Dietary Flaxseed Enhances the Inhibitory Effect of Tamoxifen on the Growth of Estrogen-Dependent Human Breast Cancer (MCF-7) in Nude Mice

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ABSTRACT

Purpose: **This study determined the effect of 10% dietary flaxseed (FS) and tamoxifen (TAM), alone and in combination, on the growth of estrogen-dependent human breast cancer (MCF-7) in athymic mice with or without 17β-estradiol (E2) supplementation.**

Experimental Design: **Ovariectomized mice received injection with MCF-7 cells, were implanted with an E2 pellet (1.7 mg), and fed the basal diet (BD). When tumor reached ~40 mm^2, the E2 implant was removed, and mice were randomized to the following groups and maintained at either low (E2 pellet removed) or high E2 level (new E2 pellet implanted) for 6 weeks: (*a*) positive control with new E2 pellet, fed BD, (*b*) negative control with no E2 implant, fed BD, (*c*) TAM group with TAM pellet (5 mg) implant, fed BD, (*d*) FS group fed 10% FS, (*e*) FS+TAM group with TAM implant, fed 10% FS. Tumor growth was monitored weekly.**

Results: **At low E2 level, FS regressed the pretreatment tumor size by 74%. TAM regressed tumor initially but later induced an increase so that the tumor size was finally similar to the pretreatment size. A tumor regression >53% was induced by FS+TAM than by TAM alone. At high E2 level, FS, TAM, and FS+TAM inhibited the tumor growth by 22, 41, and 50%, respectively, compared with the positive control. Decreased tumor size was attributable to reduced tumor cell proliferation and increased apoptosis.**

Conclusions: **FS inhibited the growth of human estrogen-dependent breast cancer and strengthened the tumor-inhibitory effect of TAM at both low and high E2 levels.**

Received 6/10/04; revised 8/11/04; accepted 8/16/04.

Grant support: In part by Natural Sciences and Engineering Research Council of Canada Grant A9995, and NIH Grant R21 CA100639-01 (L. Thompson).

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INTRODUCTION

Flaxseed (FS) is the richest source of the phytoestrogen secoisolariciresinol diglycoside (SDG), a plant lignan that can be metabolized by bacteria in the animal or human colon to the mammalian lignans enterodiol and enterolactone (Fig. 1; ref. 1). It is also a very rich source of α-linolenic acid (ALA; ref. 2). Because these compounds have been suggested to have anticancer effects (3, 4), FS is a food that has a very high potential to reduce cancer risk.

Therefore, for more than a decade, our laboratory has been studying the effects of FS and its components on the different stages of carcinogenesis, particularly breast cancer. These studies have shown that dietary FS can reduce the tumor incidence, number, and size when fed to carcinogen-treated rats at initiation, promotion, or late stages of carcinogenesis (5–7). A decrease in cell proliferation and an increase in apoptosis have also been observed in breast cancer patients given FS during the time of diagnosis to surgery (8). Furthermore, a reduction in tumor size and metastasis was observed after feeding FS to athymic mice with established human estrogen receptor (ER)-negative tumors (MDA-MB-435; ref. 9, 10). The antitumorigenic effect of SDG, the major mammalian lignan precursor in FS, was similar to that of FS (7, 11), indicating that the effect of FS is largely dependent on its SDG. Despite these observations, very little is known about the effect of FS on human ER+ breast cancer under controlled conditions of high or low levels of estrogen simulating conditions in pre- and postmenopausal women, respectively. Neither has there been a study on the interaction of dietary FS with breast cancer drug such as tamoxifen (TAM).

TAM (Fig. 1) is a well known adjuvant therapy for breast cancer particularly for those that are ER+ (12, 13). However, TAM possesses a few problems. Some patients develop TAM resistance (*i.e.*, tumors start growing again after prolonged TAM intake; ref. 13). There is also a small risk of endometrial cancer in some individuals (13). Because TAM is an antiestrogen, it also causes menopausal-like symptoms such as hot flashes. Thus many breast cancer patients taking TAM report increased use of complementary dietary supplements including phytoestrogen-rich foods such as soy and FS, instead of taking hormone replacement therapy, to reduce menopausal-like symptoms and also to supplement the tumor-reducing effect of TAM (14). However, we question whether phytoestrogen-rich foods such as soy and FS interfere with the effectiveness of TAM. Studies with soy are controversial because some studies have shown complementary effect of TAM with soy products such as miso (15) whereas other studies have shown interference by genistein, a soy isoflavone (16). Nevertheless, we hypothesized that FS complements rather than antagonizes the tumor reducing effect of TAM because some of the mechanisms whereby TAM and FS and its lignans reduce tumorigenesis are similar and/or

