The Role of Apoptosis in the Modulation of Colon Carcinogenesis by Dietary Fat and by the Organoselenium Compound 1,4-Phenylenebis(methylene)selenocyanate

Hanan S. Samaha, Rachid Hamid, Karam El-Bayoumy, Chinthalapally V. Rao, and Bandaru S. Reddy


Abstract
Studies in laboratory animals have demonstrated that dietary supplements of organoselenium, 1,4-phenylenebis(methylene)selenocyanate (p-XSC) inhibit colon carcinogenesis. Diverse chemopreventive agents and clinically used anticancer drugs have been shown to induce apoptosis in colon tumors. Inducing apoptosis is a key mechanism for the effectiveness of some chemopreventive agents; however, failure of apoptosis is now believed to contribute to the development of human cancer. In this study, we determined the number of apoptotic bodies in the colon tumors of rats fed a low-fat (LF) or a high-fat (HF) diet with or without p-XSC treatment. At 5 weeks of age, male F344 rats were divided into four groups, which were then maintained on one of the following diets: LF, 5% corn oil; HF, 23.5% corn oil; and LF and HF supplemented with 20 ppm p-XSC. In addition, the LF or HF diet with p-XSC supplements was administered either during the initiation stage or postinitiation. At 7 weeks of age, all rats except those intended for vehicle (normal saline) treatment were given 15 mg/kg of body weight of azoxymethane once weekly for 2 weeks. The animals were sacrificed 38 weeks after carcinogen treatment, and their colonic tumors were examined for appearance of apoptosis. The LF diet significantly increased the percentage of apoptosis as compared to the HF diet; the percentage of apoptosis in LF and HF diets were 12.4 and 2.9. The colon tumors that were present in the groups fed p-XSC together with a LF or a HF diet after carcinogen administration (postinitiation period) had a higher number of apoptotic bodies than those that were present in the animals fed p-XSC before carcinogen treatment (initiation period). The extent of apoptosis was weak when p-XSC was given with a HF diet (4.4%) during the initiation phase, but it was highly significant when p-XSC was administered with LF diet (25.2%). Taken together, our data suggest that administration of LF diet supplemented with p-XSC increases apoptosis as compared to a HF diet alone.

Introduction
Colorectal cancer is the second leading cause of cancer deaths in the United States (1). Since Wynder et al. (2) in 1969 first suggested that high intake of dietary fat is a risk factor in colon cancer, substantial progress has been made in understanding the relation between dietary fat intake and development of colorectal cancer. Epidemiological studies and animal model studies confirm that dietary fat can be considered a contributor to the risk for colon cancer (3). Case-control studies, as well as animal model studies, provided evidence that the colon tumor-promoting effect of dietary fat depends not only on the amount but on the type of fat (4, 5). The tumor promoting effect of dietary fat may be mediated through elevation of colonic luminal secondary bile acids that act as tumor promoters in the colon (3, 6). Patients with colon cancer have been found to excrete more secondary bile acids than healthy individuals (7). High intake of dietary fat may also induce colon tumor promotion through multiple mechanisms, including activation of TPK and ornithine decarboxylase (8, 9). Samaha et al. (10) have demonstrated that deoxycholic acid (a secondary bile acid) inhibits apoptosis in the tumors of colon cancer patients and also in the normal-appearing mucosa of patients with benign polyps or colon cancer.

