Dietary Soy Isoflavones and Estrone Protect Ovariectomized ERαKO and Wild-Type Mice from Carcinogen-Induced Colon Cancer.1,2

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ABSTRACT Consumption of soy foods has been weakly associated with reduced colon cancer risk. Colon cancer risk is influenced by estrogen exposure, although the mechanism through which this occurs is not defined. Conversion of estradiol (E2) to estrone (E1) may be protective in the colon. We hypothesized that dietary phytoestrogens, or E1, would reduce colon tumorigenesis via an estrogen receptor (ER)-dependent mechanism. Ovariectomized ERαKO or wild-type (WT) female mice were fed diets containing casein (Casein), soy protein without isoflavones (Soy-IF), soy protein + genistein (Soy+Gen), soy protein + NovoSoy (Soy+N/Soy) or soy protein + estrone (Soy+E1) from weaning. Colon tumors were induced with azoxymethane. Tumor incidence was affected by diet but not genotype. Colon tumor incidence was lower in ERαKO and WT mice fed the Soy+E1 diet compared with those fed the casein or Soy-IF diets. Mice fed Soy+N/Soy had a lower tumor incidence than mice fed casein, but not Soy-IF. Genistein did not affect tumor incidence. Soy protein, independently of phytoestrogens or E1, significantly reduced relative colon weight, tumor burden and multiplicity. Relative colon weight was lower (P = 0.008) in mice fed Soy+E1 than in the other soy-fed groups. Tumor incidence in this group was lower than in the casein and soy-IF-fed groups and tended to be lower than in the others (P = 0.020). Hence, soy protein and N/Soy protect mice from colon cancer, and E1 further reduces colon tumorigenesis in mice, independently of ERα. J. Nutr. 134: 179–182, 2004.

KEY WORDS: colon cancer; genistein; isoflavone; azoxymethane; estrone

Colon cancer is the third most common form of cancer in the U.S. population. In 2001 estimated new cases of colon cancer were 46,200 for men and 52,000 for women (1). Controversy exists concerning the role of diet in colon cancer. Based on an extensive review of the existing literature, the World Cancer Research Fund and American Institute for Cancer Research concluded that there was convincing evidence that the risk of colon cancer was reduced by physical activity and vegetable intake, and that there was probable evidence that the consumption of red meat and alcohol increased the risk of colon cancer (2). Other factors such as the amount of dietary fiber, total fat and sugar intake and high body mass were poorly correlated with risk. Consumption of soy has been found to reduce colon cancer risk in some human populations and animal studies, but the evidence is not substantial (3). In those studies, the specific components of soy, including soy protein and the isoflavones, were not carefully controlled. The soy isolate (IF) glycosides, genistein, daidzin and glycitein, and the corresponding aglycones, genistein, daidzein and glycitein, are phytoestrogens that bind with low affinity to both forms of estrogen receptors (ER), but tend to have a higher affinity for ERβ (4).

Although colon cancer has not been considered a hormone-responsive cancer, evidence suggests that estrogens may play a role in this disease. In the early 1970s, a transient decrease in colon cancer incidence occurred among women aged 35–44 y, but not among men (5). This observation correlated with a peak in fertility and the use of high dose oral contraceptives during the preceding decade. The authors concluded that either high fertility or exposure to exogenous steroid hormones protected the women from colon cancer. Based on a meta-analysis of 18 epidemiologic studies, use of hormone replacement therapy (HRT) by postmenopausal women was associated with a 20% decrease in colon cancer risk (6). Recently, one phase of the Women's Health Initiative study was prematurely halted due to increased risk of invasive breast cancer among the group receiving conjugated equine estrogen and medroxyprogesterone acetate; however, colon cancer incidence was reduced among the women receiving HRT compared with the placebo group (7).

The most active mammalian estrogen is 17β-estradiol (E2), which can be generated from estrone (E1) via 17β-hydroxy-

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steroid dehydrogenase (17βHSD). Two isozymes of 17βHSD, type 2 and 4 are localized in the colon mucosa, and may provide a barrier for ingested steroids (8). Normal human colon tissue converts E2 to E1 at a high rate, and this activity is reduced in colon tumors (9). Incubation of human colon tumor cells (Caco-2) with E2 increased cell growth, whereas E1 inhibited proliferation (10). Hence, E1 may be an antiproliferative agent in colon (11). The primary types of HRT (Premarin/Preservak) contain 8-8-E1 sulfate, which may explain in part their protective effects on colon cancer.

In this study, we investigated the response of azoxymethane-induced colon tumors in ovariectomized, female mice with intact (wild type, WT) or disrupted ERα (EraK0) to dietary soy protein, genistein, Novasoy or E1. We hypothesized that phytoestrogens and E1 would protect WT mice from colon cancer, but the absence of ERα would eliminate the protective effect.

