Flaxseed Alone or in Combination with Tamoxifen Inhibits MCF-7 Breast Tumor Growth in Ovariectomized Athymic Mice with High Circulating Levels of Estrogen

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Flaxseed (FS) is rich in mammalian lignan precursors and α-linolenic acid, which have been suggested as having anticancer effects. Previous studies have shown that 10% FS inhibits the growth of human estrogen–dependent breast cancer (MCF-7) in athymic mice, and it enhances the inhibitory effect of tamoxifen (TAM). This study determined whether the effect of FS, alone or in combination with TAM, is dose dependent, and it explored the potential mechanism of action. Ovariectomized athymic mice with estradiol (E2) supplementation (1.7 mg/pellet, 60-day release) and established MCF-7 tumors were treated with basal diet control (0FS), 5% FS (5FS), 10% FS (10FS), and TAM (TAM/0FS; 5 mg/pellet, 60-day release), alone or in combination (TAM/5FS and TAM/10FS) for 8 weeks. Compared with control, 5FS and 10FS significantly inhibited tumor growth by 26% and 38%, respectively. TAM/0FS had an effect similar to the 10FS. TAM/5FS and TAM/10FS, respectively, induced significant 48% and 43% reductions in tumor size compared with 0FS, and 18% and 10% reductions compared with TAM/0FS. The relative uterine weight was significantly lower in all TAM groups compared with the control. The reduction of tumor growth resulted from decreased cell proliferation and increased cell apoptosis. TAM/5FS caused a significantly higher expression of estrogen receptor-α (ERα) compared with 5FS and TAM/0FS, whereas TAM/10FS had a higher ERα than 10FS and TAM/0FS. Compared with the control, progesterone receptor (PgR) expression was significantly reduced in all treatment groups, but insulin-like growth factor-1 (IGF-1) expression was reduced only by 10FS, TAM/5FS and TAM/10FS. Tumor cell proliferation was significantly positively associated with expression of PgR and IGF-1 and negatively associated with apoptosis and ERα. Apoptosis was only associated with ERα. In conclusion, FS inhibited MCF-7 tumor growth in a dose-dependent manner and enhanced the inhibitory effect of TAM due to the modulation of ER and growth factor signal transduction pathways. Exp Biol Med 232:1071–1080, 2007

Key words: flaxseed; tamoxifen; breast cancer; lignan; athymic mice

Introduction

For more than three decades, tamoxifen (TAM) has remained the standard endocrine therapy in premenopausal breast cancer patients (1, 2), particularly those with hormone-dependent (estrogen receptor–positive, ER^+) breast cancer (2). The principal mechanism by which TAM protects against ER^+ breast cancer is by competing with estradiol (E2) for binding to the ER, thereby reducing E2-stimulated breast cancer growth (3). However, some women experience premature menopause and other side effects from TAM treatment (4, 5), and therefore use complementary and alternative medicine (CAM) to supplement their medical treatment or to enhance their overall health (6–9). A recent survey in Canada showed that more than 80% of all women with breast cancer use CAM, with 41% using it to manage their breast cancer (9). Many take drugs, such as TAM, with phytoestrogen-rich foods, such as flaxseed (FS) and soy, comprising 12.4% and 5.1% of total CAM products used, respectively (9). A total of 18% of health care professionals also report taking FS (10). All of these data raise concern on whether phytoestrogen-rich foods may interfere with the effectiveness of TAM treatment.

Phytoestrogens are compounds that are structurally similar to natural E2 and include the isoflavones, rich in soy, and the lignans, rich in FS. Secoisolariciresinol diglycoside

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(SDG), the major plant lignan in FS, is metabolized by intestinal microbiota to the mammalian lignans, enterodiol (ED) and enterolactone (EL; Ref. 11). Because ED and EL are biphenolic compounds structurally similar to E2, they have been hypothesized to have estrogenic and/or antiestrogenic effects, as well as anticancer properties (12). FS is unique because it is not only the richest source of the phytoestrogen lignans but also is abundant in α-linolenic acid (ALA). ALA and its metabolites, eicosapentaenoic acid and docosahexaenoic acids, also have been shown to have anticancer effects (13). Because lignans are structurally similar to TAM and may compete with E2 for binding to ER (14), they may interfere with the effectiveness of TAM for breast cancer treatment. However, our previous study in ovariectomized athymic mice with established ER^+ human breast cancer (MCF-7) showed that 10% FS did not interfere but instead enhanced the tumor inhibitory effect of TAM at high circulating E2 level (15). Nevertheless, it is unclear whether the interactive action of FS and TAM is dose dependent. Also, their mechanism of action requires further elucidation.

Because FS has been shown to downregulate the expression of epidermal growth factor receptor (EGFR), HER2, insulin-like growth factor–1 (IGF-1), and vascular endothelial growth factor (VEGF) in breast cancer cells (16–18), and the lignans have been shown to bind to ER (14), we hypothesize that dietary FS may synergistically enhance the ability of TAM to inhibit tumor growth at high circulating E2 levels through the modulation of estrogen-related and signal transduction pathways. Thus, the current study was designed to determine the dose dependency of the FS effect and the potential mechanism of FS action in ovariectomized athymic mice with supplementation of E2 to simulate premenopausal situation.