Epidemiological studies point to an increased incidence of colorectal cancer in humans in geographical regions where selenium is deficient (11). Laboratory animal model studies show that selenium supplementation in the diet or in drinking water inhibits initiation and/or postinitiation stages of colon carcinogenesis (12). Over the past several years, a major effort has been made to develop novel organoselenium compounds with high chemopreventive potential but relatively low toxicity (13). The chemical form in which selenium is administered is an important determinant of its biological activity in terms of efficacy and toxicity in both mammary and colon carcinogenesis (13-17). Because organic forms of selenium (selenomethionine) are ingested via cereals, vegetables, and grains, attention has been focused on studying the effect of organic forms of selenium in carcinogenesis (18). Studies in our laboratory have indicated that structurally defined synthetic organoselenium compounds, such as p-XSC, have great promise as chemopreventive agents (13). Dietary administration of p-XSC was...
found to inhibit chemically induced mammary, lung, and colon carcinogenesis in laboratory animal models (14–17). Apoptosis, or programmed cell death, is an active (19) physiological mode of cell death in which the cell dies by a programmed process (20–22). Apoptosis seems to be the most common morphology when cell death is physiologically determined (23). During apoptosis, nuclear condensation (forming peripheral chromatin cap) and nuclei fragmentation occur (22). The cell shrinks due to loss of cytoplasmic volume and condensation of cytoplasmic protein, and then blebbing occurs, leading to cellular fragmentation into intact membrane-bound bodies. These so-called apoptotic bodies are rapidly phagocytosed by neighboring cells (21). Unlike necrotic cell death, apoptotic cell death does not elicit an inflammatory response; therefore, there is no secondary damage to adjacent cells (22). Colorectal cancer proceeds through a cascade of specific genetic alterations. Studies of the mechanisms by which these genetic changes lead to malignant transformation have focused on the deregulation of cell proliferation. However, colorectal epithelial homeostasis is dependent not only on the rate of cell production but also on the rate of cell loss or apoptosis. Hall et al. (24) have concluded that apoptosis might account for the bulk of cell loss in the gut and that it is a central feature of the regulation of cell number in adult tissues. Many tumor promoters and chemopreventive agents have been shown to inhibit or induce apoptosis respectively (10, 25–28). In view of the importance of apoptosis in tumorigenesis, the present study was designed to examine the effects of amount of dietary fat and p-XSC administered during initiation and postinitiation stages on modulation of apoptosis in colon tumors. In this report, our data demonstrate that LF diet supplemented with p-XSC enhances apoptosis in colon tumors as compared to HF diet.

Materials and Methods

Animals, Diets, and Carcinogen. Weaning male F344 rats were purchased from Charles River Laboratories (Kingston, NY). AOM (CAS:25843-45-2) was obtained from Ash Stevens (Detroit, MI). All ingredients of the semipurified diet were purchased from Dyets, Inc. (Bethlehem, PA) and stored at 4°C until the diets were prepared for feeding. The composition of the LF and HF semipurified diets is shown in Table 1. The control and experimental diets supplemented with p-XSC were prepared weekly in our laboratory and were then stored in a cold room.

Organoselenium Compound. p-XSC was synthesized as described previously (14). The purity of p-XSC was more than 99.9% based on high-performance liquid chromatography analysis (14). The stability of p-XSC in the diet at room temperature was confirmed by high-performance liquid chromatography analysis (14). The amount of p-XSC supplementation chosen for these assays was based on our previous study in which dietary administration of 20 ppm p-XSC (10 ppm as selenium) induced significant inhibition of colon carcinogenesis in this model system (16).

Experimental Procedure. The experimental design and protocol was described in detail in our previous publication (29). Male F344 rats received at weaning were quarantined for 10 days and had free access to the modified AIN-76A control diet. Following quarantine, the rats were randomly distributed by weight into various groups and were kept in an animal holding room. Animals had access to food and water at all times, and food cups were replenished with fresh diet three times weekly. Experiments were designed to study the effects of p-XSC administered in HF and LF diets, during the initiation and postinitiation phases, on apoptosis in the AOM-induced colon tumors in male F344 rats (29). The experimental design of the study is represented in Fig. 1. When the rats were 5 weeks old, groups of animals were fed the LF, HF, or one of the experimental diets containing 20 ppm p-XSC (Table 1). At 7 weeks of age, all animals except the vehicle-treated groups received s.c. injection of 15 mg/kg of body weight of AOM once weekly for 2 weeks. Vehicle-treated animals received an equal volume of normal saline. Three days after the second injection of AOM or normal saline, groups of animals receiving the experimental diets containing 20 ppm p-XSC were switched to LF or HF control diet regimen (without p-XSC) and continued on these diets until termination of the experiment (initiation stage). Additional subgroups thus far fed the LF or HF control diets were then transferred to the experimental diets containing p-XSC and continued to receive these diets until the termination of the study (postinitiation stage). The experiments were terminated during the 38th week after the AOM treatment (Fig. 1) when the rats were sacrificed by CO₂ euthanasia. All organs, including the intestines, were examined grossly under the dissection microscope. Colon tumors that were larger than 0.3 cm in diameter were excised, and a piece of each tumor was fixed in 3% glutaraldehyde for 3 h in a refrigerator. The reason for not processing the tumors smaller than 0.3 cm for apoptosis was that both histopathological evaluation and measurement of apoptosis cannot be performed in these small tumors. The remainder of the tumors were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and processed by routine histological methods with H&E stains (29). The histological criteria used for intestinal tumor classification were described previously (30).

