

Dietary Flaxseed Inhibits Human Breast Cancer Growth and Metastasis and Downregulates Expression of Insulin-Like Growth Factor and Epidermal Growth Factor Receptor

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Abstract: Recent studies indicate that diets rich in phytoestrogens and n-3 fatty acid have anticancer potential. This study determined the effect of flaxseed (FS), the richest source of lignans and α -linolenic acid, on growth and metastasis of established human breast cancer in a nude mice model. Estrogen receptor-negative human breast cancer cells, MDA-MB-435, were injected into the mammary fat pad of mice (*Ncr nu/nu*) fed a basal diet (BD). At Week 8, mice were randomized into two diet groups, such that the groups had similar tumor size and body weight. One continued on the BD, while the other was changed to BD supplemented with 10% FS, until sacrifice at Week 15. A significant reduction ($P < 0.05$) in tumor growth rate and a 45% reduction ($P = 0.08$) in total incidence of metastasis were observed in the FS group. Lung metastasis incidence was 55.6% in the BD group and 22.2% in the FS group, while the lymph node metastasis incidence was 88.9% in the BD group and 33.3% in the FS group ($P < 0.05$). Mean tumor number (tumor load) of total and lymph node metastasis was significantly lower in the FS than in the BD group ($P < 0.05$). Metastatic lung tumor number was reduced by 82%, and a significantly lower tumor trend ($P < 0.01$) was observed in the FS group. Lung weight, which also reflects metastatic tumor load, in the FS group was reduced by 20% ($P < 0.05$) compared with the BD group. Immunohistochemical study showed that Ki-67 labeling index and expression of insulin-like growth factor I and epithelial growth factor receptor in the primary tumor were lower in the FS ($P < 0.05$) than in the BD group. In conclusion, flaxseed inhibited the established human breast cancer growth and metastasis in a nude mice model, and this effect is partly due to its downregulation of insulin-like growth factor I and epidermal growth factor receptor expression.

Introduction

Breast cancer is the most prevalent malignancy and the second leading cause of death from cancer in American

women (1). The death rate is primarily associated with metastasis, particularly for those patients with estrogen receptor (ER)-negative breast cancer. Therefore, a search for therapeutic modalities for prevention of metastasis is one of the primary areas in cancer research.

Diets rich in phytoestrogens and/or n-3 fatty acid have exhibited anticancer activity in several studies (2–4). Flaxseed is the richest source of the plant lignan secoisolariciresinol diglycoside (SDG), a phytoestrogen, and α -linolenic acid (α -LA), an n-3 fatty acid (2). The mammalian lignans enterolactone (EL) and enterodiol are formed by colonic bacterial action on SDG. Case-control studies have shown a significant inverse relationship between breast cancer risk and the urinary EL levels (5,6). Our previous studies showed that diet supplementation with flaxseed or its lignan SDG inhibits the initiation, promotion, and progression of mammary carcinogenesis in rats (7–10). Flaxseed also can inhibit experimental metastasis of murine melanoma cells in mice (11,12). α -LA in flaxseed can be metabolized to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). DHA and EPA in fish oil have been shown to inhibit ER-negative human breast cancer growth and metastasis in nude mice (4). Thus it is of interest to determine the effect of flaxseed on human breast cancer metastasis.

The mechanism by which flaxseed inhibits breast cancer is not clear, but it can include the weak estrogenic and antiestrogenic properties (2,3) and inhibition of activities of several steroid-metabolizing enzymes, such as aromatase, 5α -reductase, and 17β -hydroxysteroid dehydrogenase (13–16), by its lignan component. There is also evidence showing that the lignans exhibit nonestrogenic activities, such as antioxidant activity (17,18) and inhibition of cell membrane ATPase (19,20) and angiogenesis (21). In addition, flaxseed and SDG can downregulate the secretion of plasma insulin-like growth factor I (IGF-I) level in rats (22). However, there is no direct evidence showing that flaxseed can modulate tumor signal transduction pathways to regulate growth and metastasis in human breast cancer cells.