Materials and Methods

Cell Line and Cell Culture. Human ER^+ breast cancer cell line MCF-7 (ATCC, Manassas, VA) was maintained in Dulbecco's minimum essential medium/F12 with 10% fetal bovine serum and 1% antibiotic antimycotic solution (penicillin G 10,000 units U/ml, streptomycin sulfate 10 mg/ml, and amphotericin B 25 μg/ml, Sigma-Aldrich Chemical Co., St. Louis, MO). Cells were given fresh medium 1 day prior to trypsinization. For injection, cells at 70%–90% confluence were trypsinized and resuspended in serum-free medium with 1:1 Matrigel (vol/vol) at a concentration of 1×10^7 cells/ml on ice. Over 90% cell viability before and after cell injection was obtained by trypan blue exclusion assay.

Animal and Diets. BALB/c nu/nu athymic mice (Charles River Canada, St-Constant, PQ, Canada), 5–6 weeks old, were ovariectomized and maintained in microisolator cages (four mice per cage) within a pathogen-free isolation facility with 12:12-hr light:dark cycle at 22°C–24°C and 50% humidity. Animal care and use followed the *Guide to the Care and Use of Experimental Animals* (19), and the experimental protocol was approved by the University of Toronto Animal Care Committee.

The basal diet (BD) was based on the AIN-93G formulation (20) modified to have 7% corn oil instead of soybean oil. The FS diets were the BD supplemented with 5% or 10% freshly ground FS (Linott variety) corrected for the contribution of FS to fat, fiber, and protein components such that the energy values of the diets were the same (11, 21, 22). The 5% FS and 10% FS contained, respectively, 0.1 g/kg and 0.2 g/kg SDG and 18.27 g/kg and 36.53 g/kg flaxseed oil (FO), 57% of which was ALA. Diets were prepared by Dyets Inc. (Bethlehem, PA) and were sterilized with ^{60}Co radiation by Isomedix Corp. (Whitby, ON, Canada).

Experimental Design. After 1-week acclimatization while being fed the BD, mice were anesthetized intraperitoneally with ketamine/zylazine mixture. A 2- to 3-mm incision was made over the skin in the interscapular region, and a sterilized 17β-E2 pellet (1.7 mg, 60-day release, produce 3–4 n*M* E2 blood level; Innovative Research of America, Sarasota, FL) was implanted subcutaneously, followed by sealing of the incision with tissue adhesive Vetbond (3M Animal Care Products, St. Paul, MN). A 50-μl cell suspension containing 5×10^5 cells was subcutaneously injected into each of the four mammary fat pads, thus producing four sites of mammary tumor per mouse. Tumors were palpated weekly. The tumor cross-sectional area was calculated using the formula (length/2 × width/2) × π. At Week 6, when tumor area reached 40 mm^2, the mice were randomly divided into seven groups such that their tumor size and body weight were similar ($n = 30$ to 38 tumors per group, 8 to 10 mice per group).

The existing E2 pellet was removed under general anesthesia, and a new E2 pellet was implanted in all six treatment groups (Groups 1–6) to increase the circulating E2 level to 3–4 n*M*, which is within the range of physiologic level seen in premenopausal women, except that the negative control (–E2) had a placebo pellet and was fed BD. Group 1 (0FS) was fed the BD only to serve as positive control. Group 2 (5FS) was fed 5% FS diet, while group 3 (10FS) was fed 10% FS diet. Group 4 (TAM/0FS) was fed the BD and subcutaneously implanted with a TAM pellet (5 mg, 60-day release, produce 3–4 ng/ml blood level; Innovative Research of America). Group 5 (TAM/5FS) was implanted with a TAM pellet and fed 5% FS diet, while Group 6 (TAM/10FS) was implanted with a TAM pellet and fed 10% FS diet. The mice in Groups 1–3, which had no TAM pellet implant, received a placebo pellet (Innovative Research of America) implanted subcutaneously. The food intake, body weight, and palpable tumor size were monitored weekly. Mice were sacrificed by CO_2 asphyxiation at Week 14 (8 weeks after treatment). At necropsy, body weight, primary tumor weight and volume, and weights of major organs, including uterus, were recorded.

Table 1. Effects of FS, Alone or in Combination with TAM, on Food Intake, Body Weight, and Uterine Weight in Ovariectomized Athymic Mice with Established MCF-7 Xenografts[a]

	Food intake (g/mouse)	Body weight (g)	Uterine weight (mg/g body weight)
0FS	142.6 ± 5.6	19.1 ± 0.8	12.8 ± 3.6*
5FS	133.7 ± 3.7	18.4 ± 1.2	11.2 ± 1.5*
10FS	135.9 ± 4.4	19.2 ± 1.1	9.3 ± 1.6*,#
TAM/0FS	138.8 ± 6.6	18.1 ± 0.7	5.8 ± 0.9#
TAM/5FS	134.8 ± 8.4	17.7 ± 0.9	4.9 ± 0.8#
TAM/10FS	135.7 ± 4.1	18.9 ± 0.7	5.0 ± 0.6#

[a] Values are means ± SEM. Different symbols indicate significant difference at $P < 0.05$ among treatment groups by Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test. 0FS, positive control with basal diet; 5FS, 5% FS; 10FS, 10% FS; TAM/0FS, TAM pellet and basal diet; TAM/5FS, TAM pellet and 5% FS; TAM/10FS, TAM pellet and 10% FS. $n = 8$ to 10 mice per group.

Primary tumor volume was calculated based on the formula (length/2 × width/2 × thickness/2) × π.

