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Soy processing influences growth of estrogen-dependent breast cancer tumors

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Soy-based products consumed in Asian countries are minimally processed whereas in the USA many of the soy foods and soy ingredients are highly processed. Soy foods contain complex mixtures of bioactive compounds, which may interact with one another. The objective of this study was to evaluate the ability of various soy products containing genistin, the glycoside form of genistein, to affect growth of MCF-7 cells transplanted into ovariectomized athymic mice. Products investigated included soy flour, two crude extracts of soy (soy molasses and Novasoy®), a mixture of isoflavones and genistin in pure form. Each of the soy flour-processed products was added to the diet to provide equivalent amounts of genistein aglycone equivalents (750 p.p.m.). Tumors in the negative control animals regressed throughout the study while the tumors in the soy flour-fed animals remained basically the same size (neither grew nor regressed). In animals consuming soy molasses, Novasoy®, mixed isoflavones or genistin alone, tumor growth was stimulated when compared with animals consuming a control diet devoid of soy. These same dietary treatments resulted in increased cellular proliferation. Changes in mRNA expression of gene targets (estrogen responsiveness, cell cycle progression, apoptosis and aromatase activity) in tumors induced by the different diets were evaluated. The relative expression of pS2, progesterone receptor and cyclin D1 was increased in animals consuming the Novasoy®, mixed isoflavones and genistin. Bcl2 mRNA expression was low in most of the dietary treatment groups compared with positive (estradiol implant) controls. Aromatase expression was not affected in any of the treatment groups. The degree of soy flour processing affects the estrogenicity of products containing a constant amount of genistein. Collectively, these findings suggest that for postmenopausal women with estrogen-dependent breast cancer, the consumption of foods containing soy flour is more advisable than consuming isoflavones in more purified forms.

Introduction

The incidence of chronic diseases, such as coronary heart disease and breast cancer is lower in Asian countries than that in the USA. Differences in disease incidence are often attributed to increased consumption of soy-containing products by Asian women. Soybeans contain numerous biologically active compounds that may be important in breast cancer. These include: protease inhibitors (the Bowman–Birk inhibitor), phenolic acids, phytic acid, phytosterols, lignans, saponins and isoflavones. Soy foods consumed in Asian countries, in general, are made from minimally processed soybeans or soy flour by different cooking processes and not by extraction or purification of soy components. Alternatively, in the USA, isoflavones and soy proteins have received the majority of attention in research evaluating the beneficial effects of soy consumption. Food and dietary supplement industries are producing highly processed soy products such as soy protein isolates (80–90% protein) and/or isoflavone-enriched products, which contain 40–70% isoflavones. These enriched products have probably lost some of the beneficial biologically active components and may not have the same health benefits as the soy foods consumed in Asian countries. In fact, the highly refined isoflavone-containing soy products may have detrimental effects. The study presented here will address the estrogenic effects of diets containing similar genistein content in the presence or absence of many of the biologically active components in soy.

Isoflavones are a group of flavanoid compounds that are structural mimics of endogenous 17β-estradiol (E_2) (1). Isoflavones found in soy are genistein, daidzein and glycitein. The compounds exist naturally in soy in several glycoside forms (2), but it is the aglycone form of isoflavones that is biologically active. Isoflavone aglycones bind to the estrogen receptors (ER) α and β resulting in estrogenic responses at the cellular level and these responses are consistent with other estrogen agonists (3). Estrogenic activity of isoflavones is of interest to older women, because endogenous estrogen is believed to be critical to bone health (4). Isoflavones have also been found to have non-hormonal activities independent of the ER (5). A majority of non ER-mediated actions of isoflavones are anti-proliferative at higher concentrations. Genistein has been shown to suppress the growth of numerous types of cancer cells *in vitro* (6–8). However, the anti-proliferative effects have not been observed *in vivo*. The lack of clinical or animal data demonstrating suppression of tumor cell proliferation is probably due to blood levels of isoflavones that are below a critical concentration. At low concentrations *in vitro* (<10 μM), genistein and other isoflavones act solely as estrogen agonists (3,9), while at higher concentrations (>10 μM) genistein has multiple biological effects on cells, in addition to estrogenic activity, with the end result often being suppression of cellular growth (10). Blood concentrations of total genistein reported in humans are 0.5–5 μM (11–13). It is unlikely that dietary isoflavone consumption will result in plasma genistein concentrations required for anti-proliferative effects reported *in vitro*.

An association between soy consumption and lower cancer risk (including breast cancer) is well-documented (14–17). In

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; ER, estrogen receptors; PR, progesterone receptor.

one epidemiologic study, women who reported having consumed soy at least once a week during adolescence showed a statistically significant reduced risk of breast cancer (16). In Yamamoto *et al*., the authors report that increased miso soup and/or isoflavone consumption was associated with a reduced risk of breast cancer in Japanese women (17). Pre-clinical studies have also been designed to focus on isoflavone consumption and breast cancer development. Genistein administered to pre-pubertal rats reduces the number of mammary tumors that form after subsequent exposure to a chemical carcinogen (18–20). Soy-based products such as protein isolates (21), miso (22) and tofu (23) have also been found to reduce chemically induced tumorigenesis in rats. These findings are consistent with epidemiological data that associate lifetime, and particularly pre-adolescent, consumption of soy with a decreased risk of breast cancer development in humans (16). However, not all studies have reported such protection against tumor development. Intact female rats fed soy protein isolate following carcinogen exposure had no difference in tumor incidence (24). ERα wild-type mice fed 1000 p.p.m. genistein from weaning throughout the study developed malignant mammary adenocarcinomas following exposure to a chemical carcinogen, while the wild-type animals on a control diet void of isoflavones developed only benign adenomas (25). Timing of genistein exposure appears to be critical in determining its impact on development of breast cancer (5).