Detection of Apoptosis. The number of colon tumors examined for apoptosis in each group was as follows: HF, 23; LF, 14; HF with p-XSC and LF with p-XSC during initiation, 7 each; HF with p-XSC and LF with p-XSC during postinitiation, 18 and 5, respectively. Methods described previously were used for detection of apoptosis (10, 22, 31–33). Apoptosis is also characterized by DNA fragmentation and cleavage into 180–200 bp internucleosomal-sized fragments. The appearance of a “ladder” of nucleosomal-sized fragments on agarose gel electrophoresis has been used as hallmark of apoptosis (34, 35). However, it should be noted that DNA cleavage is not univer-

### Table 1 Percentage composition of experimental diets

<table>
<thead>
<tr>
<th>Diet ingredient</th>
<th>Corn oil diets</th>
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<tbody>
<tr>
<td></td>
<td>LF</td>
</tr>
<tr>
<td>Casein</td>
<td>20.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Corn starch</td>
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</tr>
<tr>
<td>Dextrose</td>
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<tr>
<td>Alphacel</td>
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</tr>
<tr>
<td>Corn oil</td>
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</tr>
<tr>
<td>Mineral mix</td>
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</tr>
<tr>
<td>Vitamin mix</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
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</tbody>
</table>

*This diet was formulated on the basis of the American Institute of Nutrition standard reference diet with the modification of varying sources of carbohydrates (5).Corn oil was added at the expense of starch. The composition of the HF diet was adjusted so that animals in all dietary groups would consume approximately the same amounts of protein, minerals, vitamins, fiber, and calories (5). p-XSC was added to LF and HF diet at 20 μg/g of diet.
Fig. 1. Experimental protocol and schematic presentation of initiation and postinitiation studies.

Animals: Male F344 (30 rats/group)
Carcinogen: Azoxymethane (AOM), s.c. at a dose of 15 mg/kg body weight
Experimental diets: 20 ppm p-XSC in AIN-76A diet

sally found in apoptosis (32, 36–39). A ladder of DNA fragments is also associated with necrosis in some type of cells (40, 41). To emphasize the occurrence of apoptosis, a 1-μm epoxy section was taken from each of the tumors as described previously (10). The tumors were fixed in 3% glutaraldehyde for 3 h, and then the fixative was exchanged for a phosphate buffer with 1% sucrose and kept in the refrigerator until it was processed. Then the tissues were postfixed in 1% osmium tetroxide for 1.5 h, immersed in buffer for 2 min, dehydrated in a graded series of alcohol, and then infiltrated and embedded in fresh pure epoxy. Finally, a 1-μm epoxy section was taken and stained with 1% toluidine blue. The 1-μm toluidine blue-stained epoxy sections represent a bridge to ultrastructural examination, due to the increased resolution achieved in semithin sections, and can therefore be very informative (10, 31).

Crypts were chosen randomly, and 200 cells were counted by an observer blinded to the animal treatment group the H&E-stained paraffin-embedded sections using light microscopy. The apoptotic cells were identified by cell shrinkage, nuclear condensation (which in some cells leads to formation of crescents), and formation of apoptotic bodies. The apoptotic indices were calculated as the percentage of cells showing apoptosis.

Statistical Analysis. Students t test was used to determine whether mean values were significantly different.

