ethanol-induced tumorigenesis, we assessed both apoptosis and cell proliferation. Early in colon carcinogenesis, apoptosis is inhibited, enabling the otherwise short-lived colonocytes to acquire mutations requisite for neoplastic transformation. Proliferation is concomitantly increased, allowing clonal expansion of the genetically initiated cells (10). Factors that modulate colon cancer risk often dysregulate these cellular processes (13). We did not detect alterations in apoptosis or proliferation with ethanol administration. We therefore examined cellular adduct formation, a process implicated in a wide variety of ethanol-induced disease as well as in colon carcinogenesis (20). Acetaldehyde, produced by both colonocyte and bacterial alcohol dehydrogenase, has been demonstrated to be mutagenic in many systems (21). Several lines of evidence suggest that acetaldehyde may be involved in the promotion of colon carcinogenesis by ethanol. For instance, alcoholics with mutations in acetaldehyde dehydrogenase have a higher risk of rectal cancer (21). Furthermore, in the carcinogen rat model, pharmacological inhibition of acetaldehyde production led to blunting of ethanol-induced tumorigenesis (22). Malondialdehyde, a genotoxic end product of lipid peroxidation, has been implicated in colon carcinogenesis (23), potentially through formation of DNA adducts in a cyclooxygenase 2-dependent pathway (24). Acetaldehyde and malondialdehyde can combine to form the MAA adduct, which is even more reactive and avidly binds to DNA. MAA adducts, with up to four reactive aldehyde moieties, are able to cause critical protein-protein, protein-DNA, and intrastrand DNA-DNA cross-links (25). The potential importance of MAA adducts in colon carcinogenesis is underscored by the demonstration that mutagenic DNA adduct formation promotes neoplasia in patients with familial adenomatous polyposis, the human equivalent of the MIN mouse (26). We detected strong MAA adduct immunoreactivity in both the cytoplasm and the nucleus of the uninvolved intestinal epithelium. The presence of MAA adducts in both the ethanol-treated and control animals is consistent with reports demonstrating that whereas acetaldehyde mediated ethanol's promotion of acetoxymethyl-methylnitrosamine-induced colon carcinogenesis, colon acetaldehyde concentrations were not increased in the ethanol-supplemented rats (22). There are a number of other potential mechanisms, including aberrant DNA methylation, that were not assessed in this study.

In summary, we have demonstrated, for the first time, that ethanol potentiates tumorigenesis in MIN mice. This provides additional support for the epidemiological data implicating ethanol consumption as a risk factor for colorectal cancer. Our data suggest that modulation of apoptosis or cell proliferation may not be the mechanism involved in ethanol-induced intestinal tumorigenesis. The detection of MAA adducts (both nuclear and cytoplasmic) is intriguing, but the implications of this preliminary observations need further investigation.

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