Tissue-Specific Attenuation of Endogenous DNA I-Compounds in Rats by Carcinogen Azoxymethane: Possible Role of Dietary Fish Oil in Colon Cancer Prevention

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Abstract

I-compounds are bulky covalent DNA modifications that are derived from metabolic intermediates of nutrients. Some I-compounds may play protective roles against cancer, aging, and degenerative diseases. Many carcinogens and tumor promoters significantly reduce I-compound levels gradually during carcinogenesis. Colon cancer is the second leading cause of cancer death in the United States, whereas cancer of the small intestine is relatively rare. Here we have studied levels of I-compounds in DNA of colon and duodenum of male Sprague-Dawley rats treated with azoxymethane. The effects of dietary lipids (fish oil or corn oil) on colon and duodenal DNA I-compounds were also investigated. Rats fed a diet containing fish oil or corn oil were treated with 15 mg/kg azoxymethane. Animals were terminated 0, 6, 9, 12, or 24 hours after injection. I-compound levels were analyzed by the nuclease P1-enhanced 32P-postlabeling assay. Rats treated with azoxymethane displayed lower levels of I-compounds in colon DNA compared with control groups (0 hour). However, I-compound levels in duodenal DNA were not diminished after azoxymethane treatment. Animals fed a fish oil diet showed higher levels of I-compounds in colonic DNA compared with corn oil groups (mean adduct levels for fish and corn oil groups were 13.35 and 10.69 in 109 nucleotides, respectively, P = 0.034). Taken together, these results support claims that fish oil, which contains a high level of ω-3 polyunsaturated fatty acids, may have potent chemopreventive effects on carcinogen-induced colon cancer. The fact that duodenal I-compounds were not diminished by azoxymethane treatment may have been due to the existence of tissue-specific factors protecting against carcinogenesis. In conclusion, our observations show that endogenous DNA adducts may serve not only as sensitive biomarkers in carcinogenesis and cancer prevention studies, but are also helpful to further our understanding of the chemopreventive properties of ω-3 fatty acids and mechanisms of carcinogenesis. (Cancer Epidemiol Biomarkers Prev 2005;14(5):1230–5)

Introduction

I-compounds are bulky covalent DNA modifications that are presumably derived from endogenous DNA-reactive intermediates of nutrient metabolism rather than from exogenous carcinogen exposure (1-6). Profiles and levels of these adducts are varied depending on species, strain, tissue, age, and gender (1-6). Diets, chemicals, especially some carcinogens or mutagens, and sex hormones may also affect formation of I-compounds (7-9). Our previous studies suggest that I-compounds apparently do not reflect DNA damage but may rather be functionally important. Some I-compounds may play a protective role against cancer, aging, and degenerative diseases. For example, long-term dietary restriction, a powerful method to prevent cancer and extend life span in experimental systems, results in significant elevations of I-compound levels in different rodent tissues (1-3, 5). The levels of many I-compounds in liver and kidney DNA also exhibit positive linear correlations with median life span in different strains of rats and mice (3). In contrast, numerous experiments have shown that many carcinogens and tumor promoters significantly reduce I-compound levels gradually during carcinogenesis (9-14), suggesting that I-compounds may play an inhibitory role in carcinogenesis. Neoplasms display very low levels of I-compounds (15, 16), which have been shown not to be a secondary effect of increased cell proliferation and DNA replication (1, 16).

Only lung cancer exceeds colon cancer in cancer-related mortality in the United States (17); on the other hand, cancer of the small intestine is relatively rare (17, 18). This is despite the fact that the small intestine has a higher rate of cell proliferation than the colon and proliferation plays a key role in the genetic propagation of mutations arising from DNA adducts, formed upon exposure of humans or animals to an alkylating or oxidizing agent. Proliferation is also required to promote mutated cells to malignant cells (19). There are a variety of possible differences in epithelial cells from the small intestine and large intestine that may increase the likelihood of colon carcinogenesis. These include differences in substrate metabolism (20), enhanced DNA damage in colon stem cell populations (21), reduced apoptotic removal of damaged colon cells (21, 22), and effects of luminal contents on these cells (23, 24).

