The influence of flaxseed and lignans on colon carcinogenesis and β-glucuronidase activity

Mazda Jenab and Lilian U. Thompson

Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

To whom correspondence should be addressed

Flaxseed, the richest source of mammalian lignan precursors, such as secoisolariciresinol diglycoside (SD), has been shown over the short term to decrease some early markers of colon cancer risk. This study determined whether over the long term flaxseed still exerts a colon cancer protective effect, whether its effect may, in part, be due to its high content of SD and whether any change in β-glucuronidase activity plays a role in the protective effect. Six groups of male Sprague–Dawley rats were fed for 100 days either a basal high fat (20%) diet (BD), BD supplemented with 2.5 or 5% flaxseed or 2.5 or 5% defatted flaxseed (equivalent to the respective flaxseed diets) or BD with a daily gavage of 1.5 mg SD. All rats were injected with a single dose of azoxymethane (15 mg/kg body wt) 1 week prior to commencing the dietary treatments. Urinary lignan excretion, which is an indicator of mammalian lignan production, was significantly increased in the flaxseed and defatted flaxseed groups. The total activity of cecal β-glucuronidase was significantly increased in a dose-dependent manner by the flaxseed and defatted flaxseed diet groups. Compared with the control the number of aberrant crypts per focus was significantly reduced in the distal colon of the treated rats. Four microadenomas and two polyps were observed in the control group, but not in the treated groups. The total activity of β-glucuronidase was positively correlated with total urinary lignan excretion and negatively with the total number of aberrant crypts and the total number of aberrant crypt foci in the distal colon. There were no significant differences between the flaxseed and the corresponding defatted flaxseed groups. It is concluded that flaxseed has a colon cancer protective effect, that it is due, in part, to SD and that the protective effect of flaxseed is associated with increased β-glucuronidase activity.

Introduction

Diet has been suggested to be the most important environmental factor in the development of colon cancer, with one high in fiber and low in fat associated with a decreased risk (1). Despite research efforts on the specific role of fat and fiber in colon carcinogenesis, the results are inconsistent (2), suggesting the involvement of other substances associated with fiber-rich foods, such as lignans.

Lignans are constituents of higher plants, many with a 2,3-dibenzylbutane skeleton structure which is formed from the oxidative coupling of p-hydroxyphenylpropene units (3). Mammalian lignans, primarily enterolactone (EL*) and enterodiol (ED) are formed from plant lignans in the diet by facultative bacteria in the colon (4). ED and EL have been suggested to be protective in both breast and colon cancer (5–8), because of their anti-oxidant (5,9), anti-proliferative (10), weak estrogenic/anti-estrogenic (11), anti-angiogenic (12) and anti-aromatase (13) activities. They can also inhibit 7α-hydroxylase activity (14) and increase sex hormone binding globulin synthesis (15).

Flaxseed is the richest source of mammalian lignan precursors, such as secoisolariciresinol diglycoside (SD; 16) and therefore has been used to demonstrate the protective effect of mammalian lignans on carcinogenesis. Flaxseed has been shown to significantly reduce nuclear aberration in mammary epithelial cells (7,17) and mammary tumor size at the promotion (7) and later stages of carcinogenesis (18). Purified SD produced similar effects as flaxseed (18), suggesting that the mammary cancer protective effect of flaxseed may, in part, be due to its lignans.

Since the mammalian lignans are produced in the colon, they could be expected to exert their primary effects at that site. In a 28 day feeding study in carcinogen-treated rats flaxseed and defatted flaxseed have been shown to significantly reduce epithelial cell proliferation and the number of aberrant crypts (AC) and aberrant crypt foci (ACF) (6), which are deemed to be early markers of colon cancer risk (19). The effect of flaxseed at the 5 and 10% levels was not dose related (6), but it remains to be determined whether similar results can be seen at levels less than 5% and at feeding periods longer than 28 days. The effect of SD on colon cancer has not yet been studied, so it remains to be established whether the effect observed with flaxseed can be attributed to its lignans.

The mammalian lignans undergo enterohepatic circulation, i.e. they are absorbed through the colon, conjugated with glucuronic acid or sulfate in the liver, excreted back into the colon via the bile duct, deconjugated through the action of β-glucuronidase and re-absorbed. β-Glucuronidase is an inducible enzyme which is also involved in the enterohepatic circulation and activation of procarcinogens, carcinogens, mutagens and toxins and may be associated with an increased risk for colon cancer (20). It is of interest to observe how the mammalian lignans will influence β-glucuronidase activity.