Immunohistochemistry. The formalin-fixed, paraffin-embedded tumor tissue sections ($n = 8$ to 10 per group) were deparaffinized and rehydrated followed by 3% H_2O_2 treatment to block endogenous peroxidase. The antigen was retrieved by heating in 0.01 *M* citrate buffer at pH 6.0 for 20 mins in a microwave oven. The primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, except where otherwise indicated) were rabbit anti-human polyclonal (except where otherwise indicated), and diluted as follows: Ki-67 (H-300) at 2 μg/ml; Cyclin D1 (H-295), 1 μg/ml; ERα (HC-20), 0.5 μg/ml; PgR (C-19), 0.5 μg /ml; IGF-1 (goat anti-human; R&D Systems, Minneapolis, MN), 2.5 μg/ml; IGF-1Rα (N-20), 1 μg/ml; HER2 (Dako, Mississauga, ON, Canada), 0.25 μg/ml; ERβ (PA1–311; Affinity BioReagents, Golden, CO), 4 μg/ml. The antibodies were diluted with the Diluent Buffer (Dako) that blocks nonspecific antigens. The sections were incubated at 4°C overnight, followed by biotinylated specific IgG against the primary antibody (Dako). Streptavidin–horseradish peroxidase and AEC substrate (3-amino-9-ethylcarbazole) chromogen (Dako) were used to demonstrate the antigens. All slides were read blindly under a light microscope at ×400 magnification. More than 1000 cells from different fields were counted for Ki-67 labeling index (LI), which was calculated as percentage of positive cells over total cells counted. To assess the expression of tumor cellular biomarkers, the Allred scoring method was used (23), which is the sum of intensity score (range: 0 = negative to 3 = strong staining) and proportion score (range: 0 = 0% positive to 5 = 100% positive), with a maximum score of 8 (range: 0–8).

ApopTag Detection Kit (Chemicon, Temecula, CA) was used to detect apoptosis and was run based on the manufacturer's protocol (12). Briefly, after pretreatment with proteinase K (20 μg/ml) the sections were incubated with terminal transferase and digoxigenin dUTP at 37°C for 1 hr, followed by incubation with antidigoxigenin antibody coupled to horseradish peroxidase. The slides were incubated with diaminobenzidine for 6 mins and counterstained with methyl green. The number of apoptotic cancer cells was counted blindly and expressed as apoptotic cell number/mm^2 at ×400 magnification.

Statistical Analysis. Data are presented as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) with general linear model repeated measures was used to determine palpable tumor growth difference among treatment groups over treatment time, followed by *post hoc* Tukey test. The differences among groups in food intake, body weight, and relative organ weights were analyzed by one-way ANOVA on Ranks followed by Dunn's test. The final tumor volume and weight and various biomarkers were analyzed by one-way ANOVA followed by *post hoc* Tukey test. Linear regression was used to analyze the relationship of several tumor biomarkers to cell proliferation and apoptosis. Because of the low E2 supplementation, the –E2 group just served as a negative control for the model system; results from this group were not compared with the other treatment groups. All statistical analyses were done by SPSS (Chicago, IL), and the significance level was set at $P < 0.05$.

Results

Food Intake, Body Weight, and Organ Weight. There were no significant differences in food intake and body weight among treatment groups (Table 1). The control group (0FS) had the highest uterine weight, followed by the 5FS and 10FS groups, which did not differ significantly (Table 1). Compared with the control, the uterine weight was significantly reduced by all TAM treatment groups (Table 1), but there was no significant difference among these groups. There was also no significant difference among groups in the weight of major organs, such as liver, lungs, kidneys, and brain (data not shown).

Palpable Tumor Growth. The mean pretreatment tumor size was 41 mm^2 at Week 6 (Fig. 1). The palpable tumor area of the positive control (0FS) increased consistently, and at the end of the study (Week 13, 7 weeks after treatment) was 355% of the pretreatment value and was significantly higher than all of the other treatment groups. Because MCF-7 tumors require estrogen for continued growth, the negative control (–E2 group) with E2 pellet removed had tumors that regressed to 87% of pretreatment tumor size. With E2 supplementation, compared with 0FS, 5FS and 10FS significantly inhibited tumor growth by 26% and 38%, respectively. TAM alone (TAM/0FS) significantly inhibited tumor growth to a level that did not differ significantly from that with 10FS (Fig. 1). The combination of TAM with 5FS or 10FS induced significant 48% and 43% reductions in tumor growth, respectively. Although the tumor growth in the TAM/5FS group did not differ from that in the TAM/10FS group, it was significantly lower than

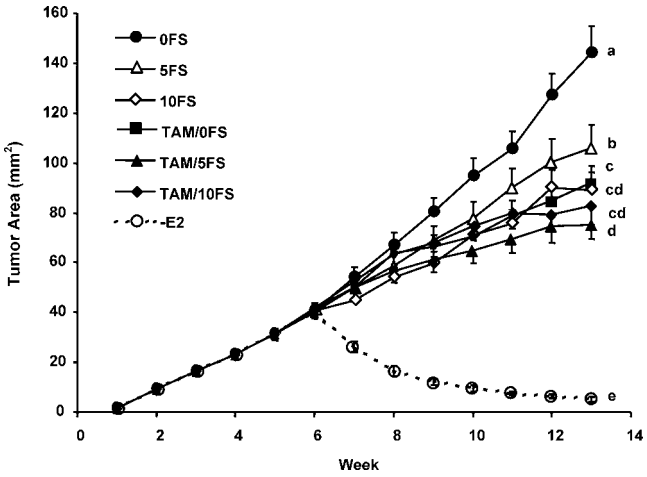

Figure 1. Effects of FS, alone or in combination with TAM, on palpable tumor growth over treatment time of MCF-7 breast cancer in ovariectomized athymic mice. The mice with established MCF-7 tumors at Week 6 were reimplanted with an E2 pellet to produce high E2 levels (1.7 mg of E2 pellet, 60-day release), except that the negative control group (–E2) received a placebo pallet. All mice were treated with basal diet (0FS; positive control), 5% FS (5FS), 10% FS (10FS), and TAM (TAM/0FS; TAM pellet: 5 mg/pellet, 60-day release), alone or in combination (i.e.,TAM/5FS and TAM/10FS), for 8 weeks. Different letters (a–e) indicate significant difference at $P < 0.05$ among groups by general linear model ANOVA repeated measures followed by Tukey test ($n = 30$ to 38 tumors from 8 to 10 mice per group).

in the 5FS and TAM/OFS groups (Fig. 1). The final tumor volume and weight at Week 14 (8 weeks after treatment; Fig. 2) followed a pattern similar to the palpable tumor growth rate (Fig. 1).