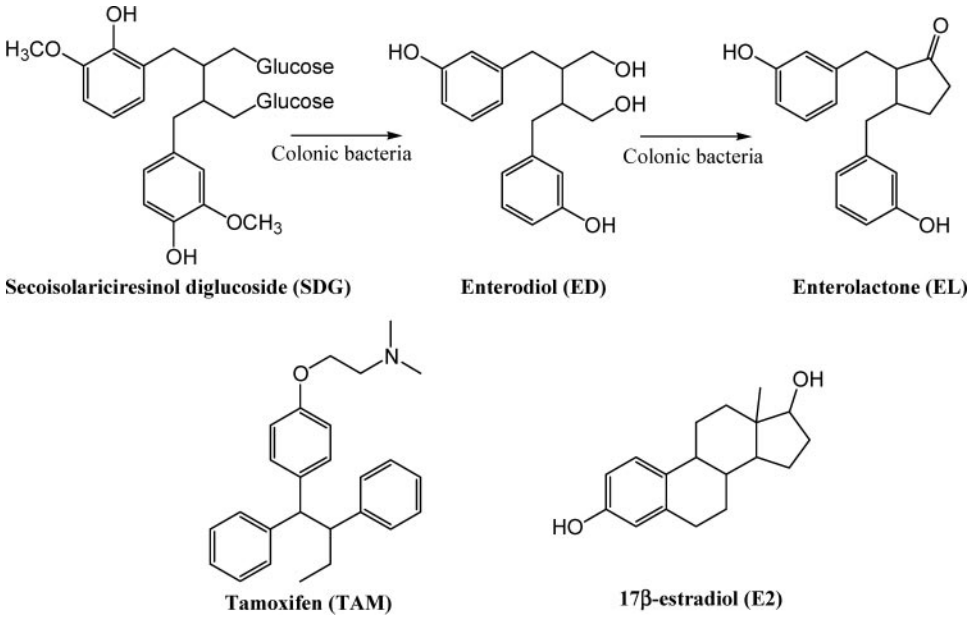

Fig. 1 Metabolism of plant lignan, SDG, to mammalian lignans, enterodiol (ED) and enterolactone (EL), and chemical structures of tamoxifen (TAM) and 17β-estradiol (E2).

complementary (*e.g.*, hormone-related mechanisms such as antiestrogen and antiaromatase activities); nonhormone-related mechanisms such as antioxidation, antiangiogenesis, increasing apoptosis, and modulating expression of growth factors (9, 10, 13, 17–21). In particular, the antiaromatase activity of the FS lignans is of interest in light of recent studies, which showed that some aromatase inhibitors are more effective in reducing tumor growth than TAM (22).

Therefore the overall objective of this study was to determine the effect of FS and TAM, alone and in combination, on the growth of ER+ human breast cancer cells (MCF-7) and uterus in athymic mice in the presence of high or low levels of circulating estrogen, simulating pre- and postmenopausal conditions, respectively. This study has important clinical implication because breast cancer patients taking TAM may consume FS for its anticancer potential or as an alternative therapy to alleviate menopausal symptoms caused by TAM.

MATERIALS AND METHODS

Cell Line and Cell Culture. MCF-7, estrogen-dependent human breast cancer cell line (The American Type Culture Collection, Manassas, VA), was maintained in DMEM/F12 supplemented with 10% fetal bovine serum and 1% antibiotics. The cells were grown to 70–90% confluence in T-150 flasks and fed with fresh medium a day before cell harvest. For injection, the cells were trypsinized and resuspended in serum-free medium with 1:1 Matrigel at a concentration of 1×10^7 cells/mL on ice. Cell viability, >95%, was determined by trypan blue exclusion assay.

Animal and Diets. Two batches (for two separate experiments) of ovariectomized athymic mice ($n = 40$/experiment; BALB/c nu/nu, Charles River Canada, St-Constant, PQ, Canada), aged 5 to 6 weeks old, were maintained in micro-isolator cages (4/cage) within a pathogen-free isolation facility with 12 light/dark cycle at 22 to 24°C and 50% humidity. Animal care and use conformed with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1984), 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 (23) modified to have a high fat content (20% corn oil) at the expense of corn starch. The FS diet was the BD supplemented with 10% freshly ground FS (Linnott Variety; Omega Products, Melfort, Saskatchewan, Canada) corrected for the contribution of FS to fat, fiber, and protein components so that the energy values of the diets were the same as described previously (9, 10). Diets were prepared by Dyets Inc. (Bethlehem, PA), and sterilized by Co^{60} radiation by Isomedix Corp. (Whitby, ON, Canada).

Experimental Design. In both Experiments 1 and 2, mice were anesthetized intraperitoneally with ketamine/zylazine mixture after 7-day acclimatization while being fed the BD. A-3 mm incision was made over the skin in the interscapular region, and a sterilized 17β-estradiol (E2) pellet (1.7 mg, 60-day release, produce 0.3–0.4 nmol/L 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 2- to 3-mm incision on the right flank was cut and the mammary fat pad was exposed. A 50-μL cell suspension containing 5×10^5 cells was injected into the mammary fat pad followed by closing the incision with Vetbond. The same procedure of skin incision and cell injection was done on the left thoracic and right and left abdominal mammary fat pads, thus producing four sites of mammary tumor growth per mouse. Tumors were palpated weekly. The tumor surface area was calculated with the formula (length/2 × width/2) × π. When tumor area reached ~40 mm^2 (at week 7 in experiment 1, week 6 in experiment 2), the mice were divided into five groups such that their tumor size and body weight were similar.