Therefore, the purpose of this study was to determine, over the long term, whether: (i) flaxseed has a colon cancer protective effect without causing any gross toxicity; (ii) its effect is, in part, due to its mammalian lignan precursor (SD); (iii) its effect is dose dependent at low levels of intake; (iv) the mammalian lignans result in an increase in cecal β-glucuronidase activity.

Materials and methods

Isolation of SD

The SD was isolated from flaxseed as described previously (21). Briefly, flaxseed was defatted with petroleum ether and chloroform and then extracted...
with dioxane:ethanol (1:1 v/v). The residue was dried and degraded with 1.6% sodium methoxide in anhydrous methanol. The extract was concentrated and then acidified to pH 3.0 with 2 M sulfuric acid and applied to a silica gel column (0.063-0.200 mm, Kieselgel 60; Merck) and then eluted with the lower phase of the solvent mixture chloroform:methanol:water (65:35:10 by volume). The crude SD was purified on a second silica gel column and eluted using chloroform with increasing amounts of methanol (10-30%). Its purity was 99.1% as determined by co-thin layer chromatography with an authentic standard, HPLC, proton- and 13C nuclear magnetic resonance, UV spectroscopy, FAB-mass spectroscopy and gas chromatography-mass spectroscopy (GC-MS) measurements.

**Experimental design**

Sixty one male Sprague-Dawley rats (40 days old; Charles River Inc., Montreal, Canada) were maintained in individual stainless steel cages at an ambient temperature of 22-24°C on a 12 h light/dark cycle. They were acclimatized for 2 weeks on the AIN-76A high corn oil (20%) basal diet (BD) (22) and then injected with 15 mg/kg body wt of the colon carcinogen azoxymethane (Sigma Chemical Co., St Louis, MO). One week later they were randomized into six groups with 16 rats in the control group and nine rats in each of the treatment groups, such that the mean weight of each group was equal. They were fed either the BD (control group), BD plus a daily gavage of 1.5 mg SD in 1.0 ml saline solution (SD group) or BD supplemented with either 2.5% flaxseed (2.5F group), 2.5% defatted flaxseed (2.5DF group), 5% flaxseed (5.0F group) or 5% defatted flaxseed (5.0DF group). The BD consisted of the following by weight: 20% casein, 20% corn oil, 24.63% corn starch, 2% sodium chloride, 5% alphacel, 4% AIN-93G mineral mix, 1.2% AIN-76A vitamin mix, 0.24% choline bitartrate and 0.3% DL-methionine. All diet ingredients were from ICN Biochemicals (St Laurent, Quebec, Canada), except the flaxseed (Linott variety; Omega Products, Melfort, Saskatchewan). Defatting was by the supercritical CO2 extraction process of Vitamins Inc. (Chicago, IL). The flaxseed or defatted flaxseed were ground prior to use and added to the diet, correcting for the amount of fat, protein and fiber in them so that all the diets were isocaloric. All rats not in the SD group were gavaged daily with 1 ml saline. The defatted flaxseed diets contained the same amount of non-flaxseed constituents as the respective flaxseed diets. Thus the effect of the oil may be differentiated from that of the non-flax constituents of flaxseed. The 1.5 mg SD corresponded to the amount of SD ingested by the 2.5DF group. The 1.5 mg SD corresponded to the amount of SD ingested by the 2.5DF group.

After 100 days treatment the rats were sacrificed by CO2 gassing. All the cecal content was diluted 1:5 with 0.2% CuSO4 solution (16), modified so that detection was by a GC-MS technique (Brinkman Instruments Sonicator; Brinkman Ultrasonics, Danbury, CT) and centrifuged at 10 000 r.p.m. (RC-5B Refrigerated Superspeed Centrifuge; DuPont Co., Wilmington, DE) for 20 min at 4°C to remove particulate matter. The supernatant was collected and stored in 1 ml aliquots at -70°C until needed. Before analysis extracts were thawed for 15 min at room temperature, after which 0.1 ml was incubated with 0.1 ml phenolphthalein β-D-glucoronide (Sigma Chemical Co.) in 0.8 ml phosphate-buffered saline, pH 7.0, for precisely 1 h at 37°C. After incubation the reaction was terminated by the addition of 2.5 ml alkaline glycine solution, 1.0 ml 5% trichloroacetic acid solution and 1.5 ml distilled water. The color was allowed to develop for 10 min and absorbance was measured at 540 nm. The phenolphthalein released was estimated based on a phenolphthalein standard curve. The specific activity was calculated as nmol phenolphthalein released/mg cecal protein/min, while total activity of β-glucuronidase was nmol phenolphthalein released/cecum/min. Protein was determined using a biinchoninic acid kit from Pierce Chemical Co. (Rockford, IL).