Cell Proliferation and Apoptosis. All treatment groups differed significantly from the control (0FS) in tumor cell proliferation, but they did not differ significantly from each other, except TAM/5S, which exhibited the lowest value and differed significantly from 5FS (Fig. 3A). Consistent with the tumor growth rate (Fig. 1), TAM/5FS had the lowest KI-67 LI and highest apoptosis (Fig. 3). An inverse relationship was observed between cell proliferation and apoptosis (i.e., groups that had higher Ki-67 LI had lower apoptosis; Fig. 3, Table 2).

Cellular Biomarkers. The FS and TAM alone groups had ERα expression that did not differ from the control and each other (Fig. 4A). However, the combination of TAM with FS showed a higher expression of ERα, particularly compared with 10FS and TAM alone groups (Fig. 4A). There was no significant difference in ERβ expression among groups (Fig. 4B). PgR expression was significantly reduced in all treatment groups compared with the control (Fig. 4C). The combination of TAM with FS tended to reduce the PgR expression as FS level increased. No significant difference in tumor cyclin D1 expression was observed among groups (Fig. 4D).

IGF-1 expression was significantly reduced by 10FS, whereas TAM had a level similar to the control, which was reduced by combining it with 5FS or 10FS (Fig. 5A). The IGF-1Rα expression did not differ among groups (Fig. 5B). HER2 expression was significantly higher in the TAM-treated tumors compared with the FS alone–treated tumors, whereas all other groups were intermediate (Fig. 5C). Ki-67 index was also significantly negatively associated with ERα and positively associated with PgR and IGF-1 (Table 2). Apoptosis was positively associated only with ERα.

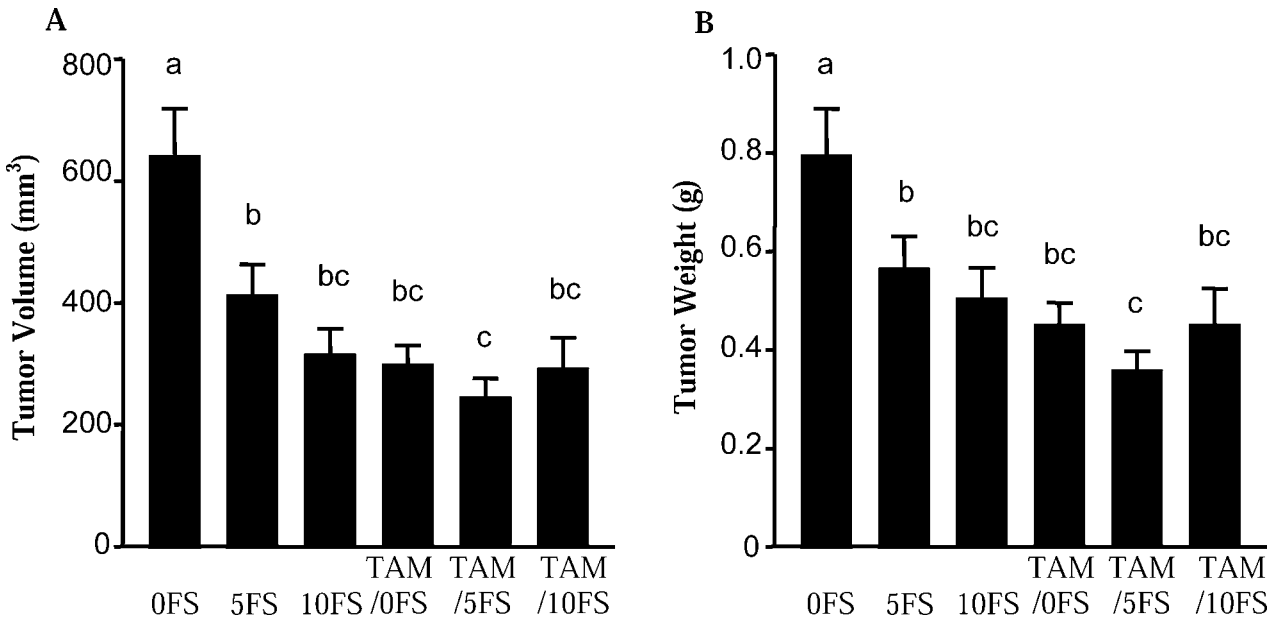

Figure 2. Effects of FS, alone or in combination with TAM, on (A) final tumor volume and (B) final tumor weight of MCF-7 breast cancer in ovariectomized athymic mice. 0FS, positive control with basal diet; 5FS, 5% FS; 10FS, 10% FS; TAM/0FS, TAM pellet and basal diet; TAM/5FS, TAM pellet and 5% FS; TAM/10FS, TAM pellet and 10% FS. Different letters (a–c) indicate significant difference at $P < 0.05$ among groups by one-way ANOVA followed by Tukey test ($n = 30$ to 38 tumors from 8 to 10 mice per group).