The objective of this study was to determine the effect of flaxseed on the growth and metastasis of ER-negative breast

cancer cells in a nude mice model and to explore the possible mechanisms associated with this effect.

Materials and Methods

Animals and Diets

Twenty-four female athymic nude mice (Ncr *nu/nu*) aged 3–4 wk were purchased from Simosen Laboratories (Gilroy, CA) and maintained in microisolator cages (4 per cage) within a pathogen-free isolation facility with a 12:12-h light-dark cycle at 22–24°C and 50% humidity. Animal care and use conformed with the *Guide to the Care and Use of Experimental Animals* (23), and the experimental protocol was approved by the University of Toronto Animal Care Committee. Animals were given free access to a semisynthetic, high-fat basal diet (BD) alone or supplemented with full-fat flaxseed (FS diet). The BD was based on the AIN-93G formulation modified to have a high fat content (20% corn oil) at the expense of cornstarch. The FS diet was the BD supplemented with 10% freshly ground flaxseed (Linott variety, Omega Products, Melfort, SK, Canada) corrected for the flaxseed's contribution to fat, fiber, and protein components so that the energy values of the diets were the same. Diets were prepared by Dyets (Bethlehem, PA) and sterilized by irradiation with ⁶⁰Co (Isomedix, Whitby, ON, Canada). Prepared diets were stored at 4°C until use. Fresh diet was provided every 2 days, and the food intake was recorded. The animal body weight was monitored weekly.

Cell Line

The estrogen-independent MDA-MB-435 human breast cancer cell line (a kind gift from Dr. Janet Price, Dept. of Cell Biology, M. D. Anderson Cancer Center, Houston, TX) was maintained in Iscove's modified Dulbecco's medium (Life Technologies, Burlington, ON, Canada) supplemented with 5% fetal bovine serum (Sigma, Oakville, ON, Canada) plus penicillin (100 U/ml) and streptomycin (1 µg/ml). The cells were grown to 70–90% confluence in T-75 flasks and fed fresh medium 1 day before cell harvest. For injection, the cells were trypsinized and resuspended in serum-free medium at 2×10^7 cells/ml on ice. Cell viability, >95%, was determined by trypan blue exclusion assay.

Experimental Design

After 7 days of acclimatization, mice were anesthetized with an intraperitoneal injection of ketamine-xylazine (24). A 2- to 3-mm incision on the right flank was cut, and the mammary fat pad was exposed. A total volume of 50 µl of cell suspension containing 1×10^6 cells was injected into the mammary fat pad, and the incision was closed with Vet-bound (3M Animal Care Products, St. Paul, MN). The mammary fat pad tumors were palpated weekly. Tumor surface area was calculated using the following formula: $(\text{length}/2 \times \text{width}/2) \times \pi$. At Week 8, the mice were randomized into two

groups ($n = 12$), such that the groups had similar tumor size and body weight; one continued on the BD, while the other was changed to the FS diet. Body weight and mammary fat pad tumor were monitored weekly. Three mice of each group died at Week 14 because of severe tumor ulceration and terminally ill condition. Because the data from these mice were similar, they were excluded in the data calculation for mice sacrificed at Week 15. All remaining mice were sacrificed at Week 15 with CO₂ asphyxiation. At necropsy, body weight, primary tumor weight and volume, and weights of major organs, including lungs, were recorded. Primary tumor volume was calculated on the basis of the following formula: $(\text{length}/2 \times \text{width}/2 \times \text{thickness}/2) \times \pi$. The number of metastatic tumors in the lymph nodes (only those in the distant lymph nodes, not those fused with the primary tumor) and other organs was observed. After fixation in 10% buffered formalin, the number of lung tumors was counted under the stereomicroscope.