MATERIALS AND METHODS

Animals, diets and study design. The experimental protocol was approved by the University of Missouri Animal Care and Use Committee and was conducted according to the NRC guidelines. EraK0 and WT littermates were bred in our colony under standard husbandry conditions. Breeding pairs were fed an AIN93G diet containing casein as the protein source throughout breeding and lactation. At weaning, female mice were randomly assigned to one of 5 diet groups and consumed that diet throughout the study. The diets were based on the AIN93G formula and were isocaloric; the groups were casein, soy without IF (Soy−IF), soy with Novasoy (Soy+NSoy), soy with genistein (Soy+Gen) and soy with estrone (Soy+E1). Soy protein, obtained from Archer Daniels Midland (Decatur, IL), was treated by the manufacturer to remove the majority of the IF. The same lot and batch were used for all of the diets containing soy protein. The dose of genistein (250 mg/kg diet) was based on our previous finding of delayed mammary tumors in mice fed this concentration (12). The dose of NSoy was calculated to provide the equivalent of 250 mg genistein/kg diet based on the manufacturer’s analysis. The dose of E1 was extrapolated from the typical human HRT dose for Premarin (0.625 mg/d). Phytate was added to the casein diet at a concentration equivalent to that found in the soy protein based on the manufacturer’s analysis. A total of 15 mice per diet and genotype were obtained over a 6-in period. At 8 wk of age, the mice were ovariectomized to reduce differences in circulating estrogen between EraK0 and WT mice. Beginning at wk 10, mice were injected experimentally with 15 mg azoxymethane (AOM; Ash-Stevens, Detroit, MI)/kg body weight once a week for 6 wk. AOM injections were done at the same time of day throughout the study. Mice were killed between 0800 and 1200 h during wk 50, and blood was collected by cardiac puncture. Serum was separated by centrifugation (1000 × g for 10 min) and stored frozen. A midultracentrifugation was made and the intestinal tract removed. The small intestine and colon were rinsed and weighed, then opened longitudinally and examined for tumors. The location of each tumor was recorded before it was excised, weighed and snap-frozen in liquid nitrogen. The small intestine was divided into three sections, duodenum, jejunum and ileum, and each segment was rinsed, blotted and weighed. Abdominal fat, including the inguinal and peritoneal pads, was removed and weighed. Serum genistein was analyzed by HPLC with CoulArray detection as previously described (13).

Statistical analyses. Tumor incidence data were analyzed using the CATMOD procedure of SAS (SAS Institute, Cary, NC). All other data were analyzed as a 2 × 5 factorial design with 2 genotypes (EraK0 and WT) and 5 diets using the General Linear Models (GLM) procedure. Differences among means were determined by the least-square (LSMEANS) component of GLM. Main effects and interactions were considered significant at P < 0.05.

RESULTS

The final body weight of EraK0 mice was less than that of WT mice (26.4 ± 0.3 vs 25.3 ± 0.4 g, P = 0.035). There was no interaction between diet and genotype but diet affected body weight. The mice fed Soy+Gen, Soy+NSoy or Soy+E1 weighed more at the end of the study than mice fed Soy−IF or casein (Table 1). There generally was more abdominal fat (g/100 g) in the heavier mice, which may partially explain the difference in body weight. Genistein was highest in serum from mice fed Soy+Gen, elevated in mice fed Soy+NSoy and very low in the other groups (Table 1).

Tumor incidence was not affected by genotype, nor was there a diet × genotype interaction. However, diet affected tumor incidence. Tumor incidence in EraK0 and WT mice was between 70 and 80% of that in mice fed casein or Soy−IF (Fig. 1). Mice fed Soy+Gen had a tumor incidence that did not differ from mice fed Soy−IF and casein. Tumor incidence was lower in mice fed Soy+NSoy than in mice fed casein but not compared with those fed Soy−IF or Soy+Gen. Mice fed estrone (Soy+E1) had lower tumor incidence than those fed Soy−IF or casein. Tumor incidence did not differ among mice fed Soy+Gen, Soy+NSoy or Soy+E1.