In Experiment 1, to mimic the low circulating E2 level in postmenopausal women, the existing E2 pellet was removed. Group 1 had a new E2 pellet (1.7 mg, 60 day release, produce 0.3–0.4 nmol/L E2 blood level) implanted and fed BD to serve as the positive control (+E2). The other four groups (Groups 2–5) did not have the E2 pellet replaced, resulting in about 35 pmol/L of circulating E2 in the ovariectomized nude mice (16). Group 2, the negative control (−E2), was fed the BD only. Group 3, FS group (FS), was fed the 10% FS diet. Group 3, TAM group (TAM), was fed the BD and subcutaneously implanted with a TAM pellet (5 mg, 60 day release, producing 3–4 ng/mL blood level, Innovative Research of America, Sarasota, FL). Group 5, the combination group (FS+TAM), was fed the FS diet and implanted a TAM pellet. The food intake, body weight and tumor size were monitored weekly. Mice in the +E2 group were sacrificed at week 13 because of high tumor burden. All remaining mice were sacrificed at week 14 by CO_2 asphyxiation. At necropsy, body weight, primary tumor weight and volume, and weights of major organs including uterus were recorded. Primary tumor volume was calculated based on the formula (length/2 × width/2 × thickness/2) × π.

In Experiment 2, to simulate the premenopausal women condition with high E2 level, the existing E2 pellet was removed and a new E2 pellet (1.7 mg, 60-day release, producing 0.3–0.4 nmol/L blood level) was implanted in all mice, except in the negative control group. The grouping and treatments of mice were the same as in experiment 1 except that the positive control and all treatment groups had a new E2 pellet. The treatment was terminated at week 12. The weekly tumor palpation, body weight and food intake monitoring, and tissue collection at sacrifice were the same as in experiment 1.

Ki-67 Labeling Index and Apoptosis. For Ki-67 immunohistochemistry, the 5-μm sections of formalin-fixed paraffin-embedded tumor tissue were deparaffinized and rehydrated. Endogenous peroxidase was blocked with aqueous 3% H_2O_2. The antigen was retrieved by heating in 0.01 mol/L citrate buffer (at pH 6) for 20 minutes in a microwave oven. The rabbit antihuman Ki-67 at 5 μg/mL was diluted in the diluent buffer that blocks nonspecific antigens, incubated at 4°C overnight followed by incubation with biotinylated swine antirabbit IgG. Streptavidin-horseradish peroxidase and 3-amino-9-ethylcarbazole substrate chromogen were used to show the antigens. All immunostaining agents were from Dako (Mississauga, ON, Canada). All slides were read blindly under a light microscope at 400× magnification. Over 1,000 cells from different fields were counted. Ki-67 labeling index (LI) was calculated as percentage of positive cells over total cells counted.

In situ terminal deoxynucleotidyl transferase-mediated nick end labeling assay was used to show DNA fragmentation by ApopTag Detection kit (Intergen, Purchase, NY) and ran based on the manufacturer's protocol. Briefly, deparaffinized and rehydrated sections were pretreated with proteinase K (20 μg/mL) for 15 minutes. The sections were incubated with terminal transferase and digoxigenin dUTP at 37°C for 1 hour. After washing, the sections were incubated with antidigoxigenin antibody coupled to horseradish peroxidase for 30 minutes at room temperature. The slides were then incubated with diaminobenzidine for 6 minutes, and counter-stained with methyl green. The number of breast carcinoma cells showing positive nuclear immunoreactivity was counted and expressed as apoptotic cell number per mm^2 at 400× magnification. All assays were done blind to the treatment groups.

Statistical Analysis. All data are presented as mean ± SEM. ANOVA with general linear model-repeated measures procedure was used to determine palpable tumor growth difference among treatment groups over treatment time followed by post hoc Tukey test. The differences in tumor area between the pre- and post-treatments in the same group were assessed by Student *t* test or Mann-Whitney *U* test. One-way ANOVA followed by post hoc Tukey test was used to determine the differences among groups within the same experiment in food intake, body weight, relative organ weighs, final tumor volume and weight, Ki-67 LI, and apoptosis. All statistical analyses were done by SPSS (Statistical Package for Social Sciences, Inc., Chicago, IL), and the significant level was set at $P < 0.05$.

RESULTS

Food Intake, Body and Organ Weights. In Experiment 1 (no E2 implant; low E2 level), food intake was significantly lower in the positive control (+E2 group) than the other groups, but all treatment groups including the negative control did not differ significantly (Table 1). Consequently, a lower body weight was seen in the +E2 group compared with the other groups, which may have been caused by the breast tumor deteriorating general health condition in mice. All treatment groups did not differ significantly in body weight from each other. As expected, the +E2 group had a significantly higher uterine weight than all other groups (Table 1). FS had no effect on the uterine weight. However, a significant 39% higher uterine weight was detected in the TAM group compared with the negative control (−E2 group; Table 1), indicating an estrogenic effect of TAM. When TAM was combined with FS, this uterotropic effect was reduced, and the uterine weight was only >29% −E2 controls, or was <10% the effect caused by TAM alone ($P < 0.05$).

In Experiment 2 (with E2 implant; high E2 level), no significant differences in food intake and body weight were observed among groups (Table 1). The uterine weight was not significantly changed among treatment groups but all were significantly higher than the negative control (−E2 group). No other major organs showed significant changes among groups, at either low or high E2 level (data not shown).