In contrast to correlations of soy consumption and breast cancer development, exposure of pre-existing estrogen-dependent tumors to estrogenic effects of dietary genistein may have a negative outcome by stimulating growth. This is particularly true in women who have low circulating estrogen levels such as postmenopausal women. Genistein increases MCF-7 cell growth in cell culture and following implantation into athymic mice (9). Additionally, dietary genistin, the glycoside form of genistein, stimulates growth of MCF-7 tumors transplanted in ovariectomized athymic mice (26). This is critical, because ~99% of genistein and the other isoflavones are present in soy as glycosides (27). Soy protein isolates containing increasing concentrations of isoflavones have also been found to stimulate dose-dependent growth of MCF-7 tumors transplanted into athymic mice (28). Data demonstrating that dietary genistein stimulates estrogen-dependent mammary tumor growth is not limited to the athymic transplant model. Genistein has been shown to stimulate the growth of chemically induced mammary tumors in ovariectomized rats (29). The fact that dietary genistein stimulated chemically induced tumors is important, because contrary to MCF-7 cells transplanted in athymic mice, chemically induced mammary tumors invade surrounding tissues and metastasize to other organs. Allred *et al*. demonstrated that genistein-stimulated growth of estrogen-dependent mammary tumors that have pathological characteristics similar to those found in women (29). Therefore, while soy isoflavones may be protective against the development of breast cancer, other studies indicate that consumption of isoflavones after a tumor has formed may in fact stimulate its growth.

The effect of dietary genistein, as a component of a soy food has not been adequately addressed regarding estrogen-dependent breast cancer tumor growth. We have demonstrated that soy protein isolates (85–90% soy protein) stimulate the growth of estrogen-dependent tumors. However, soy protein isolates do not contain many of the bioactive components present in whole soy. Soy foods are most often associated with improved health and it is critical to understand the activities of genistein in the context of whole soy. Isoflavones are consumed in Asia as traditional soy foods and not in pure or enriched forms (30). The objective of this study was to evaluate a series of products that are produced when soy is refined. These products include soy flour, which is produced when soybeans are ground, defatted and toasted. Other products evaluated were derived from soy flour and included two crude extracts of soy (soy molasses and Novasoy®), a mixture of crystallized isoflavones and purified genistin. Each product was produced by successive purification of the previous one leading to a more refined or purified product. All products evaluated were added to a phytoestrogen-free diet to provide equal concentrations of genistein (aglycone equivalents). Additionally, diets were isocaloric and nutritionally balanced for fat, protein and carbohydrate contents. Pre-clinical and epidemiological studies have demonstrated the protective effect of whole soy products against breast cancer. In this study, we evaluated the effect of different degrees of soy processing on the growth of pre-existing tumors.

Materials and methods

Tumor growth model

Animals. Female athymic nude mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and delivered at 28 days of age. Mice were ovariectomized at 21 days of age by the vendor and allowed a week to recover prior to delivery. A 2 mg E_2 pellet was placed under the skin of each mouse before MCF-7 cells were transplanted into the animal. Cells (1×10^5 cells/site) were then injected into the four flanks on the back of each animal. Average tumor cross-sectional area reached 40 mm^2 4 weeks after the E_2 implantation. Animals were then randomly assigned to one of eight treatment groups. The treatment groups were: (i) positive control; (ii) negative control; (iii) soy flour + mixed isoflavones; (iv) molasses; (v) Novasoy®; (vi) mixed isoflavones and (vii) genistin. The E_2 pellet was removed from all of the animals and a new E_2 pellet (2 mg) was implanted into animals in the positive control group. Negative and positive controls were given American Institute of Nutrition 93 growth diet (AIN 93G) as a control diet. The remaining animals were put on one of five dietary treatments, which were individually formulated and balanced for energy and genistin concentration. Tumor area and body weight were measured weekly. Food intake for each mouse was determined over several 24 h periods randomly throughout the study. At necropsy, tumors and plasma samples were collected for analysis.

Diet formulation. Each product tested (provided by Archer Daniels Midland, Decatur, IL) was derived by successive purification of the previous product which allowed us to evaluate if biologically active compounds were removed by the purification steps AIN 93G semi-purified diet, with corn oil substituted for soy oil, was selected as a basal diet for control animals because it has been established as meeting all of the nutritional requirements of mice (31). The soy flour and molasses products contained macronutrients and each was substituted into an AIN 93G diet (Table I). All other products were also substituted into the AIN 93G diet, but they provided no nutritive value. All diets meet the nutritional requirements of the mouse. In each case, it was possible to substitute the products into the diet without affecting caloric content. The soy flour and molasses diets were similar to that of the basal AIN 93G diet with regard to total fat, total carbohydrates and total protein (Table I). Soy products were added to each isocaloric diet to provide 750 p.p.m. genistein equivalents (see Table II), a dose that is sufficient for stimulating growth of MCF-7 tumors *in vivo* (9). However, when substituting the maximum amount of soy flour this diet contained only 433 p.p.m. genistein equivalents. Therefore, soy flour was supplemented with the mixed isoflavone product at a level that would provide a final concentration of 750 p.p.m. genistein.