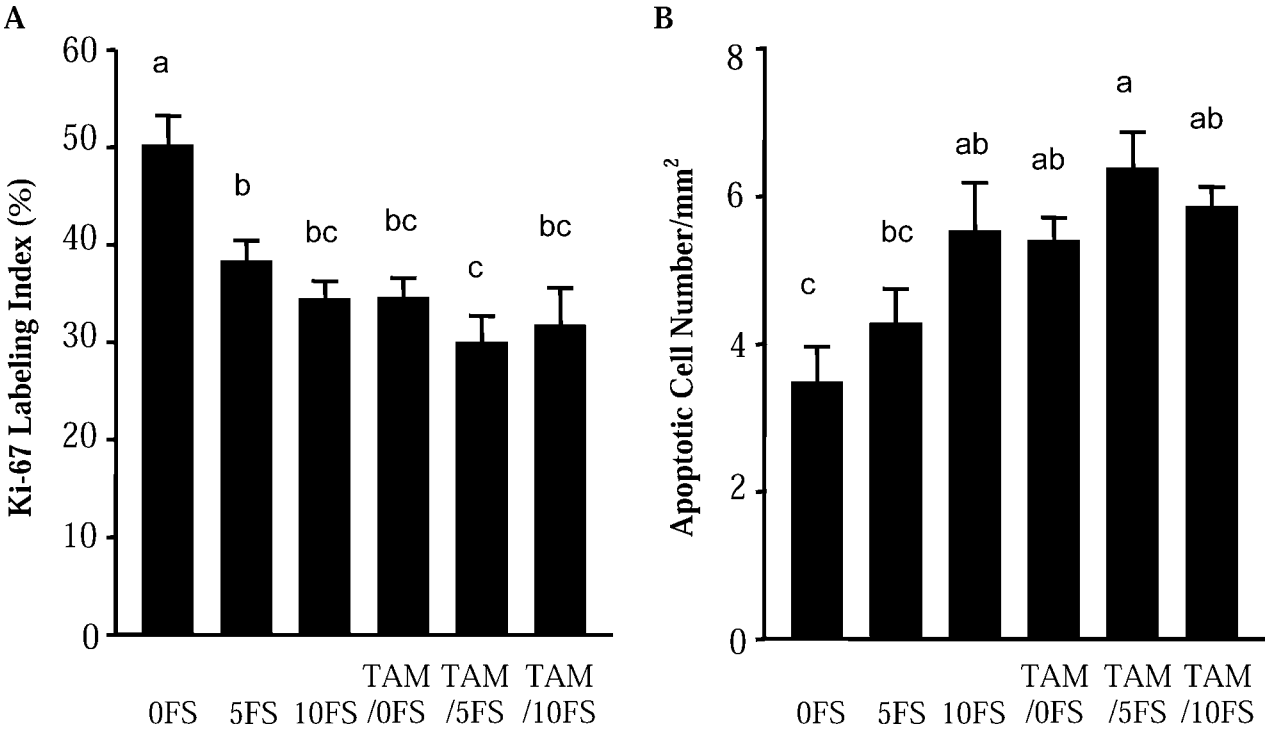

Figure 3. Effects of FS, alone or in combination with TAM, on (A) cell proliferation (Ki-67 labeling index) and (B) apoptotic index of MCF-7 breast cancer in ovariectomized athymic mice. 0FS, positive control with basal diet; 5FS, 5% FS; 10FS, 10% FS; TAM/0FS, TAM pellet and basal diet; TAM/5FS, TAM pellet and 5% FS; TAM/10FS, TAM pellet and 10% FS. Different letters (a–c) indicate significant difference at $P < 0.05$ among groups by one-way ANOVA followed by Tukey test ($n = 8$ to 10 tumors per group).

Discussion

This study demonstrated that 5FS and 10FS diets can inhibit the growth of ER^+ breast cancer MCF-7 xenografts in athymic mice supplemented with estrogen. The 10FS diet alone had a greater inhibitory effect than the 5FS diet alone, suggesting a dose-dependent effect. This study also showed that when combined with TAM treatment, both 5FS and 10FS had a similar agonistic-suppressing effect on tumor growth, indicating that FS does not interfere with the effectiveness of TAM, with 5FS being more effective than 10FS. These results confirm the findings from previous studies, which showed that a 10FS diet inhibits breast tumor growth and strengthens the tumor inhibitory effect of TAM at high circulating E2 levels (15). Moreover, this study suggests that the possible mechanisms of the anticancer effect of FS and its interaction with TAM in athymic mice with MCF-7 tumors include the modulation of the expression of estrogen-related gene products and down-regulation of IGF-1 expression.

The mammalian lignans, ED and EL, derived from SDG in FS, possess weak estrogenic and/or antiestrogenic activities, which is hypothesized to be ER mediated (14). *In vitro* studies have shown that at low E2 levels, lignans at low concentrations act as weak estrogens that stimulate MCF-7 cell proliferation (24–26), but when combined with E2, EL suppresses the stimulatory effects of E2 (25), suggesting an antiestrogenic effect. These *in vitro* studies indicate that the effect of lignans on MCF-7 cell proliferation depends on the dose and E2 status. However, *in vivo*, FS or the mammalian lignans ED and EL do not induce estrogenic, tumor-promoting effects on mammary tumorigenesis. Dietary FS and the mammalian lignans have been shown to inhibit the growth of carcinogen-induced,