Immunohistochemistry

The excised primary tumors were preserved in 10% buffered formalin, embedded in paraffin, and cut into 5-µm sections. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with aqueous 3% H₂O₂ for 10 min. The antigen was retrieved by heating sections in 0.01 M citrate buffer at pH 6.0 for 20 min in a microwave oven. The following primary antibodies were diluted optimally in diluent buffer (Dako, Mississauga, ON, Canada) that blocks nonspecific antigens: rabbit anti-human Ki-67 at 5 µg/ml (Dako), goat anti-human IGF-I at 10 µg/ml (Sigma), rabbit anti-human epidermal growth factor receptor (EGFR) at 5 µg/ml, and rabbit anti-human vascular epithelial growth factor (VEGF) at 10 µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). The sections treated with primary antibodies were incubated at 4°C overnight. The biotinylated corresponding secondary antibodies, rabbit anti-goat IgG (Sigma) and swine anti-rabbit IgG (Dako), were incubated for 45 min at room temperature. Streptavidin-horseradish peroxidase and AEC substrate chromogen (Dako) were used to demonstrate the antigens. All slides were read blindly under a light microscope at $\times 400$ magnification. Over 1,000 cells from at least 5 different fields were counted. Ki-67 labeling index was calculated as follows: $\text{number of positive cells} \div \text{total cells counted} \times 100$. Expression of growth factors was assessed semiquantitatively using a 0–3 scoring system; that is, the number of stained cells was first counted, and the staining intensity was graded as negative (0), weak (1), intermediate (2), and strong (3) staining, and then the score was calculated by the following formula: $\text{score} = (1 \times \text{cell number} + 2 \times \text{cell number} + 3 \times \text{cell number}) / \text{total cell number}$.

Statistical Analysis

Values are means \pm SE. Analysis of variance with general linear model repeated-measures procedure was used to de-

Table 1. Body Weight and Food Intake of Nude Mice Fed Basal Diet or 10% Flaxseed^a

	Body Weight, g			Food Intake, g/day/mouse
	Week -1 ^b (n = 12)	Week 8 ^c (n = 12)	Week 15 ^d (n = 9)	
BD	12.0 ± 0.3	23.3 ± 0.4	27.2 ± 1.1	3.67 ± 0.31
FS	12.5 ± 0.2	23.2 ± 0.5	27.2 ± 0.4	3.72 ± 0.34

a: Values are means ± SE; n, number of mice. BD, basal diet; FS, 10% flaxseed diet.

b: Initial body weight.

c: Body weight at diet change.

d: Body weight at necropsy.

termine tumor growth difference between treatment groups over time. A χ^2 test was used to analyze the metastasis incidence. The metastatic lung tumor number was analyzed by the test of Armitage's trend in proportion (25) to determine whether a proportional trend, either upward or downward, with the ordering of the groups differs significantly. Differences between food intake, organ weight, primary tumor weight and volume, metastatic tumor number per tumor-bearing mouse or per mouse in a group, and immunohistochemistry scores were determined by *t*-test. The significance level was set at $P < 0.05$. All statistical analyses except the test of Armitage's trend in proportion were done using SPSS version 10.0 (Statistical Package for Social Sciences, Chicago, IL).

Results

Food Intake, Body Weight Gain, and Organ Weights

No significant difference in food intake or body weight change was recorded between treatment groups (Table 1). Weights of major organs, such as the liver, heart, spleen, kidney, ovary, and uterus, adjusted to body weight, were not significantly different (data not shown). However, the relative lung weight was significantly lower ($P < 0.05$) in the FS group (11.04 ± 0.51 g/kg body wt) than in the BD group (13.93 ± 0.99 g/kg body wt).

Primary Tumor Growth

Palpable primary tumor growth rate was significantly reduced ($P < 0.05$) in the FS group after 1 wk of diet separation, and this reduction continued until Week 14 (Fig. 1). At necropsy at Week 15, the final tumor volume (2.46 ± 0.23 and 3.38 ± 0.56 cm³ in FS and BD groups, respectively) and weight (3.51 ± 0.29 and 4.14 ± 0.68 g in FS and BD groups, respectively) were lowered by 27% and 15%, respectively, in the FS group, but not significantly, perhaps because of small sample size (9 per group).