E_2 pellet preparation. After 1 week of acclamation animals were implanted with E_2 pellets. Each E_2 pellet implanted contained 2 mg of E_2 mixed with 18 mg of cholesterol as a carrier. E_2 pellets were then placed subcutaneously in the interscapular region of mice.

Tumor implantation. MCF-7 cells were maintained in improved minimal essential medium (IMEM) (Biofluids) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and E_2 (1 nM) at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested using trypsin–EDTA,

and injected at 1×10^5 cells (in 40 μl Matrigel®, Becton Dickinson, Alto, CA) per site into each of the four flanks of the athymic mice.

Tumors were measured weekly and cross-sectional area was determined using the formula [length/2 × width/2 × p] (32,33). When tumors reached an average cross-sectional area of 40 mm^2, animals were randomly assigned into treatment groups with each group normalized for tumor number, tumor size and animal number. E_2 pellets were removed from all animals and mice were then placed on treatment diets. Positive-control mice were re-implanted with a fresh E_2 pellet (2 mg). Tumor area was measured weekly as described above. All animal procedures were approved by the Animal Care and Use Committee of the University of Illinois.

Table I. Formulations and estimated nutrient composition of experimental diets

Ingredient (g)	Diet		
	Soy flour + mixed isoflavones	Molasses	AIN 93G[a]
Cornstarch	318.986	361.85	397.486
Casein	NA	195.13	200
Dextrinated cornstarch	132	132	132
Sucrose	100	69.4	100
Corn oil	56.4	58.7	70
Fiber	NA	50	50
Mineral mix (AIN-93G-MX)	35	35	35
Vitamin mix (AIN-93G-VX)	10	10	10
L-Cystine	3	3	3
Methionine	2.1	NA	NA
Choline bitatrate	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014
Soy flour	340	NA	NA
Soy molasses	NA	82.4 U/kg diet	NA
Total carbohydrate, g	623.8[b]	637.3[b]	643.7
Total fat, g	70[c]	70	70
Total protein, g	180.2[d]	179.11	178.6

[a]AIN 93G with corn oil substituted for soy oil was used as control diet. All other soy components (except SF and Mol) were added to AIN 93G. These components contributed no nutritive value to the diet. Nutrient composition of AIN 93G diet was taken from Reeves *et al.* (31). NA (not applicable) was entered for cells where specific ingredients were not added.
[b]Nutrient content of soy flour and molasses products was analyzed prior to diet formulation. The above values are estimates based on the amount of each product added to the diet.
[c]Fat in the soy flour-based diet was from two sources. Soy flour provided 13.6 g of fat while the remaining 56.4 g came from addition of corn oil. Corn oil was the sole source of fat in all of the AIN 93G diets.
[d]In the soy flour-based diet, soy protein is the sole source of protein. In the molasses diet, 4.86 g of protein came from soy protein while 195.13 g of casein protein was added. Casein is the only source of protein in the AIN 93G diets.

Tissue/tumor collection. At the conclusion of the study, mice were killed by cervical dislocation and tumors were collected. Tumors from each mouse were immediately frozen by submersion in liquid nitrogen for immunohistochemical staining and RNA isolation.

Tumor cell proliferation immunohistochemistry

BrdU (5-bromo-2′-deoxyuridine) analysis. BrdU incorporation into cellular DNA was used as an indicator of proliferation (34). Four hours prior to killing the animals, each mouse was injected intraperitoneally with 50 mg BrdU/kg body wt. Tumors were excised, skin and fat removed, and immediately frozen in liquid nitrogen. Then, 4 to 6 μm sections were cut from each tumor. Prepared sections were then stained for the presence of BrdU utilizing a modified immunohistochemistry protocol (28,35). Positively stained cells were counted in a given area of tissue (0.54 × 0.54 mm) and five positions were randomly selected to count cells in each tumor. Data are presented as average numbers of proliferating cells in a given area of tumor.

Analysis of mRNA expression

RNA preparation. Frozen tumors (≤200 mg) from liquid nitrogen were smashed and the coarse tumor powder was transferred into TRIZOL® (Gibco BRL, Grand Island, NY) in a 15 ml Corex® tube and was homogenized using a Polytron-Aggregate (Littau/Luzern, Switzerland). Chloroform was added into a homogenized tumor sample, shaken vigorously and then incubated for 10 min at 24°C. The reaction tube was centrifuged at 12 000 *g* for 15 min at 4°C. The upper portion was transferred into a fresh tube. An equal volume of isopropyl alcohol was added, shaken and incubated for 10 min at 24°C. The mixture was centrifuged at 12 000 *g* for 10 min at 4°C. The RNA pellet was washed with ice-cold 75% ethanol, and centrifuged at 7500 *g* for 5 min at 4°C. The RNA pellet was air-dried, then dissolved into RNase-free dH_2O. RNA was stored at −80°C and the concentration was measured at 260 nm (1 OD_{260} = 40 μg of single stranded RNA/ml).

Real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). Five mRNA targets were selected to evaluate the effect of soy products on their ability to modulate these targets. Analysis was done on pS2 and progesterone receptor (PR) to determine the effect of the products on estrogen-responsive genes in the tissues. Cyclin D1 and bcl2 were chosen to determine the actions of the products on the ER-mediated cell cycle progression and relative amounts of apoptosis, respectively. The aromatase gene was chosen to examine if different soy extracts were capable of modulating activity of aromatase at the DNA level. A change in relative abundance of aromatase mRNA would suggest that the diets were capable of influencing the concentration of endogenous E_2 in the tissues. Reagents for RT and PCR reactions were purchased from PE Applied Biosystems (Foster City, CA) and Invitrogen (Carlsbad, CA).