Diet plays an important role in promoting or preventing colon cancer. Both meat and fat intakes have been suspected as important risk factors for colorectal cancer in humans (25). In a study of U.S. nurses, the highest quintile of total fat intake was associated with an ~2-fold increased risk of colon cancer. The costs of publication of this article were defrayed in part by the payment of page charges. The costs are to be paid exclusively from the advertisement revenue of the journal. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/16/04; revised 2/15/05; accepted 3/15/05.
Grant support: National Institute of Environmental Health Sciences grants 5-P30-ES-09106 for the Center of Environmental and Rural Health; NIH grants CA61750, CA82907, and CA59034; and National Space Biomedical Research Institute grant 00202.

The authors declare no conflict of interest.

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cancer compared with women in the lowest quintile. This increase was attributable to animal fat intake and not to vegetable fat intake (26). Animal studies indicate a strong positive association of dietary fat with colorectal cancer risk (27). However, some dietary fatty acids seem to be protective against colon cancer. The long chain (ω-3) polyunsaturated fatty acids, such as eicosapentenoic acid and docosahexaenoic acid, occurring in fish oil are considered protective. Epidemiologic and experimental evidence supports the hypothesis that ω-3 polyunsaturated fatty acids play a protective role against colon cancer. For example, Alaskan and Greenland Eskimos consume a higher level of foods containing ω-3 fatty acids than other North Americans and have a lower rate of colon cancer (28, 29). Numerous experiments show that fish oil exerts a protective effect against colon cancer (30-38).

This paper reports the effects of azoxymethane, a colon-specific carcinogen on I-compounds in DNA of colon and duodenum of male Sprague-Dawley rats. As sensitive biomarkers, endogenous DNA I-compounds in colon and duodenum are used to investigate the protective role of dietary fish oil in colon carcinogenesis.

Materials and Methods

Materials. Azoxymethane was purchased from Sigma (St. Louis, MO). Corn oil was kindly donated by Degussa Bio-actives (Champaign, IL). Vacuum-deodorized Menhaden fish oil was provided by the NIH Fish Oil Test Material Program, Southeast Center (Charleston, SC). High methoxylated pectin was obtained from Grinsted (Industrial Airport, KS).

Materials for DNA extraction (39-41) and 32P-postlabeling (41, 42) have been reported previously.

Animal Experiment. Male Sprague-Dawley rats, 21 days old, 40 to 60 g, were obtained from Harlan Sprague-Dawley (Houston, TX). The animal protocol was approved by the University Animal Care Committee of Texas A&M University and confirmed to NIH guidelines. Animals were housed individually in cages at a temperature and humidity controlled facility with a daily photoperiod of 12 hours light and 12 hours dark. Thirty male rats were randomized into 10 groups of three animals each for DNA I-compound analysis. Rats were acclimatized for a week to the new environment during which the rats consumed a standard pelleted diet; then, the rats were randomly allocated to one of the two experimental diet groups. All diets contained 15% lipid by weight (Table 1). To meet essential fatty acid requirements, the fish oil diet contained 3.5 g of corn oil per 100 g of diet (43). To prevent formation of oxidized lipids, the diet was stored at −20°C in the dark. The fish oil contained 1 g/kg ω-tocopherol and 0.025% tertiarybutylhydroquinine as antioxidants. Food-grade corn oil was also supplemented with α-tocopherol, γ-tocopherol, and tertiarybutylhydroquinine to obtain antioxidant levels equivalent to those in the fish oil. Rats had free access to water and fresh diet was provided in clean food bowls daily. After 30 days of consuming the experimental diet, the rats were injected s.c. with a colon-specific carcinogen, azoxymethane, at a single dose of 15 mg/kg body weight. To minimize any diurnal variation, rats were injected in the narrow time period between 8:30 and 9:30 am. Animals were euthanized by CO2 asphyxiation and cervical dislocation before sample collection at 6, 9, 12, or 24 hours after the azoxymethane injection. Three rats served as a control for each diet and, therefore, were not injected with the carcinogen and were terminated at 0 hour. The colon samples from all time points and duodenum samples only from the 12-hour group were immediately resected, flushed with PBS, and opened longitudinally. The tissue was placed on an ice-cold glass plate and the colonic and duodenal mucosa were removed by scraping with a glass slide. Scraped mucosal samples were placed in cryo-vials, snap frozen in liquid nitrogen, and stored at −80°C until DNA extraction.