Palpable Tumor Growth. In Experiment 1 (Fig. 2*A*), the average tumor size was 37.4 mm^2 at the beginning of treatments at week 7. The palpable tumor area of the positive control (+E2) increased consistently, indicating that the model system is working and that the tumor growth was not affected by the surgical procedures. At the end of the study, it was >229% ($P < 0.01$) the pretreatment value and higher than all of the other treatment groups without E2 supplementation ($P < 0.01$). This group was killed a week earlier than the others because of their large tumor burden. After withdrawal of E2 pellet at week 7, tumors in all treatment groups regressed initially. The FS group had a similar tumor regression pattern as the −E2 control to the end of experiment, resulting in 75% reduction *versus* pretreatment value ($P < 0.01$). TAM initially regressed tumor size similar to other

Table 1 Effect of 10% FS and TAM on the body weight, food intake, and relative uterine weight *

	Total food intake (6 weeks; g/mouse)	Body weight (g)		Uterine weight (mg/g body weight)
Experiment 1 (no E2 pellet implant)		Week 7	Week 13	Week 14
+E2 †	74.1 ± 2.4[a]	18.0 ± 0.3[a]	17.7 ± 0.3[a]	5.59 ± 1.21[a]
−E2	99.7 ± 3.9[b]	18.4 ± 0.3[a]	19.3 ± 0.4[a,b]	1.13 ± 0.10[b]
FS−E2	104.2 ± 4.4[b]	17.6 ± 0.5[a]	19.9 ± 0.2[b]	1.18 ± 0.10[b]
TAM−E2	94.5 ± 2.9[b]	17.3 ± 0.4[a]	18.7 ± 0.4[a,b]	1.57 ± 0.12[c]
FS+TAM−E2	109.0 ± 4.8[b]	17.7 ± 0.3[a]	19.2 ± 0.4[a,b]	1.46 ± 0.15[b,c]
Experiment 2 (with E2 pellet implant)		Week 6	Week 12	Week 12
+E2	97.9 ± 1.0[a]	18.3 ± 0.3[a]	19.2 ± 0.6[a]	2.80 ± 0.65[a]
−E2	107.3 ± 3.4[a]	17.9 ± 0.3[a]	20.5 ± 0.5[a]	0.95 ± 0.10[b]
FS+E2	95.4 ± 3.5[a]	18.5 ± 0.2[a]	19.2 ± 0.4[a]	2.95 ± 0.17[a]
TAM+E2	96.6 ± 9.8[a]	18.3 ± 0.3[a]	19.0 ± 0.6[a]	3.25 ± 0.48[a]
FS+TAM+E2	92.0 ± 1.8[a]	18.4 ± 0.4[a]	18.4 ± 0.5[a]	3.26 ± 0.72[a]

* Data are means ± SEM. Means with different superscripts within the same column and experiment are significantly different at $P < 0.05$ (one-way ANOVA followed by Tukey test).

† Mice were sacrificed at week 13, whereas all other groups were sacrificed at week 14.

groups (38% reduction at week 10, 3 weeks after treatment, $P < 0.05$) but then started to increase the tumor size resulting in only about 8% nonsignificant reduction at week 14 *versus* pretreatment size. This late promoting effect of TAM was suppressed by the feeding of FS. The combination treatment (FS+TAM) induced greater tumor size reduction to <47% pretreatment value ($P < 0.01$) and <42% the value with TAM treatment alone ($P < 0.05$).

In Experiment 2 (Fig. 2*B*), the mean pretreatment tumor size was 38.8 mm^2 at week 6. Both the +E2 and −E2 controls had similar growth and regression patterns as seen in Experiment 1. With E2 supplementation, FS and TAM treatments did