One RT reaction contained 1× PCR buffer, 5.5 mM of $MgCl_2$, 500 μM of each dNTP, 2.5 μM of random hexamer, 8 U of RNase inhibitor, 0.16 U

Table II. Isoflavone analysis of various treatment diets

Isoflavone (p.p.m.)[a]	Diet				
	Soy flour + mixed isoflavones	Molasses	Novasoy	Mixed isoflavones	Genistin
Genistein	9.7	9.3	15.7	8.3	1.5
Genistin	750.3	1015.1	1054.3	1148.0	1178.7
Malonyl-genistein	299.3	62.4	7.0	3.0	4.0
Acetyl-genistin	196.2	120.3	118.8	26.5	8.4
Daidzein	10.8	9.1	20.2	5.6	0.0
Daidzin	502.3	804.4	798.8	619.9	134.4
Malonyl-dadzein	319.5	68.8	20.9	0.0	0.0
Acetyl-dadzin	170.6	115.4	120.9	30.8	0.0
Glycitein	3.3	4.9	16.7	2.6	0.0
Glycitin	112.1	171.2	244.3	88.7	6.0
Malonyl-glycitein	82.0	23.0	16.1	0.0	0.0
Acetyl-glycitin	21.8	11.1	11.5	2.7	0.0
Total aglycone genistein equivalents (μmol/g)	749.8 (2.8)	749.8 (2.8)	751.2 (2.8)	748.2 (2.8)	751.0 (2.8)

[a]Isoflavone profiles were determined by Archer Daniels Midland.

Table III. Relative expression (±SEM)[a] of mRNA in tumors from mice fed various soy extract products

Gene target	Treatment						
	Negative control	Soy flour + mixed isoflavones	Molasses	Novasoy	Mixed isoflavones	Genistin	Positive control
pS2	1.1(0.2)[1]	1.7(0.2)[1]	2.3(0.2)[1]	3.9(0.4)[2]	4.1(0.4)[2]	5.6(0.6)[3]	9.0(0.6)[4]
PR	1.1(0.2)[1]	0.9(0.2)[1]	1.5(0.3)[1]	2.4(0.3)[1,2]	2.4(0.5)[1,2]	3.7(0.6)[2]	7.6(0.7)[3]
Cyclin D1	1.0(0.1)[1]	1.4(0.1)[1]	1.6(0.2)[1]	2.5(0.2)[2,3]	2.4(0.2)[2]	3.1(0.3)[3]	3.8(0.2)[4]
Bcl2	1.2(0.2)[1,2]	1.1(0.1)[1]	1.2(0.1)[1]	1.4(0.2)[1,2]	1.2(0.2)[1]	2.0(0.2)[2]	2.9(0.3)[3]
Aromatase	2.0(0.4)	1.9(0.3)	2.4(0.4)	2.5(0.5)	2.0(0.3)	2.2(0.4)	2.3(0.4)

[a]Seven tumors per treatment group were analyzed and relative mRNA expression was quantified using RT–PCR. GAPDH was used as a standard. SEM represents the standard error of the mean. Cells within a row that have different superscript numbers are significantly different ($P < 0.05$).

reverse transcriptase and tumor RNA (10 ng). Thermal cycling for the RT reaction was incubation for 10 min at 25°C, reverse transcription for 30 min at 48°C, and reverse inactivation for 5 min at 95°C. The pS2, PR, cyclin D1, bcl2 and aromatase primers and fluorescence-labeled probes were designed using Primer and Probe Design Express (PE Applied Biosystems) and were purchased from Integrated DNA Technologies (Coralville, IA) and Synthegen (Houston, TX). PCR and analysis of PCR products were performed using the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems) as described in company protocols. Briefly, total volume of a PCR reaction mixture was 25 μl containing 12.5 μl of Taqman Universal Master PCR mix, 1 μl of cDNA, 100 nM of fluorescence-labeled probe, and 40–200 nM of forward/reverse primers. Data were analyzed using a Ct cycle method (Bulletin, PE Applied Biosystems).

The parameter Ct was defined as the point at which the amplification plot, representing the fractional cycle number of fluorescence generated by cleavage of the probe (ΔRn), passed a fixed threshold above baseline. Ct was reported as the cycle number at this point. A comparative Ct method detected relative gene expression. Amplicons were run as triplicates in separate tubes to permit quantification of target genes normalized to a control, human GAPDH. The data are presented in Table III. Individual gene targets are represented in each row. Within rows treatments that caused significant changes in mRNA expression are notated by different numbers.

Statistical analysis

Tumor area data were analyzed according to a completely randomized design with a one-way or repeated measures analysis of treatment according to the characteristics of the data set. Gene expression (pS2, PR, Cyclin D1, bcl2 and aromatase) and cellular proliferation data were analyzed according to a randomized design with a one-way analysis of treatment. If the overall treatment F-ratio was significant ($P < 0.05$), differences between treatment means were tested with Fisher's least significant difference (LSD) test. Error bars on all graphs represent standard error of the mean. All statistical analysis was done using the SAS program (SAS, Cary, NC, 1985).