Urinary mammalian lignans (ED and EL) were analyzed according to Thompson et al. (16), modified so that detection was by a GC-MS technique instead of GC with a flame ionization detector.

The pH of the cecal contents was assessed using a flat surface pH electrode (Beckman Instruments Inc., Fullerton, CA). To determine the SCFA 0.35 g cecal content was diluted 1:5 with 0.2% CuSO4 solution for 15 min with a polytron (Brinkman Instruments) and then centrifuged for 10 min at 13 000 r.p.m. in a centrifuge (RC-5B Refrigerated Superspeed Centrifuge). The supernatant was analyzed for SCFA by the HPLC method of McBurney and Thompson (26).

**Statistical analysis**

All data were analyzed by analysis of variance followed by t-tests or by Duncan’s multiple range test using the SAS system (SAS Institute, Cary, NC). Regression analyses were also performed on: (i) total β-glucuronidase activity versus level of flaxseed or defatted flaxseed diet; (ii) total β-glucuronidase activity versus total urinary excretion of ED and EL; (iii) mean number of AC per focus versus total urinary excretion of ED and EL; (iv) total β-glucuronidase activity versus total number of AC; (v) total β-glucuronidase activity versus total number of AC.

**Results**

**Aberrant crypt formation**

No significant differences were seen in the total number of AC in the distal colon (Table I). In the proximal sections all groups had lower total numbers of ACF than the control, but only that in the 2.5DF group was significantly lower (P < 0.02). The numbers of AC in the proximal and distal colon were lower in all the treatment groups than in the control, but only that in the distal colon in the SD group reached significance (P < 0.01).

The AC multiplicity in the proximal section did not differ significantly amongst groups (Figure 1). However, in the distal sections the control group had a significantly higher number of AC per ACF than the SD (P < 0.01), 2.5F (P < 0.04), 2.5DF (P < 0.02), 5.0F (P < 0.04) and 5.0DF (P < 0.01) groups. The treatment groups did not differ amongst themselves.

Figure 2 shows the number of ACF with a given number of AC. The control group has consistently more ACF of any size than all the other groups. In addition, the control group had four microadenomas and two polyps, neither of which were found in any of the other groups.

Significant negative correlations were observed between the levels of flaxseed and defatted flaxseed and the number of AC per focus (r = -0.38, P < 0.006; n = 52).

**β-Glucuronidase activity**

Compared with the control the specific activity of cecal β-glucuronidase was increased in the SD, 2.5DF, 5.0F and 5.0DF groups, but only the latter was significantly different (P < 0.03; Table II). All treatment groups had increased total activity compared with the control group, with the 2.5DF (P < 0.01),
5.0DF (P < 0.0004) and 5.0F (P < 0.047) groups all significantly higher. There were no significant differences amongst the treatment groups for either specific or total activity.

The total activity of β-glucuronidase had a significant negative correlation with the total number of ACF (r = -0.310, P < 0.018, n = 57) and the total number of AC (r = -0.330, P < 0.010, n = 57) in the distal colon (Figure 3).

Urinary lignan excretion

The total urinary ED and EL was significantly lower in the control than in the 2.5F (P < 0.0003), 5.0F (P < 0.0001) and 2.5DF (P < 0.0001) groups, but not in the SD group (Figure 4). The 5.0DF group also had significantly higher lignan excretion than the SD (P < 0.001), 2.5F (P < 0.001) and 2.5DF (P < 0.001) groups. More EL than ED was produced in all groups.

Total urinary ED and EL excretion had a significant positive relationship with: (i) the total activity of β-glucuronidase (r = 0.280, P < 0.036, n = 60); (ii) the intake level of flaxseed (r = 0.780, P < 0.0001, n = 57). A significant negative correlation between total urinary lignan excretion and mean number of AC per ACF was observed (r = -0.340, P < 0.036, n = 57).

Cecal SCFA production, pH, weight gain, food intake and organ size

No significant differences in the above variables were observed amongst the six groups.