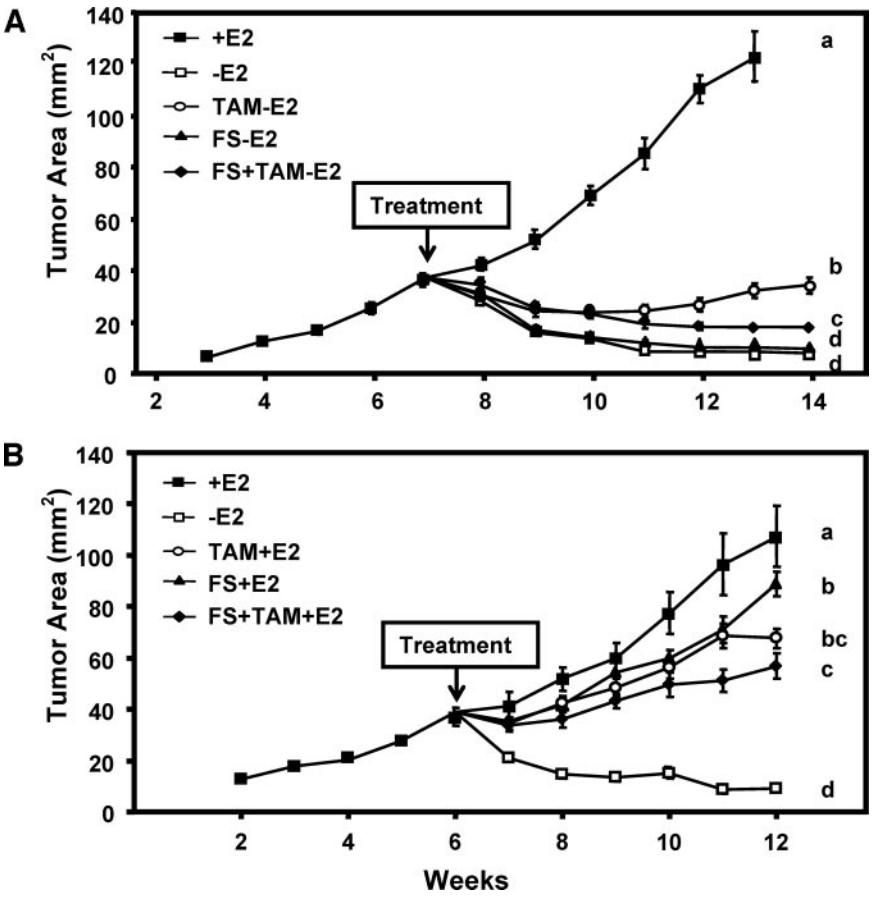

Fig. 2 Effect of 10% dietary flaxseed (FS) and tamoxifen (TAM), alone or in combination, on the palpable tumor growth of MCF-7 breast cancer in ovariectomized nude mice. *A*, Experiment 1, treatments started at week 7 after implanted estradiol (E2) pellet was removed to produce low E2 level. The positive control group (+E2) had new E2 pellet implant. *B*, Experiment 2, treatments started at week 6 after the implanted E2 pellet was removed and a new one was reimplanted to produce high E2 level. The negative control group (−E2) did not have E2 pellet reimplanted. Treatments with −E2 means E2 pellet not reimplanted; with +E2 means a new E2 pellet reimplanted. All E2 pellets have 1.7 mg of E2, 60-day release, producing 0.3 to 0.4 nmol/L E2 blood levels. TAM pellets have 5 mg of TAM, 60-day release, producing 0.3 to 0.4 nmol/L E2 blood levels. Different letters (*a-d*) indicate significant difference at $P < 0.05$ among groups by general linear model one-way ANOVA repeated measures followed by Tukey test.

not regress tumor size but retarded tumor growth by 17 and 37%, respectively, compared with the +E2 control ($P < 0.05$). There was no significant difference between the FS and TAM groups. The combined treatment (FS+TAM) induced a greater reduction (47%) of tumor growth compared with the +E2 control, and resulted in 36% smaller tumor size than with FS treatment alone ($P < 0.05$) and 16% nonsignificant smaller tumor than with TAM treatment alone.

Final Tumor Volume and Weight. At necropsy, the final tumor volumes and weights of treatment groups (Fig. 3) followed a similar pattern as seen in the palpable tumor growth. At low E2 level (Experiment 1; Fig. 3*A* and *C*), FS had a similar effect on tumor regression as the −E2 control, indicating that FS did not exert an estrogenic effect on tumor growth. Contrary to FS, TAM induced a significantly higher tumor volume (850%) and weight (375%) than the −E2 control. However, this promoting effect was attenuated by combining FS with TAM. The tumor volume and weight in the FS+TAM group were significantly lower by 62% and 39%, respectively, compared with those in the TAM group, although these were still significantly higher than those in the −E2 group. These results indicate that TAM has a weak estrogenic effect on tumor growth that can be offset by FS.

At high E2 level (Experiment 2), the lowest tumor volume and weight were induced by FS+TAM, followed by TAM, and then FS, compared with +E2 control (Fig. 3*B* and *D*). Although the tumor inhibition caused by FS+TAM was not significantly greater than by TAM alone, it was significantly stronger than by FS alone. These results suggest that, at high E2 level, FS has an agonistic effect with TAM on tumor growth.

Tumor Cell Proliferation and Apoptosis. At low E2 level (Experiment 1), the tumor Ki-67 LI in the mice fed FS was reduced to a similar extent as that of −E2 control (Fig. 4*A*), in agreement with the tumor growth pattern. TAM reduced the cell proliferation rate by 39% compared with the +E2 group ($P < 0.05$), but was 62% and 54% higher than that in the −E2 and FS groups, respectively. However, a significant 21% lower Ki-67 LI was induced after addition of FS to TAM compared with TAM alone. At high E2 level (Experiment 2), all treatments caused inhibition of Ki-67 LI compared with the positive control (Fig. 4*B*). Both FS and TAM significantly induced 30% reduction of KI-67 LI compared with the positive control. A further 12% decrease in Ki-67 LI was induced by the combination of TAM and FS.