Results

Effect of various dietary treatments on the growth of MCF-7 tumors in athymic mice

Ovariectomized athymic mice were implanted with a 2 mg E_2 pellet and subsequently transplanted with MCF-7 cells. Tumors were allowed to develop to a cross-sectional area of ~40 mm^2. At this point, E_2 pellets were removed and animals were assigned to one of the dietary treatments groups (all control mice received AIN 93G diet). Positive control animals were implanted with a new E_2 pellet and negative control mice were given AIN93G diet without estrogen supplementation. Four weeks after re-treatment with a new 2 mg E_2 pellet, the average cross-sectional area of the tumors in the positive control group was 140 mm^2 (Figures 1 and 2) and mice were killed. Eleven weeks after the E_2 pellets were removed, the negative control tumors regressed from 40 mm^2 to an average area of 16 mm^2. Soy molasses, Novasoy®, mixed isoflavone and genistin diets stimulated tumor growth to average areas of

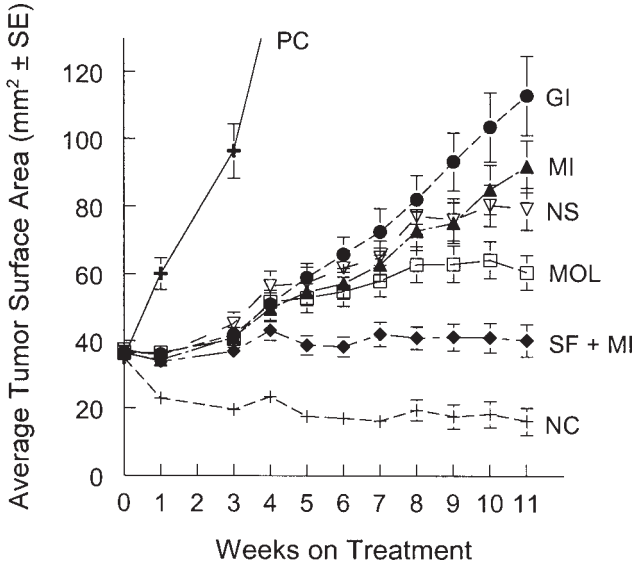

Fig. 1. Effects of soy products on MCF-7 tumor growth in athymic mice. Female ovariectomized athymic mice were implanted with a 2 mg 17β-estradiol pellet. The animals were then injected with MCF-7 cells in four locations. Subsequently, tumors developed at these four sites and were allowed to grow to an average cross-sectional area of 40 mm^2. At this time, estradiol pellets were removed from all of the mice and they were assigned to one of eight treatment groups: positive controls (PC) that were reimplanted with a new 2 mg estradiol pellet, negative controls (NC) that were fed AIN 93G rodent diet alone soy flour + mixed isoflavones (SF + MI), molasses (MOL), Novasoy® (NS), mixed isoflavones (MI) and genistin (GI). Tumors were monitored weekly. Data are expressed as average cross-sectional tumor area for each treatment. Error bars represent standard error of the mean.

60, 79, 91 and 112 mm^2, respectively (Figure 1). By week 11, the tumors in each of these groups were significantly ($P < 0.01$) larger than those in the negative control group (Figure 2). At week 11 of treatment, the average tumor area in the soy flour + mixed isoflavones group was 40 mm^2, which was also significantly ($P < 0.05$) higher than that of the negative control group; however, tumor area in this group was unchanged from the beginning of treatment.

Other differences in tumor area were observed between treatment groups at week 11. Tumors from animals fed genistin were significantly ($P < 0.01$–0.05) larger than all other treatment groups except the positive controls. Animals fed the mixed isoflavones had larger tumors than animals fed the negative control diet, soy flour + mixed isoflavones or molasses diets ($P < 0.01$). Of significant interest is the fact that the tumors from animals fed mixed isoflavones were

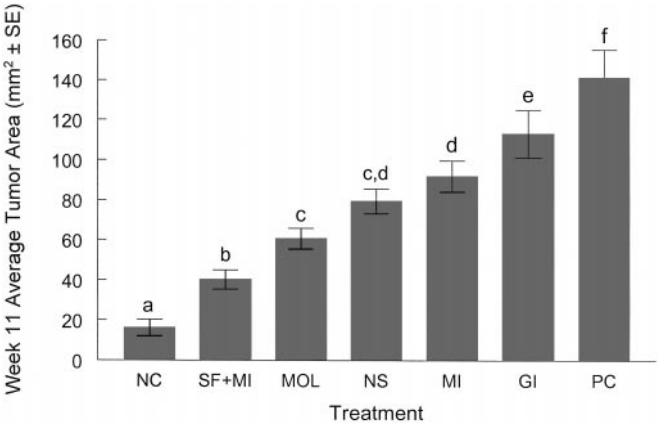

Fig. 2. Average tumor area for week 11. The graph represents the effect of dietary treatment with each of the soy products on the growth of MCF-7 tumors after 11 weeks (positive control tumors were collected at 4 weeks). Treatments included: positive control, negative control, soy flour + mixed isoflavones, molasses, Novasoy®, mixed isoflavones and genistin. All tumors from each treatment group were evaluated by measuring tumor area. Tumor areas from each group were then combined and averaged. Differences between treatment means were tested with Fisher's LSD test. Error bars represent the standard error of the mean. A lettering system has been used to represent statistical significance. Those bars with common letters are not different while those with unique letters are significantly different ($P < 0.05$) from one another.