Table 1. Diet components

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Fish oil diet (%)</th>
<th>Corn oil diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>51.05</td>
<td>51.05</td>
</tr>
<tr>
<td>Casein</td>
<td>22.35</td>
<td>22.35</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>Corn oil</td>
<td>3.50</td>
<td>15.00</td>
</tr>
<tr>
<td>Fish oil</td>
<td>11.50</td>
<td>0.00</td>
</tr>
<tr>
<td>Mineral mix, AIN-76, Harlan Teklad (Madison, WI)</td>
<td>3.91</td>
<td>3.91</td>
</tr>
<tr>
<td>Vitamin mix, AIN 76-A, Harlan Teklad</td>
<td>1.12</td>
<td>1.12</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>Pectin</td>
<td>6.00</td>
<td>6.00</td>
</tr>
</tbody>
</table>

32P-Postlabeling Analysis. DNA was isolated by solvent extraction combined with enzymatic digestion of protein and RNA (40) and stored at −80°C until analysis. The nuclelease I–enhanced bisphosphate version of the 32P-postlabeling method (41) was used for analysis. Briefly, DNA (10 μg) was enzymatically degraded to normal (Np) and modified (Xp) deoxyribonucleoside 3’-monophosphates with microcossal nuclelease and spleen phosphodiesterase (pH 6.0) and was incubated at 57°C for 3.5 hours. After treatment of the mixture with nuclelease P1 to convert normal nucleotides to nucleosides, modified nucleotides (Xp) were converted to 5’-32P-labeled deoxyribonucleoside 3’,5’-bisphosphates (pXp) by incubation with carrier-free [γ-32P]ATP and polynucleotide kinase. Radioactively labeled modified nucleotides were mapped by multidirectional anion-exchange TLC on polyethyleneimine-cellulose sheets (44). Labeled products were purified and partially resolved by one-dimensional development with solvent 1 (Table 2) overnight (D1). Bulky labeled I-compounds retained in the lower (2.8 × 1.0 cm) part of the D1 chromatogram were contact-transferred to fresh thin-layer sheets and resolved by two-dimensional TLC. The I-compounds were separated with solvents 2 and 3 (Table 2) in the first and second dimensions, respectively. 32P-labeled I-compounds were visualized by screen-enhanced autoradiography at −80°C using Kodak XAR-5 film or with the aid of an InstantImager (Perkin Elmer, Downers Grove, IL; ref. 45).

Quantitative Analysis. Radioactivities of TLC fractions from individual animals were determined with the aid of an Instant Imager (45). Appropriate blank count rates were automatically subtracted by the instrument from sample values. The extent of covalent DNA modification was estimated by calculating relative adduct labeling (RAL) values from corrected sample count rates, the amount of DNA assayed (expressed as pmol DNA monomer units or DNA-P), and the specific activity of [γ-32P]ATP according to the following formula (41):

\[ RAL = \frac{\text{DNA modification(s)} \times \text{Spec. act.} \times \text{ATP}}{\text{DNA-P} \times \text{cpm}} \]
Quantitative data represented minimum estimates because 100% recovery presumably was not achieved. Statistical analysis was done with the Sigmastat v. 2.03 software (SPSS, Chicago, IL). Comparisons of the DNA I-compound data were done by using one-way ANOVA, two-way ANOVA, or unpaired Student’s t test (46). Data are presented as mean ± SE.