At low E2 level (Experiment 1), all treatment groups significantly induced tumor cell apoptosis (Fig. 4*C*). Compared with the positive control, 155 and 278% increase were induced by treatments with TAM and FS, respectively. FS increased

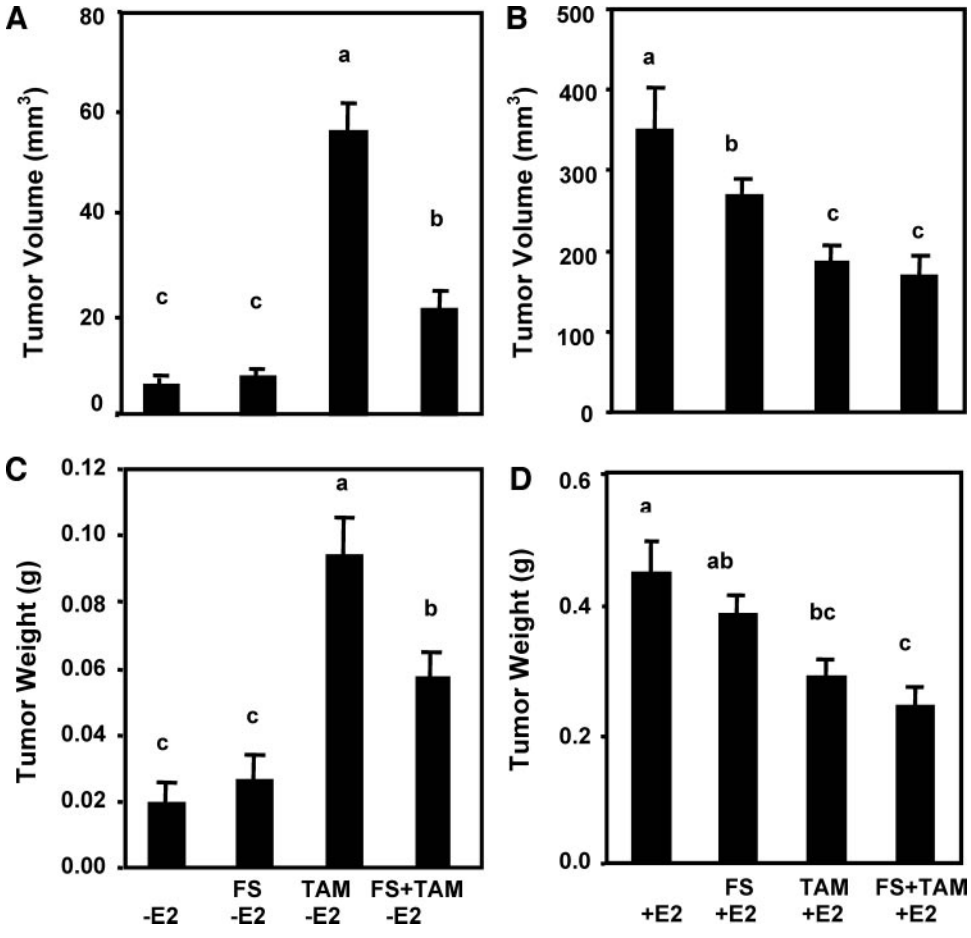

Fig. 3 Effect of 10% dietary flaxseed (FS) and tamoxifen (TAM), alone or in combination, on the final volume and weight of MCF-7 breast tumors at sacrifice in nude mice, at low estradiol (E2; Experiment 1, *A* and *C*) or high E2 (Experiment 2, *B* and *D*) levels. −E2, negative control; +E2, positive control; treatments with −E2 means no E2 pellet implant; with +E2 means with E2 pellet implant. E2 pellets have 1.7 mg of E2, 60-day release, producing 0.3 of 0.4 nmol/L E2 blood levels. TAM pellets have 5 mg of TAM, 60-day release, producing 3 to 4 ng/mL blood levels. Different letters (*a-c*) indicate significant difference at $P < 0.05$ among groups by one-way ANOVA followed by post hoc Tukey test. Data are means ± SEM.