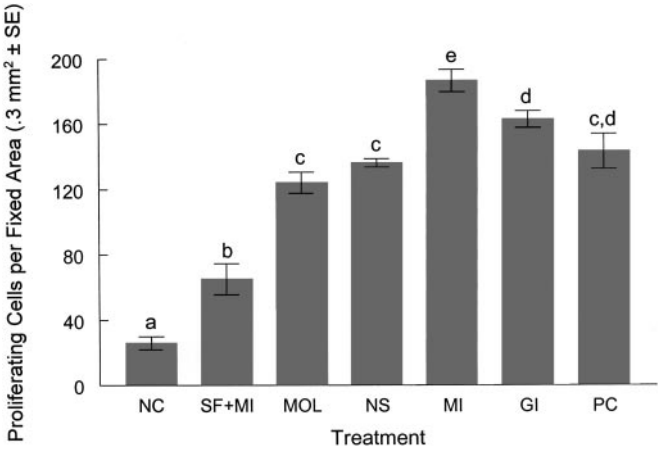

Fig. 3. Effect of soy products on the cellular proliferation within MCF-7 tumors. Tumors were removed from the mice and collected for immunohistochemical analysis. Incorporation of BrdU into cellular DNA was utilized as a marker of cellular proliferation. Immunohistochemistry was utilized to stain for cells containing BrdU. Positively stained cells were counted in a given area of tissue (0.54 × 0.54 mm) and five positions were randomly selected to count cells in each tumor. The data are presented as the average number of proliferating cells in a given area of tumor. Differences between treatment means were tested with Fisher's LSD test. Error bars represent standard error of the mean. A lettering system has been used to represent statistical significance. Those bars with common letters are not different while those with unique letters are significantly different ($P < 0.05$) from one another.

significantly ($P < 0.01$) larger than tumors from animals fed the soy flour + mixed isoflavones. Tumors from animals fed soy molasses were also significantly ($P < 0.01$–0.05) larger than negative control and soy flour + mixed isoflavones groups by week 11. No significant difference was observed in food intake of animals consuming the various dietary treatments (data not shown). Plasma concentrations of total genistein (aglycone genistein + genistein conjugates) were <5 μM (<5% of the total genistein is agalycone, which is estrogenically active form) in all treatment groups (data not shown), which are similar to total genistein concentrations reported in humans who consume soy-containing products (11–13).

Cellular proliferation in MCF-7 tumors

Cellular incorporation of BrdU was utilized as an indicator of cellular proliferation in MCF-7 tumors. Total numbers of proliferating cells in a given area in five randomly selected locations within the tumor were counted and final values were expressed as average number of proliferating cells. The average number of proliferating cells for the negative control group was 25 (Figure 3). Positive control animals had a significantly ($P < 0.01$) higher number of cells proliferating when compared with the negative control group with a value of 143. Mice consuming soy flour + mixed isoflavones, molasses, Novasoy®, mixed isoflavones and genistin had average numbers of proliferating cells of 65, 124, 136, 186 and 162, respectively. These values were significantly ($P < 0.01$) higher than the negative control. Average number of proliferating cells in the tumors from mice fed genistin, soy molasses and Novasoy® products were not significantly different from the positive control group. The value from the animals fed the mixed isoflavones product was significantly ($P < 0.01$) different from the positive control group. The mice consuming the mixed isoflavones had a significantly greater number of proliferative cells when compared with the animals consuming soy flour + mixed isoflavones ($P < 0.01$).

Effect of various dietary treatments on mRNA expression in MCF-7 tumors

To evaluate the ability of various soy extracts to modulate mRNA expression of selected genes, we conducted quantitative RT–PCR analysis using mRNA isolated from tumors in each treatment group. Tumors were collected from the positive control group 4 weeks after re-treatment with a new E_2 pellet, while tumors were collected from all of the other groups 11 weeks after the treatment began.

pS2

Expression of pS2 was higher ($P < 0.01$) in animals continually treated with E_2 when compared with the negative control group (Table III). Also, animals that were consuming Novasoy®, mixed isoflavones and genistin products had significantly ($P < 0.01$) higher levels of pS2 expression when compared with negative controls. The relative level of pS2 expression in tumors from the mixed isoflavones group was also significantly ($P < 0.01$) higher than that seen in tumors from mice fed soy flour + mixed isoflavones.

PR

As in the case of pS2, the relative expression of PR was significantly higher ($P < 0.01$) in tumors from positive control animals compared with tissues excised from negative control mice (Table III). PR expression in tumors from animals in the soy molasses, Novasoy® and mixed isoflavones groups was higher than that of tumors in negative control mice, but this difference did not reach statistical significance. Genistin induced significantly ($P < 0.01$) higher expression of PR in their tumors when compared with negative controls.

Cyclin D1

Expression of cyclin D1 in tumors from animals in the negative control group was significantly ($P < 0.01$) lower than that

of the positive control group (Table III). Animals in the Novasoy®, mixed isoflavones and genistin treatment groups had increased ($P < 0.01$) expression of cyclin D1 in their tumors when compared with negative control. Mice fed soy flour + mixed isoflavones had significantly ($P < 0.01$) lower cyclin D1 expression than that found in tumors from animals in the mixed isoflavones group.

bcl2

Unlike some of the other genes chosen for analysis, dietary treatment with the soy products did not greatly modulate expression of bcl2 mRNA, an apoptotic marker (Table III). No significant difference was observed between any treatment groups fed soy products and negative control animals. Continuous treatment with E_2 in the positive control animals did however, result in a significantly ($P < 0.01$) higher expression of bcl2 mRNA when compared with tumors from negative control animals and all of the dietary treatments.