Results
The body weight of animals treated with vehicle or AOM and fed LF or HF diets containing p-XSC were comparable among the dietary groups (29). In vehicle-treated animals, p-XSC did not produce any gross changes in liver, kidney, intestines, or lungs that were attributable to toxicity (29). LF diet and supplementation of p-XSC diminished the colon tumor incidence in this study (29). Briefly, the colon tumor incidences (percent-age of animals with tumors) in the LF and HF groups were 53 and 90%, respectively. The colon tumor incidences in the LF group with p-XSC and the HF group with p-XSC during the initiation period were 50 and 62%, respectively, and during the postinitiation period, they were 27 and 52%, respectively (29).

The number of colon tumors in each treatment was as follows: LF, 25; HF, 49; LF with p-XSC during initiation, 22; LF with p-XSC postinitiation, 9; HF with p-XSC during initiation, 33; and HF with p-XSC postinitiation, 24 (29).

Some of the tumors seen in this study show well-differentiated goblet cells; these can be treatment-related effects. Differentiation of the colonic epithelial cells into goblet cells is not a feature of dysplasia (30). Apoptosis becomes very low or is absent in well-differentiated adenocarcinoma (Fig. 2), which indicates that p-XSC selects only for the tumors with dysplastic lesions (poorly differentiated adenocarcinoma) without affecting the neighbor cells.

As shown by representative micrographs in Fig. 3, A and B, p-XSC administered together with a LF diet induced the classical morphological characteristics of apoptosis, including cell shrinkage, nuclear condensation, and formation of apoptotic bodies (Fig. 3B). However, it is noteworthy that there was an absence of apoptotic cells in the colonic tumors of rats that were given the HF diet alone (Fig. 3A). Similar morphological characteristics were also observed in 1-μm epoxy sections under the light microscope (Fig. 4): we observed shrinkage of colonic epithelial cells, irregular shape, and nuclear condensation, with some cells in the process of forming apoptotic bodies.

The effects of p-XSC during the initiation or postinitiation phases together with HF or LF diets on induction of apoptosis

Fig. 2. Adenocarcinoma from the colon of a rat fed a LF diet supplemented with p-XSC, showing well-differentiated goblet cells. Note the absence of apoptotic cells. (Four-μm paraffin sections, H&E stain).
Modulation of Apoptosis by Fat and Organoselenium

Fig. 3. Comparison of the morphology of colonic epithelial cells of rat tumors in the absence and presence of p-XSC (4-μm paraffin sections, H&E stains). A, adenocarcinoma from the colon of a rat fed a HF diet in the absence of p-XSC, showing dysplastic epithelium and absence of goblet cell differentiation. Note the absence of apoptotic cells. B, adenocarcinoma from the colon of a rat fed a LF diet supplemented with p-XSC, showing dysplastic epithelium and absence of goblet cell differentiation. Many cells show shrinkage, nuclear condensation, and apoptotic bodies (arrows).

Fig. 4. Adenocarcinoma from the colon of a rat fed a LF diet, showing dysplastic epithelium and absence of goblet cell differentiation. Many cells are condensed and irregular in shape and have condensed nucleus, and some cells were captured in the process of forming apoptotic bodies (arrows). (1-μm epoxy section, toluidine blue stain).

Fig. 5. Effect of p-XSC and HF and LF diets on percentage of apoptosis in colonic epithelial cells of rat tumors. Columns, average; bars, SE. The number of tumors examined for apoptosis is described in “Experimental Procedure.”

in colonic tumors are summarized in Fig. 5. Rats on the LF diet exhibited a significant increase in the apoptotic index (percentage of apoptosis) as compared to those with a high dietary fat intake (12.4 versus 2.9; P < 0.0001). The colonic tumors of animals fed p-XSC in a LF diet during the initiation phase had a higher number of apoptotic bodies than those that were not fed p-XSC (25.2 versus 12.4; P < 0.001); however, p-XSC had no measurable effect when given in the HF diet during the same period (4.4 versus 2.9; P < 0.3). Interestingly, when p-XSC was administered in either the LF or HF diet during postinitiation, a significant increase in apoptosis was observed as compared to LR or HF diets without p-XSC (21.1 versus 12.4; P < 0.02, and 14.4 versus 2.9; P < 0.0001, respectively.