Discussion

This study has shown that over the long term flaxseed and defatted flaxseed can cause a significant reduction in AC multiplicity, suggesting their protective effect against colon cancer. That the oil level in the 2.5 and 5% flaxseed diets was...
not responsible for the effect seen is evident from the similar effects of flaxseed and defatted flaxseed. SD had an effect on AC formation that did not differ significantly from that of flaxseed and defatted flaxseed, indicating that the protective effect of flaxseed can be attributed to SD. A significant correlation between urinary lignan excretion and AC further supports the hypothesis that the mammalian lignans derived from SD are partly responsible for the cancer protective effects of flaxseed.

ACF formation was used as an indicator of protective effects in this study, since ACF are thought to be valid putative preneoplastic markers for colon carcinogenesis (19). (i) They have a mean proliferative activity that is three to four times greater than that in adjacent normal crypts and is comparable with that in benign and malignant colon tumors (27); (ii) they have mutations in the p53 tumor suppressor gene (28) and the k-ras oncogene (29), which are also found in many colon tumors; (iii) their induction and distribution is correlated with formation of colon tumors (30).

The protective effects of flaxseed in this longer term (100 day) study are in agreement with the short-term (28 day) study of Serraino and Thompson (6). However, the latter study showed a significant reduction (41–58%) in the numbers of AC and ACF with flaxseed intake, while in this study significant reductions were seen primarily in AC multiplicity, as opposed to the numbers of AC and ACF. Since AC multiplicity has been shown to be a more important and consistent predictor of tumor incidence than the numbers of ACF or AC (31), this study is an even better predictor of the cancer protective effect of flaxseed. In fact, the control group, with significantly higher AC multiplicity (Figure 2), was shown to be progressing faster towards cancer formation, since it already had a total of four microadenomas and two polyps, while the treatment groups had none.

The SD group received a dose of 1.5 mg SD/day, which is about equivalent to the daily SD intake of the 5.0F and 5.0DF groups. However, urinary lignan excretion by the SD group was not significantly different from that of the control group and was significantly lower than that of the flaxseed and defatted flaxseed groups, even though SD had the same colon cancer protective effects as the flaxseed. This could be due to a balance of two conflicting, although equally plausible, possibilities. First, since β-glucuronidase activity induced by SD was not significantly different from that caused by flaxseed and defatted flaxseed, a possibility exists that pure SD is better metabolized to ED and EL by colonic bacteria and are then circulated enterohepatically more efficiently, retained more by the body, excreted less in the urine and thus able to be protective by virtue of their presence in the colon. Second, 1.5 mg pure SD was given daily to the rats in a single bolus, whereas the SD from the flaxseed diets was continuously ingested by the rats, along with other dietary components, such as fiber. The single bolus of SD may have overwhelmed the bacteria’s ability to effectively metabolize it to mammalian lignans. Any mammalian lignans formed from this SD bolus may not have undergone efficient enterohepatic circulation, due to the absence of other dietary components (32), and so were excreted more in the feces than in the urine. However, the presence of SD or secoisolariciresinol (hydrolyzed SD) in the colon may nonetheless confer cancer protection in that region without them necessarily being re-absorbed into the bloodstream. Since either one of these two mechanisms is feasible and both may even occur simultaneously, further studies are necessary to determine what is indeed occurring.

Mammalian lignans may exert their protective effects on the colon in a number of ways. Because of their diphenolic structure, they may act as anti-oxidants, by acting as free radical scavengers and inhibitors of lipid peroxidation (5,8,9). Extracts of flaxseed which contain mammalian lignan precursors have been shown to have an anti-oxidant effect (33). The anti-oxidative effect of some lignans has been shown to be more potent than that of vitamin E at the same concentrations (9).

Lignans have also been shown to be anti-mitotic (34) and anti-proliferative in vitro (10,35). Both ED and EL modulate the activities of 7α-hydroxylase, which is the rate limiting enzyme in the synthesis of bile acids from cholesterol (14). They also modulate the activity of acyl CoA:cholesterol acyltransferase, which is involved in the production of cholesterol esters (14). Modulation of these two enzymes could affect cholesterol catabolism and the production of secondary bile acids, which are thought to be even more active promoters (36).

Both estrogen and progesterone receptors have recently been identified in some normal human colonic mucosa (37), in some colon tumors (38) and in some colon cancer cell lines (39).