Aromatase

No significant differences were observed between any of the treatment groups when relative expression of aromatase mRNA was measured in the tumors (Table III).

Discussion

The objective of this study was to determine if the matrix of compounds present in soy flour impacts the ability of diets with equal amounts of genistein as aglycone equivalents to stimulate the growth of estrogen-dependent breast tumors *in vivo*. This comparison is critical because soy products in the Asian diet are primarily made from either soybeans or soy flour. This is different than the way soy products and isoflavones are consumed in the USA. In general, soy that is consumed in the US has many of the bioactive compounds in soy removed by processing. Therefore, it is possible that the protective effects of many of the components in soy are not available from soy products produced and consumed in the USA. To evaluate this possibility we utilized soy flour, crude extracts (soy molasses and Novasoy®), purified mixtures of isoflavones, and genistin for their potential to stimulate estrogen-dependent tumor growth in a well-established preclinical tumor growth model.

We have reported previously that the rate of tumor growth in athymic mice increases in a dose-dependent manner as dietary genistein concentration increases (36). Diets in this study were prepared using soy components that were produced from progressive processing and purification. Each product produced by successive purification was incorporated into the AIN 93G diet and each dietary treatment with equal genistein content was used to evaluate the effect on growth of estrogen-dependent tumors. Soy molasses, Novasoy®, mixed isoflavones and genistin-containing diets all stimulated the growth of MCF-7 tumors. However, these diets do not stimulate tumor growth equally. Previous studies from our laboratory indicate that soy isoflavones stimulate MCF-7 tumor growth (28). In this study, diets were formulated so each soy product-containing diet would contain equal concentrations of genistein (750 p.p.m. aglycone genistein equivalents). If the level of dietary exposure to genistein was the principal factor that influenced tumor growth, various dietary treatments would have resulted in similar tumor growth. This was not the case. The matrix or composition of soy-derived compounds in the diet influenced genistein-stimulated tumor growth. The specific compounds that are responsible for these protective effects are currently unknown. However, the results here demonstrate that there is a significant difference in how soy processing can alter potential health benefits of soy foods.

The fact that biologically active components are lost during processing of soy flour is most evident when one compares the results from consuming the mixed isoflavones and mice consuming soy flour + mixed isoflavones. These two diets altered tumor growth and various other biomarkers of estrogenic effects and tumor growth in a dramatically different manner. Specifically, soy flour + mixed isoflavones and mixed isoflavones-containing diets each had identical aglycone genistein equivalents, but differed in the presence of the numerous other bioactive compounds originally present in the soy flour. Both diets meet the nutrient requirements of growing mice and food intake was not altered by any of the dietary treatments. Differences in tumor growth observed for these two diets are not due to the nutritive content, but are more likely due to the effects of non-nutritive components in the soy flour-containing diet. Tumors neither grew nor regressed over the treatment period in animals fed the soy flour + mixed isoflavones diet. This is of particular interest because the minimally processed soy flour (defatted toasted soybeans), as was used in these diets, is more representative of soy-containing foods consumed in the Asian diet. Conversely, diets in this study containing purified isoflavones are more representative of the use of dietary supplements containing isoflavones, which is how many Americans consume these compounds.

Diets that stimulated growth of the MCF-7 tumors also resulted in higher amounts of cellular proliferation when compared with negative controls. A difference in tumor growth between mice fed the mixed isoflavones and animals fed soy flour + mixed isoflavones was observed. It was important to determine if the diets impacted cellular function of MCF-7 cells differently at the mRNA level. Several gene targets were identified and used to evaluate each soy product for its ability to stimulate estrogenic effects (pS2 and PR), to influence cell cycle progression (cyclin D1), to modulate the apoptotic pathway (bcl2) and to affect production of endogenous estradiol (aromatase) at the tissue level. Therefore, the biomarkers of estrogenic effects and tumor cell growth were lower in the soy flour diets when compared with the diets containing genistin, mixed isoflavones or the soy extracts (molasses and Novasoy®).

The estrogen-responsive gene pS2 serves as a reliable reporter of estrogenic activity as transcription of the gene is induced by estrogen (37) and inhibited by anti-estrogens in MCF-7 cells (38). Dietary genistein and genistin increase mRNA expression of pS2 in MCF-7 tumors transplanted into athymic mice (9,26,28). Women consuming textured vegetable protein derived from soy in the form of bread rolls had increased expression of pS2 in their nipple aspirates (39). In the present study, products that significantly increased pS2 expression were Novasoy®, mixed isoflavones and genistin. Each of these products also increased relative concentration of PR mRNA, but only the mRNA levels in the genistin-fed animals reached significance when compared with negative control tumors. Relative concentration of PR has also been shown to be a marker of estrogenic activity in MCF-7 cells (40). Cyclin D1 mRNA expression was also measured. Synthesis of cyclin D1 occurs during the G_1 phase of the cell cycle prior to activation of cyclin-dependent kinase 2, which progresses

cells into S phase. E_2 has been found to increase cyclin D1 synthesis in MCF-7 cells (41). At concentrations as low as 0.5 μM, genistein also increases the concentration of cyclin D1 in MCF-7 cells *in vitro* (42). In this study, animals consuming Novasoy®, mixed isoflavones and genistin diets had increased cyclin D1 mRNA expression relative to negative controls. It is probable that cellular proliferation in tumors from these animals is the result of estrogenic action of compounds found in these various soy extracts. If cellular proliferation occurred without an increase in cyclin D1, then pathways independent of the estrogen receptor may be responsible for the proliferation. Collectively, data from these three target genes suggest that several of the soy products have sufficient estrogenic activity to increase cellular proliferation and such activation correlates with increases in tumor growth.