Tumor burden (total tumor weight/mouse) and tumor multiplicity (tumors/mouse) were not affected by genotype, nor was there a diet × genotype interaction. However, both were affected by dietary treatment. Mice fed soy protein diets,

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### TABLE 1

Final body weight, body fat and intestine relative weights and serum genistein concentration in ovariectomized mice fed diets containing casein (Casein), soy without IF (Soy−IF), soy with genistein (Soy+Gen), soy with Novasoy (Soy+NSoy) or soy with E1 (Soy+E1).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight</th>
<th>Abdominal fat</th>
<th>Duodenum weight</th>
<th>Jejunum weight</th>
<th>Ileum weight</th>
<th>Colon weight</th>
<th>Genistein2</th>
<th>n</th>
<th>g/100 g body</th>
<th>nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>26</td>
<td>24.5 ± 0.58b</td>
<td>1.85 ± 0.24b</td>
<td>2.47 ± 0.13</td>
<td>2.17 ± 0.12a</td>
<td>1.95 ± 0.05a</td>
<td>1.90 ± 0.10a</td>
<td>4.6 ± 4.6c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy−IF</td>
<td>30</td>
<td>24.9 ± 0.54a</td>
<td>2.49 ± 0.33a</td>
<td>2.21 ± 0.12</td>
<td>1.71 ± 0.07b</td>
<td>1.56 ± 0.04b</td>
<td>1.37 ± 0.10b</td>
<td>19.1 ± 5.0c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy+Gen</td>
<td>23</td>
<td>26.4 ± 0.62a</td>
<td>3.01 ± 0.26a</td>
<td>2.11 ± 0.14</td>
<td>1.71 ± 0.13b</td>
<td>1.64 ± 0.07b</td>
<td>1.12 ± 0.10b</td>
<td>1250.4 ± 43.0b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy+NSoy</td>
<td>30</td>
<td>26.7 ± 0.54a</td>
<td>3.04 ± 0.27a</td>
<td>2.35 ± 0.12</td>
<td>1.78 ± 0.16b</td>
<td>1.40 ± 0.08b</td>
<td>1.29 ± 0.10b</td>
<td>232.8 ± 54.0b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy+E1</td>
<td>27</td>
<td>26.8 ± 0.57a</td>
<td>3.29 ± 0.32a</td>
<td>2.28 ± 0.13</td>
<td>1.58 ± 0.05b</td>
<td>1.46 ± 0.07b</td>
<td>1.08 ± 0.10b</td>
<td>33.3 ± 22.0c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-value³</td>
<td>0.007</td>
<td>0.011</td>
<td>0.362</td>
<td>0.0003</td>
<td>0.0037</td>
<td>0.0008</td>
<td>0.0013</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Means in a column with superscripts differ, P < 0.05 (ANOVA and LSMEANS).
2 Data were log transformed.
3 Effect of diet, P ≤ 0.05 was considered significant.
regardless of the addition of estrogenic compounds, had lower tumor burden and multiplicity compared with mice fed casein (Fig. 2). The addition of E1 to the soy protein diet tended to further reduce colon tumor weight (P = 0.008). The relative weights of the colon, jejunum and ileum, but not duodenum, were significantly less in mice fed soy protein compared with casein (Table 1). An exception was the relative ileum weight in mice fed Soy+Gen, which was not different from the casein-fed mice.

DISCUSSION

Soy consumption is increasing in the United States due to an awareness of the health benefits of soy. The FDA recently approved a health claim for reducing cardiovascular disease that recommends 25 g of soy protein/d (21 CFR 101.82). Periand postmenopausal women are using soy and soy extracts containing IF as an alternative to HRT, and NSoY or similar commercial soy extracts are increasingly being marketed in food products. In this study, we attempted to separate the effects of soy protein, genistein and a soy extract on colon tumorigenesis. We observed no protection from AOM-induced colon tumorigenesis due to soy protein alone. The addition of NSoY to soy protein, however, reduced tumor incidence compared with casein-fed mice. This finding agrees with two previous reports. Reduced tumor incidence was observed in AOM-treated rats fed soy protein isolate containing a similar amount of IF (14). A reduction in aberrant crypt foci, purported precancer lesions, was observed in AOM-treated rats fed soy flake or soy flour containing IF compared with soy concentrate without IF (15). Two other studies, however, were not in agreement. More aberrant crypt foci were observed in AOM-treated rats fed soy protein with IF compared with soy protein without IF (16), and no difference in tumorigenesis was reported in Apc<sup>Minn</sup> mice fed low IF or IF-rich soy protein (17). In our study, genistein added to a soy protein diet did not protect mice from colon tumorigenesis. In contrast, a smaller dose of genistein (167 mg/kg diet) with soy protein reduced aberrant crypt foci by 50% in AOM-treated rats (15). A previous report also found that genistein added to a casein diet did not affect tumorigenesis in AOM-treated rats (18), but tumor multiplicity was higher in rats fed genistein compared with rats fed a casein diet without genistein. We found genistein to have no effect on tumor multiplicity, although soy protein had an independent protective effect that may have superceded a response to genistein. Furthermore, our study was done using ovariectomized female mice, whereas most previous studies have used male rats (14-18). It is possible that the response to AOM and/or the dietary treatments differs between males and females and between rats and mice.