Results

Figure 1 depicts typical autoradiograms of nonpolar I-compounds analyzed by the nuclease P1–enhanced 32P-postlabeling assay. Colon and duodenum DNA of rats exhibited qualitatively similar profiles of I-compounds. The I-compound patterns in colon and duodenum DNA of rats fed corn oil and fish oil diets were also comparable. There were no extra bulky DNA adducts detectable by 32P-postlabeling assay in the animals treated with azoxymethane compared with control (data not shown).

Quantitative analysis indicated that the levels of total I-compounds in colon DNA of rats fed fish oil diet seemed to be higher in most groups compared with those maintained on the corn oil diet (Fig. 2). However, these data, except for the 24-hour time point, were not statistically significant. The P values obtained for the four time points by one-way ANOVA were 0.795 and 0.354 for fish oil and corn oil groups, respectively. When data from all four time points were combined (Total), the differences between the fish oil and corn oil groups were statistically significant (Fig. 2). Mean
Table 3. Differential effects of two diets on colonic total I-compounds in rats treated with azoxymethane (RAL × 10³)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>Azoxymethane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>Fish oil</td>
<td>3</td>
<td>17.20</td>
</tr>
<tr>
<td>Corn oil</td>
<td>3</td>
<td>15.25</td>
</tr>
</tbody>
</table>

*Data were analyzed by unpaired Student's t test.

RAL values for fish and corn oil groups in colon tissues were 13.35 ± 1.88 × 10³ (13.35 adducts in 10³ nucleotides) and 10.69 ± 1.42 × 10³, respectively, P = 0.034.

Although total I-compound levels in both fish oil and corn oil groups seemed to be lower in colon DNA of rats treated with azoxymethane, significant attenuation of I-compounds was only observed in corn oil (Table 3) but not the fish oil group.

Further statistical analysis was carried out to compare the effects of azoxymethane treatment and diets on colonic I-compounds. Two-way ANOVA displayed a statistically significant difference (P = 0.019) in colon DNA of rats treated with azoxymethane (12 hours) compared with control (Table 4). However, this difference was not observed in duodenal DNA (Table 5).

Although total I-compound levels in duodenal tissues of untreated rats were lower in both fish oil and corn oil groups compared with those of colon tissues (P = 0.038), azoxymethane treatment did not diminish levels of the I-compounds in the duodenum (Table 6). In contrast, 12 hours after azoxymethane treatment, I-compound levels in colon DNA were significantly lower than those in duodenum DNA (P = 0.028). Data from two different diet groups at the 12-hour time point were combined because there were no statistical differences in the I-compound levels in these two diet groups. Colonic I-compound levels in azoxymethane-treated rats were decreased 39.19% and 23.15% at 12 hours for fish oil and corn oil groups, respectively. However, I-compound levels were quickly recovered to 93.55% (16.09/17.20) of control levels in both diet groups, respectively. However, I-compound levels were decreased 39.19% and 23.15% at 12 hours for fish oil and corn oil groups, respectively. However, I-compound levels were quickly recovered to 93.55% (16.09/17.20) of control levels in both diet groups.