Table 2. Relationship of Tumor Biomarkers to Cell Proliferation and Apoptosis in Ovariectomized Athymic Mice with Established MCF-7 Xenografts

	r	*P*
Ki-67 *versus*		
Apoptosis	−0.544	<0.003
Cyclin D1	0.357	0.063
ERα	−0.431	0.022
ERβ	0.198	0.968
PgR	0.707	<0.001
IGF-I	0.530	0.003
IGF-IRα	0.009	0.963
HER2	0.186	0.269
Apoptosis *versus*		
Cyclin D1	−0.250	0.119
ERα	0.399	0.010
ERβ	−0.121	0.433
PgR	−0.288	0.103
IGF-1	−0.155	0.353
IGF-1Rα	0.031	0.860
HER2	0.079	0.650

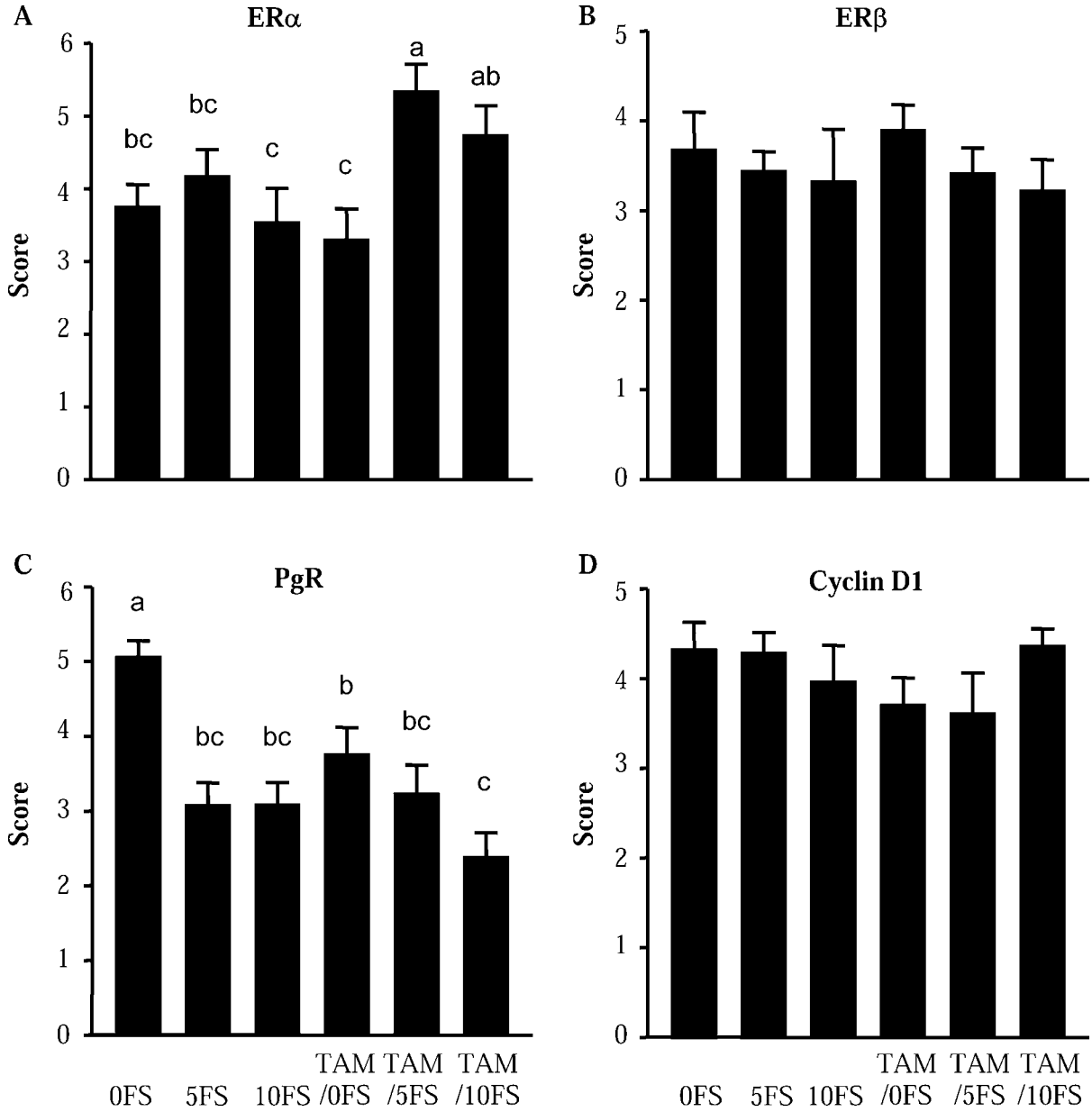

Figure 4. Effects of FS, alone or in combination with TAM, on the expression of estrogen-sensitive gene products of MCF-7 breast cancer in ovariectomized athymic mice. (A) ERα. (B) ERβ. (C) PgR. (D) Cyclin D1. 0FS, positive control with basal diet; 5FS, 5% FS; 10FS, 10% FS; TAM/0FS, TAM pellet and basal diet; TAM/5FS, TAM pellet and 5% FS; TAM/10FS, TAM pellet and 10% FS. Different letters (a–c) indicate significant difference at $P < 0.05$ among groups by one-way ANOVA followed by Tukey test ($n = 8$ to 10 tumors per group).

hormone-sensitive tumors in intact rats (27–29) and human breast cancer (MCF-7) xenografts in ovariectomized mice (15, 30–32).