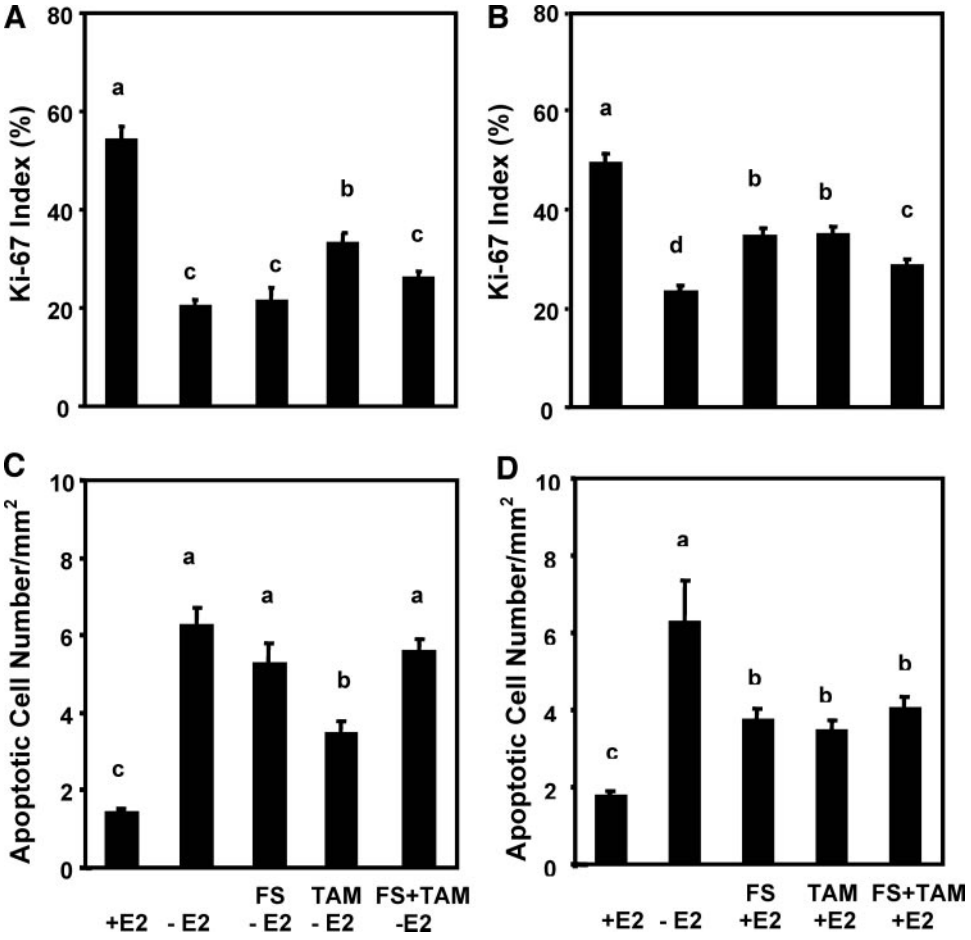

Fig. 4 Effect of 10% dietary flaxseed (FS) and tamoxifen (TAM), alone or in combination, on Ki-67 labeling and apoptotic indices of MCF-7 breast tumors, at low estradiol (E2; Experiment 1, *A* and *C*) or high E2 (Experiment 2, *B* and *D*) levels. −E2, negative control; +E2, positive control; treatments with −E2 means no E2 pellet implant; with +E2 means with E2 pellet implant. All E2 pellets have 1.7 mg of E2, 60-day release, producing 0.3–0.4 nmol/L E2 blood levels. TAM pellets have 5 mg of TAM, 60-day release, producing 3 to 4 ng/mL blood levels. Different letters (*a-d*) indicate significant difference at $P < 0.05$ among groups by one-way ANOVA followed by post hoc Tukey test. Data are means ± SEM.

apoptosis by 48% compared with TAM alone. A 300% increase in apoptosis was induced by the combination of TAM and FS, which was 56% higher than with TAM alone ($P < 0.05$). When treatments were conducted at high E2 level (Experiment 2; Fig. 4*D*), 94, 109, and 126% higher apoptosis was induced by treatments with FS, TAM, and FS+TAM, respectively, compared with the positive control. There were no significant differences among treatment groups (FS, TAM, or FS+TAM) at high E2 level.

DISCUSSION

This study showed for the first time that dietary FS can inhibit the growth of human ER+ breast cancer, and enhance rather than antagonize the anticancer effect of TAM in nude mice in the presence of low or high E2 level, which mimics the hormone status of post- and premenopausal women, respectively. The anticancer effect of FS is consistent with our previous studies, which showed that FS inhibits the growth of mammary tumors induced by chemical carcinogens in rats (3, 5–7) and −ER human breast cancer in nude mice (9, 10). It also agrees with the results from a double-blinded placebo-controlled clinical trial, which showed that 25 g of FS reduces the tumor cell proliferation of preoperative breast cancer in postmenopausal patients (8).

The mammalian lignans enterodiol and enterolactone, derived from flaxseed SDG, possess weak and/or antiestrogenic activities, which may be ER-mediated. Therefore their effect on MCF-7 cells varies in the absence or presence of E2 (24–26). Although lignans promoted MCF-7 tumor cell growth *in vitro* with E2 deprivation because of weak-estrogen activity (24), this was not observed in ovariectomized athymic mice in this study. In fact, the present study showed that FS regressed the tumors to a similar extent as the negative control, and failed to stimulate cell proliferation and uterus development in mice after E2 pellet withdrawal, suggesting that FS does not elicit an estrogenic effect on the tumor and uterus.

In addition to ER-mediated effects, lignans can modulate the activities of enzymes involved in estrogen biosynthesis and metabolism. Lignans have been shown to moderately inhibit the activity of aromatase, an estrogen synthetase converting androgen to estrogen (27), in human tissues and MCF-7 cells (28–30). Recent study in our laboratory also found that both enterodiol and enterolactone dose-dependently modulate the 17-β hydroxysteroid dehydrogenase activities, decreasing E2 production in MCF-7 cells (30). Moreover, in humans, FS supplementation increased urinary excretion of 2-hydroxyestrone, a less estrogenic metabolite of E2 (31), in pre- and postmenopausal women (32–34). Taken

together, lignans in FS may either reduce the synthesis of E2 in cancer cells or convert E2 to less active metabolites, thereby reducing the bioavailability of E2 for MCF-7 cells. This in turn may explain, in part, the lack of estrogenic effect of FS after E2 withdrawal in this study.