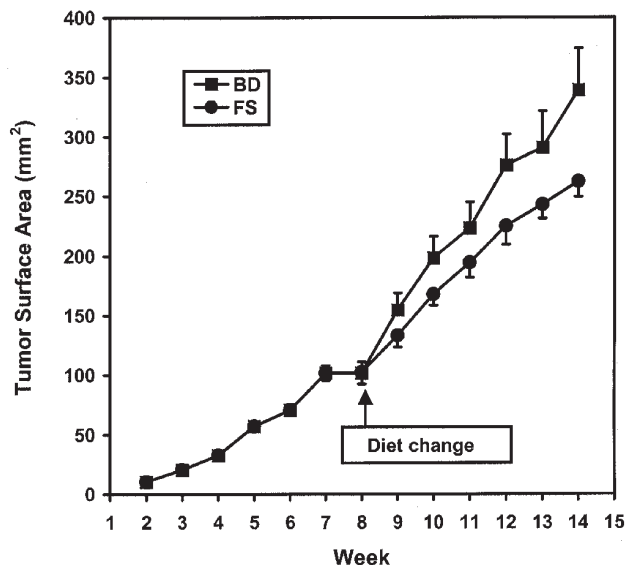


Figure 1. Effect of 10% flaxseed (FS) vs. basal diet (BD) on growth of established human breast cancer, MDA-MB-435, in nude mice. Values are means ± SE (n = 12). $P < 0.05$, FS vs. BD over time of treatment.

Metastasis

Figure 2 shows reductions in the metastasis incidence and the tumor number per mouse in the FS group. There was a strong tendency for reduction in total metastasis incidence in the FS group (44% reduction, $P = 0.08$). However, the reducing effect of the FS diet (66%) reached statistical significance ($P < 0.05$) only in the case of lymph node metastasis incidence. Although the 82% reduction in mean number of lung tumor in the FS group was not significant, a significant decrease of 20% in lung weight ($P < 0.05$), which also re-

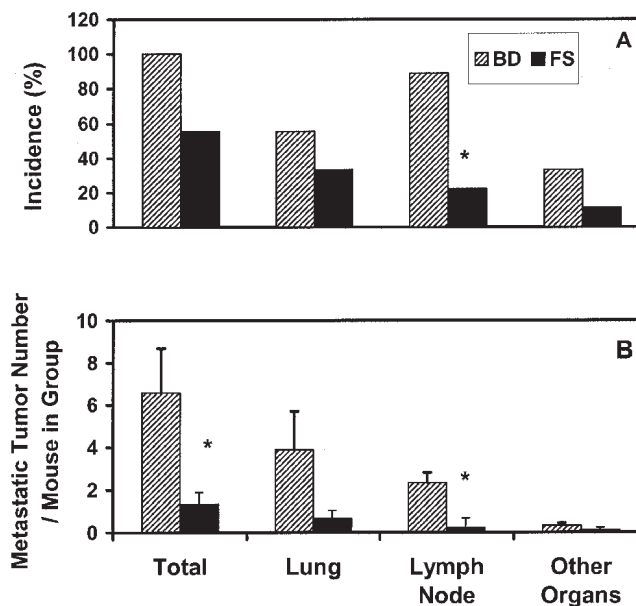


Figure 2. Effect of FS vs. BD on metastasis of established human breast cancer, MDA-MB-435, in nude mice. A: metastasis incidence. B: mean metastatic tumor number per mouse. Values are means ± SE. *, $P < 0.05$.