An increase in cellular proliferation was observed in tumors from mice fed all of the different dietary treatments when compared with negative control animals, but significant increases in tumor growth were only observed in mice fed molasses, Novasoy®, mixed isoflavones and genistin diets. It is possible that apoptosis was occurring concurrently with cellular proliferation within the tumors. To evaluate this, relative levels of bcl2 mRNA was measured in tumor tissue. It has been proposed that bcl2 protects cells from apoptosis (43). A decrease in bcl2 expression in MCF-7 cells is related to growth inhibition and apoptosis (44). Bcl2 protein levels in MCF-7 cells treated with 400 μM genistein decreased to 30% of the levels observed in controls in this study (45). Plasma concentrations of total genistein were <5 μM in all treatment groups (data not shown) and yet differences in bcl2 mRNA expression were observed. Since they regressed, tumors from negative control animals were assumed to contain apoptotic cells. Relative amounts of bcl2 in all of the dietary treatments were lower than positive controls, but not different from negative control animals. This suggests that while apoptosis occurs in these tumors, the relative amount of cellular proliferation caused by the consumption of each product overwhelmed apopotosis resulting in increased tumor growth. To verify the effect of the soy diets on apoptosis within breast tumors further experimentation exploring potential mechanisms of action will be required.

The relative level of aromatase activity was also measured in the tumors excised from mice consuming the various soy extract diets. *In vitro*, genistein has been shown to decrease aromatase activity of MCF-7 cells resulting in lower concentrations of estradiol in the media surrounding the cells, which in turn reduces the pS2 expression (46). This action is observed with high concentrations of genistein ($\geq$10 μM). The ability of genistein to act as an aromatase inhibitor has not been reported *in vivo*. We measured relative mRNA expression of aromatase in tumors of mice fed the various soy products and found no differences in mRNA concentrations between any treatment groups. Therefore, these diets do not influence aromatase gene expression.

It is possible that the composition of bioactive compounds in soy and soy-processed products may alter genistein absorption in the intestine. A study in mice demonstrated that combining dietary fructooligosaccharides with isoflavone conjugates increased the bioavailability of isoflavones (47). It is possible that the degree of processing of soy flour-derived products could alter bioavailability and affect the estrogenic potency of the genistein content. This hypothesis has been examined and will be reported in detail in a separate scientific communication (submitted for publication). Briefly, while the degree of soy processing did affect isoflavone metabolism and bioavailability, stimulation of tumor growth was best correlated with the plasma peak concentrations of aglycone genistein produced by the diets.

Another possibility for how soy extracts differentially influenced tumor growth in this study is that other, yet unidentified, compounds antagonized the ability of genistein to stimulate the growth of the MCF-7 tumors. Soybeans contain a wide variety of compounds in addition to the isoflavones that are being explored for their biological actions. These include, but are not limited to: protease inhibitors (the Bowman–Birk inhibitor), phenolic acids, phytic acid, lignans and saponins. It is possible that one or more of these compounds is capable of antagonizing the estrogenic activity of genistein. A group of bioactive compounds of soy that are not present in the more pure products is the phytosterols. We have reported that dietary phytosterols can reduce the effect of estradiol on stimulation of estrogen-dependent tumor growth (48). Specific interactions between the bioactive components of soy and genistein have not been identified and further investigation is required to explore this potentially important research area.

Consumption of isoflavone-containing products is being encouraged for postmenopausal women based on the perception that dietary exposure to estrogenic compounds will relieve symptoms of menopause by acting as a natural alternative to hormone replacement therapy. This belief persists despite the lack of significant supporting clinical evidence (49). However, with regard to breast cancer an increase in total estrogenic load in these women is a cause for concern. Seventy-five percent of breast cancer cases are diagnosed in women over 50 years of age and the majority of these tumors are defined as estrogen-dependent. It is important to understand how the consumption of isoflavones may affect a pre-existing estrogen-dependent breast tumor.

A number of epidemiological and animal model studies have been performed to evaluate how consumption of different soy-derived products influence the development of breast cancer. The present study was designed to investigate how soy processing affects the ability of isoflavones to stimulate the growth of pre-existing breast cancer tumors. Using a well-established preclinical estrogen-dependent breast cancer model we have demonstrated that consumption of isoflavones in pure or highly enriched forms may have deleterious effects on estrogen-dependent tumor growth. However, consumption of isoflavones contained in less-processed soy foods, such as soy flour, does not have the same effect on tumor growth, even though the isoflavone content [based on our previous investigations (9,28,36)] is sufficient to stimulate growth. Eating soy foods that are made from soy flour, such as the types of soy foods that are consumed in Asian countries, may be more advisable than consuming soy-derived supplements that do not contain the full complement of bioactive compounds and nutritive components originally present in soy itself. Therefore, although pure genistein may have a negative impact on growth of estrogen-dependent breast tumors, other bioactive components contained in whole soy products may, by an as yet unidentified mechanism, reduce the estrogenic effects of genistin.

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