The facts that dietary FS inhibited tumor growth of ER+ MCF-7 at low E2 level and −ER MDA-MB-435 (9, 10) indicate that the nonhormone related properties may also play a pivotal role. The nonhormonal properties of FS include antioxidant (21), antiangiogenesis (22), inhibition of growth factors (23–25), and induction of apoptosis (8), which may also be complementary to TAM treatment on the tumor growth.

TAM is well known to have both weak estrogenic and antiestrogenic properties (12, 13). Its use was originally restricted to women with ER+ breast tumor based on the findings that TAM competes with E2 for ER and therefore inhibits ER-mediated events that lead to tumor growth (13). TAM possesses hormone-independent mechanisms for inhibiting tumor growth as summarized earlier (13, 18–21). However, tumor regrowth caused by TAM resistance usually occurs after prolonged treatment, which is a significant clinical problem.

An important finding in the present study is that FS in combination with TAM treatment had a greater inhibitory effect on tumor growth than TAM alone at both low and high circulating E2 levels. The mechanism(s) for this enhanced interactive effect is not yet clear but may be related to their complementary actions. The inhibitory effect of FS at high E2 level may be through competition of lignans with E2 for ER, thereby enhancing the effect of TAM in inhibiting E2-mediated tumor growth. After E2 deprivation, FS lignans may block the endogenous production and bioavailability of E2 to cancer cells as discussed earlier, and then reduce the tumor regrowth induced by TAM. Furthermore, lignans primarily bind to ERβ than ERα (35), whereas TAM binds to both receptors with equal affinity; thus different ER activities and effects may occur that lead to a greater inhibitory effect of TAM when combined with lignan-rich FS.

The increased anticancer effect of TAM and FS in combination may also be mediated by nonhormone-related actions. One of the possible mechanisms is modulation of signal transduction pathways. Previous studies have found that FS down-regulates insulin-like growth factor I expression in rats and breast tumors (10, 36). The cytostatic effect of TAM has been associated with insulin-like growth factor I receptor regulation as observed in MCF-7 cells (37). Epidermal growth factor receptor has also been associated with TAM resistance (38, 39), which can be prevented by combining TAM with an epidermal growth factor receptor-tyrosine kinase inhibitor (40). Previous work in our laboratory found that 10% FS reduced the −ER breast cancer epidermal growth factor receptor expression in nude mice (10), and HER2, a member of epidermal growth factor receptor family, expression in human breast carcinomas (8). All of these suggest that dietary FS supplementation during TAM treatment may result in an additive decline of transduction signal activities, ultimately delaying or retarding the development of TAM resistance and tumor growth.

Antiangiogenesis is also a potential mechanism. Lignans in FS inhibited angiogenesis *in vitro* (41), and 10% dietary FS reduced vascular endothelial growth factor expression in −ER breast cancer cells (9). TAM treatment reduced vascular endothelial growth factor expression and vascular density in MCF-7 tumors in nude mice (42). Hence the combined TAM and dietary FS treatment may enhance antiangiogenesis in decreasing tumor growth.

ALA in FS may also partly contribute to the anticancer effect of FS and enhanced effect with TAM treatment. ALA reduces the production of eicosatetraenoic acids and leukotrienes (4, 43), both of which have been shown to directly stimulate the growth of malignant cells (44). Other ALA-related anticancer mechanisms include increased lipid peroxidation (43), antiangiogenesis (45), modulation of signal transduction pathways (44), and inhibition of mevalonate pathway (46). Further, cotreatment of TAM with 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% (47).

Differential effects on tumor cell proliferation and apoptosis were induced by the treatment with FS, TAM, alone, or their combination. FS and TAM alone inhibited cell proliferation and induced apoptosis, with or without E2 supplementation. After E2 withdrawal (Experiment 1), FS with TAM not only lowered Ki-67 LI, but also, in concordance, induced a higher apoptosis as compared with TAM alone. However, with E2 implant, the combined FS and TAM only decreased Ki-67 LI and had no significant impact on apoptosis. These results suggest that the strengthened tumor-inhibitory effect induced by the cotreatment of FS with TAM was attributable to inhibition of cell proliferation at both low and high E2 levels and also to enhancement of apoptosis at low E2 level.

This study has important clinical implications as it suggests an alternative approach for the management of breast cancer with TAM treatment. There were no adverse effects induced by the combination of 10% FS and TAM on food intake, body weight, or weights of major organs. Supplementation of 10% FS in the animal diet is equivalent to human intake of 25 to 50 g of FS per day, depending on the amount of other foods consumed. At such dose, no estrogenic promoting effect on tumor growth was observed at low circulating E2 level, which is about 35 pmol/L in ovariectomized mice (16) and within the range of plasma E2 levels in postmenopausal women (48, 49). An antiestrogenic effect on tumor growth was induced by 10% FS at high plasma E2 level (0.3–0.4 nmol/L E2 produced by 1.7 mg of E2 pellet), which is also within the range of physiologic E2 level in premenopausal women (48, 49).

In conclusion, FS inhibited the growth of human ER+ breast cancer MCF-7 and enhanced rather than antagonized the inhibitory effect of TAM in nude mice in the presence of low or high circulating E2 levels. The results are encouraging and provide some scientific justification for the clinical testing of FS in both pre- and postmenopausal breast cancer patients taking TAM.

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