Table 2. Number of Mice With Lung Metastatic Tumor and Lung Tumor Number per Tumor-Bearing Mouse

	Mice With Lung Tumors				Lung Tumor Number/ Tumor-Bearing Mice	
	0 Tumor	1-4 Tumors	5-9 Tumors	>10 ⁴ Tumors	Median (Range)	Mean ± SE
BD	4	2	1	2	6 (2-15)	7 ± 2.49
FS	6	3	0	0	2 (1-3)	2 ± 0.57

a: BD vs. FS ($P < 0.01$) by Armitage's trend in proportion test (24).

flects lung tumor load, was recorded in the mice fed the FS diet (see above). A significant trend ($P < 0.01$) of lower tumor number was also found in the FS group (Table 2).

Immunohistochemistry

As shown in Fig. 3, a significant 25% lower rate of cell proliferation ($P < 0.05$), expressed as Ki-67 labeling index, was detected in the FS group compared with the BD group. The intensities of immunostaining of growth factors, IGF-I, EGFR, and VEGF, were reduced by 32.5%, 34.7%, and 17.5%, respectively, in the FS group, although only the expressions of IGF-I and EGFR in the FS group reached a significant level ($P < 0.05$).

Discussion

This study provides the first experimental evidence of an inhibitory effect of flaxseed on the growth and metastasis of established human ER-negative breast cancer in athymic nude

mice. The growth rate of solid tumor was reduced 1 wk after shift to the FS diet, and this reduction continued throughout the treatment until Week 14. Epidemiological studies have shown that the lignan EL, which is produced from flaxseed, can reduce the risk of breast cancer in pre- and postmenopausal women (5,6). Animal studies have shown that flaxseed supplementation can inhibit the progress of chemically induced mammary tumorigenesis in rats, particularly at the late stage (8), suggesting that flaxseed may also have a role in the treatment of breast cancer. Results from this study confirmed this therapeutic efficacy in ER-negative and highly malignant human breast cancer without influence on body weight gain and major organ weights.

The mechanism by which flaxseed inhibits ER-negative cancer cell growth is not clear. One of the suggested mechanisms by which flaxseed exerts its cancer-preventive effects is through the weak estrogenic and antiestrogenic properties of its lignans, which have structural similarity to estradiol. This can explain the inhibitory effects of flaxseed on rat mammary tumorigenesis induced by chemical carcinogens, most of which are ER-positive (26). However, this mechanism cannot elucidate the inhibitory effect on ER-negative cancer cells in this study. Therefore, other non-estrogen-related mechanisms may be involved, such as antioxidant activity (17,18) and inhibition of membrane ATPase (19,20), as described earlier. Our recent finding that flaxseed and SDG can reduce the plasma level of IGF-I in rats (22), however, suggests that flaxseed may also play a role in modulating the activities of signal transduction pathways. Indeed, the observed reduction in the expression of IGF-I and EGFR in the primary tumor by flaxseed supplementation in this study confirmed this and provides evidence that flaxseed may regulate breast cancer signal transduction pathways.

IGF-I and epidermal growth factor (EGF) belong to the tyrosine kinase family, known to be mitogenic, and play a crucial role in regulating breast cancer development. No study has previously shown that flaxseed and its components modulate these growth factors in human breast cancer. However, the lower plasma level of IGF-I in rats treated with flaxseed or SDG than in control rats (22) suggests that flaxseed may also have the potential to reduce autocrine expression of IGF-I in breast cancer cells. Considering that genistein, a phytoestrogen in soy with a structure similar to that of lignans, inhibits breast cancer through specific inhibition of tyrosine protein kinase, which blocks the signal transduction pathway (27), it is not surprising that flaxseed may also have such ability to in-