![Fig. 3. Correlation between total activity of β-glucuronidase and the number of aberrant crypts and the number of aberrant crypt foci in the distal colon.](image-url)

![Fig. 4. Total urinary lignan excretion. ED, enterodiol; EL, enterolactone.](image-url)
The presence of these receptors indicates a possible modulation of colonic mucosal cell proliferation and a potential role in colon carcinogenesis by estrogens. In some colon carcinomas the estrogen receptors have been identified as mostly type II receptors (39), the presence of which is indicative of the state of estrogen responsiveness of the tissues (40). Interestingly, lignans and isoflavones have high affinities for type II estrogen receptors in the rat uterus (41). This affinity could also hold for the type II estrogen receptors identified in colon carcinomas. Thus mammalian lignans may modulate the effects of estrogens on both normal and cancerous cells in the colon.

The initial weight, final weight, weight gain and food intake did not differ amongst the six groups, suggesting that none of the diets was more palatable than the others and that any effects seen are not due to variations in food intake or weight differences. Likewise, the lack of significant differences in organ weights indicates that there were no gross toxic effects from the SD or flaxseed diets.

Total β-glucuronidase activity was significantly correlated with level of flaxseed or defatted flaxseed and both were significantly associated with urinary lignan excretion. Therefore, it is likely that flaxseed and defatted flaxseed provided the ED and EL glucuronides as substrates for β-glucuronidase.

Since the SCFA and pH did not differ significantly amongst the groups, it is unlikely that the protective effect of flaxseed is related to the fermentation of its fiber, a large portion of which is mucilage (42).

Increased bacterial β-glucuronidase activity is thought to be associated with an increased risk of colon cancer, as it can increase the enterohepatic circulation of carcinogens and mutagens, as well as estrogens (20,36). However, in this experiment flaxseed or defatted flaxseed supplementation increased β-glucuronidase activity while at the same time exerting a colon cancer protective effect. It can only be speculated how this could have taken place.

Normally ~ 80% of biliary estrogen glucuronide conjugates are deconjugated and re-absorbed to undergo enterohepatic circulation (43). With flaxseed consumption, however, these biliary estrogen conjugates may have competed with large amounts of ED and EL glucuronides for β-glucuronidase, although β-glucuronidase has the same affinity for all three conjugates (44). This experiment showed that with increases in flaxseed and defatted flaxseed levels from 2.5 to 5% β-glucuronidase activity was increased at most 1.25 times for specific activity and 1.75 times for total activity, whereas total urinary lignan excretion (which represents only a fraction of biliary lignan excretion) was increased 600–1500 times. Thus the β-glucuronidase enzyme may have been overwhelmed by the dramatic increase in ED and EL glucuronides and may not have been able to act on all of them. Since the concentration of ED and EL glucuronides is presumably considerably larger than that of any other biliary conjugates, including those of the estrogens, it is possible that they compete for β-glucuronidase action and force excretion of the estrogen conjugates in the feces. This may thus reduce the levels of re-absorbed and circulating estrogens.

The increased β-glucuronidase activity could also affect enterohepatic circulation of other hormone conjugates, such as testosterone glucuronides in males, and hence affect other hormone-related cancers, like prostate cancer. It may be a factor in the observed breast cancer protective effects of flaxseed (7,17,45). The effects of flaxseed and lignans on estrogen excretion, metabolism and enterohepatic circulation need much further study and analysis.

There are no significant differences between the 2.5F and 2.5DF nor the 5.0F and 5.0DF groups in either total or specific activities of β-glucuronidase, ACF formation or urinary lignan excretion. This suggests that at the 2.5–5% levels of intake the effects of the lignan precursors in flaxseed and defatted flaxseed are more important than the effects of the oil component. However, the oil may also play a beneficial role at higher levels of intake. Flaxseed oil is one of the richest dietary sources of α-linolenic acid (ALA), an n-3 fatty acid (46). Dietary fish oils, which have high concentrations of n-3 fatty acids (especially eicosapentaenoic acid and docosahexaenoic acid) have been found to be protective against colon cancer, while oils rich in n-6 fatty acids have been found to be tumor promoting (47,48). ALA, eicosapentaenoic acid and docosahexaenoic acid directly compete for enzymes involved in the eicosanoid synthesis process and so inhibit the production of series 2 prostaglandins from arachidonic acid (49). These prostaglandins suppress many immune cell functions and may allow tumor cells to escape the immunosurveillance system and thus to proliferate and metastase (49).

It is concluded that flaxseed and defatted flaxseed have a colon cancer protective effect and that this effect is due, in part, to SD and not the oil at the 2.5–5% levels of intake. The colon cancer protective effects of flaxseed, defatted flaxseed and SD are associated with a dose-dependent increase in cecal activity of β-glucuronidase and urinary lignan excretion, but not to SCFA production or pH.

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References