Our observation that soy protein reduced colon tumor weight and multiplicity in mice compared with casein was independent of estrogenic compounds in the diet. This protective effect of soy protein may have been due to reduced cell proliferation in the colon because the relative colon weight was lower in these mice compared with those fed casein. Analysis of colonic cell proliferation and apoptosis will be presented in a subsequent report. The components of soy protein that may have provided the protective effect are not known. Dietary fiber has long been considered to reduce colon cancer development. Although our five diets contained the same amounts of cellulose, soy protein may contain other forms of fiber that may have altered gut microflora, thereby reducing tumor cell growth. It is important to note that we added phytoestrogens to the casein diet to eliminate this variable from the study; however, exogenously added phytoestrogen may have physicochemical properties that differ from endogenous phytoestrogen in the soy.

Human colon cancer cell lines have been found to express primarily ERβ (19,20). E2 (10-1000 nmol/L) consistently inhibited cell proliferation in colon tumor cells expressing ERβ (19). Furthermore, in colon cells that expressed ERβ but not ERα, E2 induced apoptosis in a dose-dependent manner, but in cells that expressed both receptors, E2 had no effect on apoptosis (20). Normal human colon expressed both ERα and ERβ, but in malignant tissue ERβ was selectively lost (20). Hence, ERβ appears to be the dominant receptor in the colon and to play a role in cell proliferation or metabolism. These two receptors may function in opposite ways, i.e., ERβ activates apoptosis whereas ERα acts as a survival factor (20). In the present study, NSoY and E1 reduced tumorigenesis in both WT and ERαKO mice; hence, this effect was independent of ERα. Several hypotheses to explain this observation are possible. One hypothesis is that both NSoY and E1 mediate their protective effects via ERβ. To examine this, we are repeating the experimental design in ERβKO mice. Another hypothesis is that NSoY and E1 alter ER-independent pathways in the colon. Soy IF, in particular genistein, are enzyme inhibitors.

FIGURE 2 Tumor burden (total tumor weight, g/mouse) and tumor multiplicity (number of tumors per mouse) in ovariectomized mice fed diets containing casein (Casein), soy without IF (Soy-IF), soy with genistein (Soy+Gen), soy with NSoY (Soy+NSoY) or soy with E1 (Soy+E1). Values are means ± SEM, n = 23-30. Means for a variable with unlike letters differ, P < 0.005 (ANOVA and least-square means).
However, our data do not support the concept that IF provide protection primarily via inhibition of intracellular signaling pathways related to tyrosine kinases because genistein did not reduce tumorigenesis. NSoy contains a mixture of compounds in addition to IF. Saponins are a major component of this product and have been reported to reduce aberrant crypt foci in mice (21) and to inhibit colon tumor cell growth in vitro (22). Bowman-Birk inhibitor (BBI) may also be present in NSoy and has been found to inhibit carcinogen-induced tumorigenesis in rats (23). The mechanisms through which saponins or BBI alter colon tumorigenesis are not known; however, it is unlikely that these effects are mediated via ER. Furthermore, the IF in NSoy are primarily in the glucoside form, which may have a very different effect from aglycones on the gastrointestinal tract (24). Perhaps the presence of slowly digested IF glucosides in the colon influenced gut microflora or contents (e.g., bile salts) which offered protection from tumorigenesis (25). It is also feasible that soy protein has an independent effect on colon microflora (25).

The most effective tumor protection in our study was oral administration of E1. This is the first report to our knowledge in which oral E1 was shown to be protective of colon tumorigenesis in an animal model. The metabolism of estrogens in the colon has not been well described, although 17βHSD activity is present in colon. Using immunocytochemistry, 17βHSD2 and 17βHSD4 were localized in the colon epithelial cells primarily in the luminal region (11). Immunoreactivity was reduced in colon tumor tissue and was further reduced in the tumor. The activity of these isoenzymes is primarily oxidative, resulting in inactivation of E2 via conversion to E1; hence inactivation of E2 may be important for colon cell regulation. It has been proposed that loss of E2 inactivation by colon tumors may lead to increased cell proliferation, supposedly via an ER-dependent pathway (9,10). Antisense oligodeoxynucleotides to ER were found to inhibit E2-stimulated growth in a mouse colon tumor cell line; unfortunately the specificity of the oligodeoxynucleotides for ERα or ERβ was not defined (26). Although our study does not specifically address the question of E2 stimulation of colon tumor cell proliferation, we showed that E1 does not require ERα to provide protection from colon tumorigenesis. Many metabolites of E2 and E1 are generated by the cytochrome P450 enzymes, which may function as local mediators of cellular events via ER-independent mechanisms (27). Hence, the observed protection from colon tumorigenesis by E1 may be mediated by such locally generated metabolites. Our observation that E1 reduced colon tumorigenesis corroborates evidence from the Women’s Health Initiative that HRT may be protective of colon cancer (7) and provides a new approach for colon cancer prevention.

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LITERATURE CITED