Discussion

I-compounds denote a special class of covalently modified nucleotides in normal tissue DNA. I-compound levels depend on species, strain, sex, tissue, and diet and are generally lowered by carcinogen exposure (1-16). As previously reported, levels of many hepatic and renal I-compounds increase linearly with age in the tissue DNA of calorically restricted, but not ad libitum fed rats (2, 3). Levels of some I-compounds in liver DNA show significant circadian rhythm (47), suggesting diurnal differences of DNA synthesis and gene expression. Neoplasms display very low levels of I-compounds, apparently independent of growth rate, suggesting a loss of the ability to form these modified nucleotides (1, 15, 16). In this study, we have shown for the first time that azoxymethane, a colon carcinogen, reduces levels of endogenous I-compounds in colonic but not duodenal DNA. We have also found that dietary fish oil containing ω-3 fatty acids, which is considered chemoprotective (48, 49), enhances I-compound levels in colon DNA. Based on all previous and current results, several very interesting issues have been raised regarding endogenous I-compounds: (a) Based on indirect evidence, some I-compounds (endogenous DNA modifications) may play protective roles against carcinogenesis (1, 6), but these modified nucleotides themselves seem not to lead to mutations during DNA replication. (b) I-compound levels can be modulated by healthy diets, such as those containing oat lipids (7, 50), fish oil, and dietary restriction (1-3, 45). (c) Current and previous (51) results showed that the levels of these endogenous DNA modifications are affected at a very early stage of carcinogenesis. (d) Once cells become malignant, the structures of these endogenous DNA modifications may be altered (15, 16). Collectively, these observations support our hypothesis that I-compounds are formed from normal intermediary metabolites. Thus far, only a few I-compounds have been linked to specific endogenous or exogenous factors. These include cholesterol precursors (1) or the presence of oat lipids in the diet (7, 50). However, the source of most of I-compounds has not been identified. Recently, the structures of type II I-compounds (DNA oxidative damage) were successfully identified as dinucleotides containing 8,5'-cyclo-2'-deoxyadenosine (52). Characterization of the structures of endogenous DNA I-compounds would provide useful information regarding the source and mechanism of these compounds. As stable, sensitive, and useful biomarkers, the possible relationships between I-compounds and cancer prevention are shown in Fig. 3.

A fish oil diet, which contains a high level of ω-3 polyunsaturated fatty acid, may exert protective effects against some common cancers, such as colon, breast, and, perhaps, prostate (48). Previous studies showed that fish oil suppresses tumor development in colon carcinoma (35, 53). Other experiments have shown that fish oil increased apoptosis and differentiation in rats with colon cancer (32, 34, 38, 54, 55). Cell oxidative DNA damage, which is associated with cancer

Table 4. Effects of azoxymethane treatment (12 hours) on colonic I-compounds in rats fed fish oil and corn oil diets (RAL × 10³)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fish oil</th>
<th>Corn oil</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>17.20</td>
<td>2.97</td>
</tr>
<tr>
<td>Azoxymethane</td>
<td>3</td>
<td>10.46</td>
<td>0.74</td>
</tr>
</tbody>
</table>

*Data were analyzed by two-way ANOVA and Holm-Sidak methods.

Table 5. Effects of azoxymethane treatment (12 hours) on duodenal I-compounds in rats fed fish oil and corn oil diets (RAL × 10³)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fish oil</th>
<th>Corn oil</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>12.94</td>
<td>0.89</td>
</tr>
<tr>
<td>AOM</td>
<td>3</td>
<td>14.06</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*Data were analyzed by two-way ANOVA and Holm-Sidak methods.

Table 6. Comparison of colonic and duodenal I-compound levels between control and azoxymethane-treated rats (RAL × 10³)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Colon</th>
<th>Duodenum</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>16.22</td>
<td>1.49</td>
</tr>
<tr>
<td>Azoxymethane</td>
<td>6</td>
<td>11.09</td>
<td>0.73</td>
</tr>
</tbody>
</table>

*Data were analyzed by unpaired Student’s t test.
In summary, the data indicate that type I I-compounds exhibit a response to nutritional factors and carcinogen exposure. As biomarkers, I-compounds have utility in understanding the chemopreventive properties of ω-3 fatty acids and mechanisms of carcinogenesis. The main results of this study are as follows: (a) rats treated with azoxymethane displayed lower levels of I-compounds in colonic DNA; (b) duodenal I-compound levels were not reduced after azoxymethane treatment; and (c) rats fed a fish oil diet showed higher levels of I-compounds in colonic DNA compared with corn oil groups.

Acknowledgments
We thank Dr. Bhagavatula Moorthy for his suggestions in the preparation of the manuscript.

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