Discussion

Epidemiological and animal model studies had suggested that high dietary fat intake is a risk factor for colon cancer (3, 42). Several studies have demonstrated that resistance to apoptosis is a part of the genesis and development of colorectal cancer (43, 10). Wright et al. (44) concluded that inhibition of apoptosis is a mechanism of tumor promotion. In this study, we show for the first time that low dietary fat intake induces apoptosis in the epithelial cells of colonic tumors, whereas a high intake of dietary fat prevents induction of apoptosis. This can be expected because a HF diet is known to act as a strong tumor promoter (45) and is in line with the observation by Reddy and Wynder (6) showing a positive association between increase in dietary fat and elevation of colonic luminal secondary bile acids which act as colon tumor promoters. Moreover, Samaha et al. (10) have reported that bile salt lacks the ability to induce apoptosis in the tumors and in the normal-appearing mucosa from colon cancer patients. However, bile salt induces apoptosis in normal mucosa of healthy subjects. In an animal model, Magnuson et al. (46) have demonstrated that cholic acid administration leads to increased resistance to apoptosis. It is, therefore, possible that the elevation of secondary bile acids, such as deoxycholic acid, may be responsible for the failure of the HF diet to induce apoptosis. In this respect, it is also noteworthy that bile acids induce the release of arachidonate from the membrane phospholipids of the colon and conversion...
of arachidonic acid to prostaglandins (47). Rao and Reddy (8) have described that high dietary fat intake increased colonic mucosal prostaglandin E₂, which is formed via the Cox pathway. Tsuji and DuBois (48), who have implicated COX-2 activity in the regulation of apoptosis of rat intestinal epithelial cells, have demonstrated that overexpression of COX-2 can cause the cells to adhere more to the extracellular matrix and make them resistant to apoptosis (48). It is possible that high dietary fat-induced COX activity may also play a role in the inhibition of apoptosis in colon tumors. Furthermore, Rao and Reddy (8) have demonstrated that a HF diet enhances intestinal TPK activity, suggesting a key role for TPK in mediating the potent inducer of apoptotic cell death than selenite in a mammary carcinoma cell line. Dive and Hickmann et al. (51) and Thompson et al. (52) have shown that p-XSC is a more potent inducer of apoptotic cell death than selenite in a mammary carcinoma cell line. Lanfear et al. (53) have observed that selenodiglutathione, the primary metabolite of selenite, induces apoptosis in mouse erythrocyturias. Dive and Hickmann (54) have suggested that the efficiency of various antitumor agents is related to the intrinsic ability of the target tumor cells to respond to these agents by induction of apoptosis. This is the first report showing that dietary administration of p-XSC induces apoptosis in the colon tumors of rats fed either LF or HF diets. This effect offers an additional explanation for the chemopreventive activity of organoselenium compounds (55). In fact, selenium induction of apoptosis parallels tumor inhibition by p-XSC and stresses the consideration of the apoptotic index as a prognostic marker for monitoring the outcome of chemopreventive efficacy in an individual patient. However, this study also suggests that p-XSC affects only the poorly differentiated adenocarcinomas and has no effect on well-differentiated adenocarcinomas. This is similar to effects of salicylate, which differ at various stages of neoplastic progression (28). Colorectal carcinomas in in vitro-transformed adenoma cells were more sensitive than colorectal adenoma cells to induction of apoptosis by salicylate (28).

In conclusion, dietary administration of p-XSC, an organoselenium, in LF or HF diets increases apoptosis in colon tumors at a rate that parallels the colon tumor inhibition by this agent. The fact that maximal induction of apoptosis could be achieved in animals by feeding p-XSC along with a LF diet is intriguing and emphasizes that a low dietary fat regimen along with chemopreventive supplements is a desirable approach for secondary prevention of colon cancer in high-risk individuals. For the successful implementation of cancer control in individuals at increased risk for cancer, a mechanism or a biomarker is needed to rapidly evaluate agents for their chemopreventive potential and future use in clinical chemoprevention trials. Traditionally, evaluation of such agents has used assays in laboratory animals as the standard, often with a reduction in tumor incidence as the measurement of the chemopreventive efficacy of a compound. Our observations and those of others emphasize that apoptosis can be a valid intermediate biomarker that helps in developing good dietary strategies for the control of colon cancer and for further evaluating chemopreventive agents.

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References


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