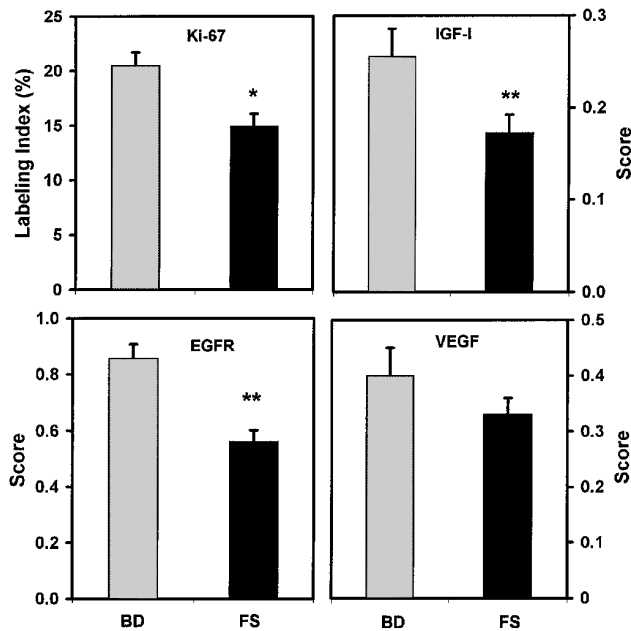


Figure 3. Effects of FS diet on cell proliferation and growth factor expression of established human breast cancer, MDA-MB-435, in nude mice. IGF-I, insulin-like growth factor I; EGFR, epidermal growth factor receptor. Values are means ± SE. *, $P < 0.05$; **, $P < 0.01$.

hibit expression of these growth factors in tumor cells, therefore inducing a reduction in cell proliferation and/or metastasis observed in this study.

Flaxseed may exhibit an antiangiogenesis potential on breast cancer because of its oil component (see below) and lignans, the latter having been reported to inhibit endothelial cell proliferation in vitro (21). The reduced level of IGF-I and EGFR in breast cancer cells by flaxseed may down-regulate VEGF expression, inasmuch as both growth factors can induce VEGF expression (28,29). However, in this study, the reduction in VEGF did not reach significance, perhaps because of the technique used in this study, inasmuch as VEGF isoform 121 diffuses freely into the extracellular space and cannot be detected by immunostaining (30,31). Indeed, in a separate study conducted similar to this study, where we used a microdialysis technique to collect extracellular fluid in the primary tumor, the VEGF measured in the microdialysate of large primary tumors showed a significantly lower level ($P < 0.05$) in the FS than in the BD group (unpublished data).

One of the mechanisms by which flaxseed exerts its inhibitory effects on breast cancer growth and metastasis may also be through its n-3 fatty acid α -LA, which can be metabolized to EPA and DHA. Rose and colleagues showed that EPA and DHA inhibit the tumor production of arachidonic acid-derived eicosanoids, including prostaglandin E_2 and 12-hydroxyeicosatetraenoic acid (12-HETE) (32), compounds known to be angiogenic in breast cancer (33). A recent study has shown that 12-HETE stimulates the production of type IV collagenase, which plays an important role in degradation of basement membrane, resulting in cancer cell metastasis (34). It has also been known that the 12-lipoxygenase pathway, which produces 12-HETE, can be activated by the EGF signal pathway (35), which in turn suggests that an inhibitor of the EGF signal pathway may reduce the production of 12-HETE and, consequently, inhibit angiogenesis.

To metastasize, a malignant cell or a group of cells must 1) initiate angiogenesis, 2) detach from the primary tumor, 3) attach to and invade/degrade the basement membrane, 4) transport through the circulation (lymphatic and systemic), 5) extravasate to a secondary organ, and 6) commence proliferation there (36). The reduction of metastasis in the mice fed the FS diet may be due to the intervention of flaxseed components on the steps of this metastasis cascade. Our previous in vitro studies have shown that the flaxseed components, α -LA and lignans (enterodiol and EL derived from SDG), can inhibit MDA-MB-435 cell adhesion to and invasion through Matrigel (a reconstituted basement membrane material) (37) and migration (unpublished data). The lower primary tumor growth rate induced by flaxseed through mechanisms described above may have contributed as well to the reduced metastasis.

In conclusion, the FS diet can reduce established ER-negative breast cancer growth and inhibit its metastasis to the distant organs, particularly lymph node and lungs, and this inhibitory effect may be partly due to the downregulation of IGF-I and EGFR expression. This suggests a poten-

tial use of flaxseed in the treatment of advanced human breast cancer with ER-negative status.

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