To explore potential mechanisms, several tumor biomarkers of estrogen action were measured, including ERα, ERβ, PgR, cyclin D1, HER2, IGF-1R, IGF-1, Ki67, and apoptosis. These biomarkers were chosen because it is well known that E2 induces its growth stimulatory effects in MCF-7 cells/tumors through the ER. It is also known that E2 treatment results in a reduction in ERα protein and RNA levels in MCF-7 cells (33). Thus, measuring the ERα and ERβ levels in the MCF-7 tumors after treatment with FS or TAM will help establish a part of the mechanism by which these treatments may interfere with E2 action in MCF-7 tumors. PgR is a classical marker of estrogenic activity, as it is regulated by the ER in tumor cells (34). Consequently, the change in PgR positivity can be used as an outcome marker for assessing the effectiveness of endocrine therapy (35). Cyclin D1 is a known estrogen-sensitive gene marker that has been shown to be regulated by E2 acting through the ER *via* the AP-1 DNA response element (36). Thus, measuring the effects of the treatments on cyclin D1 expression will also help determine the pathways in which the treatments interfere with E2-stimulated ER activation. Since cyclin D1

is a major player in the cell cycle regulation, cell proliferation and apoptosis were assessed as typical endpoint markers of estrogen action on tumor growth. HER2, IGF-1R, and IGF-1 are major players in various signal transduction pathways. It has been shown that tumor growth stimulation induced by E2 and endocrine resistance are in part due to cross-talk between signal transduction pathways and the ER pathway, such that activation of MAPK by growth factor receptor (HER2 and IGF-1R) can enhance the activation of the ER (37). Thus, assessing the levels of the components involved in growth factor signaling pathways can help understand whether FS and TAM are interfering with E2 action on tumor growth *via* modulation of growth factor/ER cross-talk mechanisms.

In this study, PgR levels were significantly reduced by all treatments compared with the control, which then significantly correlated with a decrease in Ki-67 LI. A similar pattern of results was observed in an MCF-7 xenograft model treated with TAM and Arzoxifene (38), suggesting that the tumor growth inhibition is the result of decreasing estrogenicity caused by the treatments.

This study also revealed that FS plays an important role in modulating the expression of growth factors that are involved in signal transduction pathways. All treatment groups induced a significant reduction in IGF-1 protein expression, which is in agreement with previous studies, showing that dietary FS and its lignans lower the IGF-1 levels in the plasma of carcinogen-treated rats (39) and in the ER human breast cancer cells (MDA-MB-435) in athymic nude mice (16). IGF-1 is known also to act synergistically with E2 (40, 41) to induce cancer cell proliferation; therefore, the reduction of IGF-1 expression may, in turn, suppress the stimulatory effect of E2 on breast cancer cells. FS may also have inhibited tumor growth in the present study through antiangiogenesis. Although angiogenesis was not measured here, our recent study showed direct evidence that FS and the lignans ED and EL inhibit MCF-7 tumor growth through reduction in the expression of extracellular VEGF and the formation of blood vessels (32).

A beneficial effect was induced by the combination of FS with TAM, which confirmed our previous study (15). Since the lignans are estrogen-like in structure and have been shown to bind to the ER (14), competition with E2 for binding to the ER may be a possible mechanism for this interactive effect. Although significant changes in tumor ERα expression was not observed after treatment with FS or TAM alone, when combined, ERα increased while PgR expression decreased, suggesting a greater antiestrogenic effect. The synergistic effect of the combination of TAM and FS may also be triggered by the modulation of signal transduction pathways. For example, a significant reduction in IGF-1 expression was induced when FS was added to TAM compared with TAM alone. Both estrogen and growth factors, such as IGF-1 and EGF, can result in phosphorylation of ERα (42). Because TAM can reduce the ER activation *via* binding to the ER to form a TAM-ER complex (43), and FS can reduce growth factor receptor signal transduction *via* suppression of IGF-1, their combination led to a greater reduction in E2-induced cell proliferation and tumor growth. Since both FS and TAM

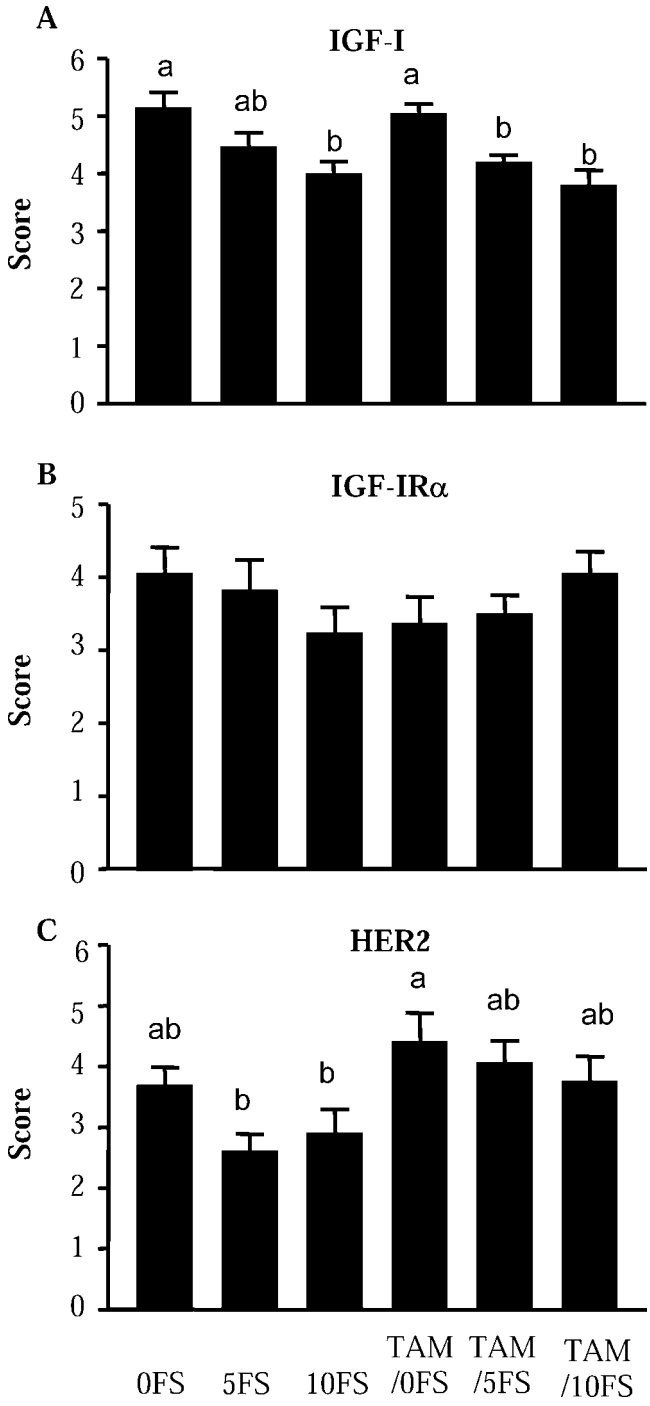

Figure 5. Effects of FS, alone or in combination with TAM, on the expression of products in the signal transduction pathways of MCF-7 breast cancer in ovariectomized athymic mice. (A) IGF-1. (B) IGF-IRα. (C) HER2. 0FS, positive control with basal diet; 5FS, 5% FS; 10FS, 10% FS; TAM/0FS, TAM pellet and basal diet; TAM/5FS, TAM pellet and 5% FS; TAM/10FS, TAM pellet and 10% FS. Different letters (a–b) indicate significant difference at $P < 0.05$ among groups by one-way ANOVA followed by Tukey test ($n = 8$ to 10 tumors per group).

can reduce angiogenesis (17, 32, 44), their combined effect may also have contributed to a greater reduction in tumor growth. Because the HER2 expression in the 5FS and 10FS groups was significantly lower than that in TAM/0FS, combining FS with TAM may supplement the inhibitory effect of TAM by downregulating HER2 expression and, hence, tumor growth. However, because there was no relationship between expression of HER2 and Ki-67 in this study, the exact role of HER2 under high E2 levels remains unclear.

ALA, which is known to have an anticancer effect (13, 45–48), may complement the action of FS lignans in counteracting the stimulatory action of E2 on tumor growth. Eicosapentaenoic acid, a metabolite of ALA, has been shown to restore TAM sensitivity and induce breast cancer cells to be more responsive to the growth-inhibitory effects of TAM by 35% (49). Recent studies found that 10% dietary sesame seed, which produces the same amount of mammalian lignans as FS *in vivo* (50), failed to inhibit tumor growth and negated the inhibitory effect induced by TAM (51). The different effects have been attributed in part to differences in their lipid components (i.e., ALA in FS and oleic acid in sesame seed). However, further studies are needed to determine the exact role of ALA in FS and its interaction with TAM in inhibiting tumor growth.

Like the breast tumors, the uterus is an estrogen-sensitive tissue in which compounds with estrogenic or antiestrogenic properties, such as TAM and FS, can influence the cell proliferation and subsequent growth. However, no significant differences in uterine weight were observed among FS groups, suggesting that FS does not act antiestrogenically or interfere with estrogen in the uterus. Previous studies also have not found dietary FS and its lignans to cause significant uterine changes (15, 27, 28, 52, 53). It is possible that different estrogen-sensitive tissues (e.g., uterus and mammary gland) respond differently to various estrogen-like compounds. This may be due to different co-regulatory proteins expressed in different tissues. Thus, estrogenic compounds binding and activating the ER would result in different genes expressed, due to the differences in transcriptional machinery components expressed in the specific tissues.

The therapeutic strategy for breast cancer in premenopausal women is ablation of ovarian estrogen production, such as oophorectomy, and TAM. This study is of clinical interest because premenopausal breast cancer patients taking TAM may use complementary dietary supplements, including such phytoestrogen-rich foods as FS, to alleviate menopausal-like symptoms and supplement the tumor-reducing effect of TAM. This study demonstrated a greater inhibitory effect on the tumor growth, without side effects on food intake, weight gain and other organ weights, induced by the combined FS-TAM treatment. The FS dose with TAM can be reduced to 5%, which may be more tolerated by patients, without decreasing the efficacy. Considering that women consume daily about 1150–2250 g diet on an as-is basis (with mean 80% moisture content), which is equal to 230–450 g on dry basis, the 5FS–10FS dry animal diet used in this study is equivalent to a human intake of about 11.5–45 g FS per day, depending on the amount of other foods consumed (21, 54).

FS as a crop accumulates heavy metals, particularly cadmium (55, 56), which also exerts estrogenic properties (57). Cadmium content was not determined in the FS used in this study. However, the 5FS and 10FS diets did not exhibit estrogenic effect on the tumor growth and uterine weight, indicating that the amount of cadmium may not be high enough to cause very significant estrogenic effects, although its role cannot be discounted in the better effect of 5FS than 10FS when combined with TAM. Nevertheless, excessive consumption of FS may be unnecessary, because this study showed that dietary exposure to 5FS provided more beneficial effects in inhibiting cancer growth when combined with TAM than 10FS.

In conclusion, FS, when used alone, inhibited the MCF-7 tumor growth in a dose-dependent manner under high circulating levels of E2. The mechanisms by which FS alone exerts this inhibitory effect include decreasing cell proliferation and increasing apoptosis due to its antiestrogenic effect and ability to downregulate IGF-1 expression. Further, an agonistic effect was induced when FS was combined with TAM, which may be attributed to the enhanced antiestrogenic effect and modulation of signal transduction pathways. These findings provide justification for further studies of FS as complementary treatment for premenopausal breast cancer patients receiving TAM.

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