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Antitumor and Antiangiogenic Activity of Soy Phytoestrogen on 7,12-Dimethylbenz[α]anthracene-Induced Mammary Tumors Following Ovariectomy in Sprague–Dawley Rats

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ABSTRACT: Soy phytoestrogen is often used as hormone replacement therapy to alleviate the symptoms of menopause in postmenopausal women. Since estrogen has been considered as an important risk factor for the development of breast carcinoma, we need to know whether it is safe for these postmenopausal women with breast cancer to take soy foods that are rich in phytoestrogen. In the present study, we investigated the efficacy of soy phytoestrogen on tumor proliferation, apoptosis, and angiogenesis in mammary tumors that had already formed in ovariectomized rats. We found that soy phytochemical extraction inhibited proliferation and induced apoptosis *in vitro* and *in vivo*, and it demonstrated better antitumor effects than single phytoestrogen. Soy phytochemical extraction also produced surprisingly good antiangiogenic effects, which were evidenced by lower microvascular density, reduced plasma vascular endothelial growth factor, and increased plasma endostatin levels. Our findings suggest that soy phytochemical extraction exerts significant antitumor and antiangiogenic activity in a postmenopausal animal model with breast cancer.

Keywords: angiogenesis, breast cancer, daidzein, genistein, soy

Introduction

When we discuss menopause and alternatives to hormone replacement therapy, phytoestrogen is often mentioned. Phytoestrogens are plant-derived hormone-like diphenolic compounds of dietary origin. These include the flavones, flavanones, flavanols, isoflavones, lignans, and coumestans, which are found in numerous plant sources (Sirtori and others 2005). A growing number of studies suggest that isoflavones found in soybeans have estrogenic activity and may safely alleviate the symptoms of menopause (Somekawa and others 2001; Messina and Hughes 2003). Statistical data show that a majority of breast cancer cases occur in postmenopausal women (Yancik and others 2001), and chemotherapy can also cause the symptoms of early menopause in breast cancer patients, including hot flashes, irritability, sleep disturbances, and vaginal dryness (Davis and others 2005). Since sex hormones, especially estrogen, have been implicated as an important risk factor for the development of breast carcinoma, we need to know whether it is safe for these postmenopausal women to consume soy foods that are rich in phytoestrogen.

An association between increased intake of soy products and reduced risks of certain cancers in Asian countries, such as breast and prostate cancers, has been suggested (Wu and others 2002). Recent studies have demonstrated that soy isoflavones, mainly genistein and daidzein, have potent antioxidant properties, and their antioxidant power can reduce the long-term risk of cancer by preventing free radical damage to DNA (Wietrzyk and others 2005). Most of these studies have focused on the relationship between soy isoflavones and cancer prevention, and there are limited data about how soy isoflavones impact breast cancer in postmenopausal women whose estrogen is only synthesized locally in peripheral target tissues by tissue-specific steroidogenic enzymes. Some researchers have suggested that phytoestrogen may act as a dietary estrogen by binding empty estrogen receptors during conditions of low circulating endogenous estrogen (Wuttke and others 2003). These phytoestrogens can also produce a wide range of non estrogen receptor-mediated intracellular responses, and may have potential as antimetastatic compounds (Boersma and others 2001). When we evaluate the overall biological effects of phytoestrogen, it is necessary to consider more than hormone-related activities. Here, we investigate the antitumor and antiangiogenic effects of genistein, daidzein, and soy phytochemical extraction on mammary tumors that have already formed in ovariectomized rats.

Materials and Methods

Cells and chemicals

Human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the Tumor Research Inst. of Harbin. Human umbilical vein endothelial cells (HUVEC) were purchased from the Cell Biology Inst. of Shanghai. Cell culture medium and additives were obtained from Gibco Co. (Shanghai, China) if not otherwise stated. The breast cancer cell lines were cultured in DMEM without phenol red supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 IU/mL penicillin-G, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. HUVEC cells were

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maintained in MCDB131 culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 units/mL heparin, 10 ng/mL epidermal growth factor, and 5 ng/mL basic fibroblast growth factor at 37 °C in 5% CO_2.

Genistein, daidzein, and soy phytochemical extraction (SPE) were obtained from Feida Biotechnology Co. (Xian, China). The purity of genistein or daidzein, as determined by HPLC, was above 98%. SPE was prepared as follows: soybeans were cracked and flaked, then extracted with hexane to remove a majority of the lipids. The resulting defatted soy flour was extracted with ethanol and purified with resin adsorption and acetone to obtain a final powder with a 40% isoflavone concentration that contained 22.5% genistein, 15.4% daidzein, and 2.1% glycitein. One gram of final soy phytochemical extraction also contains 0.12 g of protein, 0.02 g of fat, 0.08 g of ash, and 0.011 g of moisture, with the remaining matter undefined but apparently rich in saponins.

Tumor cells and endothelial cell proliferation assay

In vitro proliferative testing was performed on single-cell suspensions of MCF-7, MDA-MB-231, and HUVEC cells plated in 96-well plastic plates (1% gelatin coated for the endothelial cells) and allowed to attach overnight. Each drug concentration was represented by at least 10 wells and replicated 3 times. Cells were treated for 72 h (10^4 cells/well in 200 μL of medium) with genistein, daidzein, and SPE. At the end of the experiment, the cells were pulsed for 6 h with 2 μCi/well of methyl-[^{3}H]-thymidine (Atomic Energy Inst. of Beijing, China), as described previously by Klement and others (2000). The cells were counted and compared with controls in triplicate experiments.

Animals and diets

A total of 100 female Sprague–Dawley rats were obtained at 6 wk of age. After 1wk of acclimatization, rats were given a single dose of 20 mg/rat of 7,12-dimethylbenz[α]anthracene (DMBA, Sigma, Beijing, China) in 1 mL of corn oil by gavage. At this stage, the rats were maintained on a modified AIN-93G diet (SLAC, Shanghai, China) in which soy oil was replaced by corn oil to minimize extraneous phytoestrogens until the time of individual treatments. Palpable tumors began to appear 6 wk after carcinogen exposure. When palpable tumors reached an acceptable size (300 to 400 mm^3), the rats were bilaterally ovariectomized under anesthesia and randomly placed into 1 of 5 treatment groups. The experiment groups were as follows: (1) control, (2) genistein 50 mg/kg, (3) daidzein 50 mg/kg, (4) SPE 50 mg/kg, and (5) SPE 100 mg/kg. The rationale for selecting 50 and 100 mg/kg for the isoflavones and SPE was based on a preliminary study in rats that indicated that doses below 50 mg/kg were ineffective in tumor angiogenesis and apoptosis. The average daily intake per rat falls within the amount of soy isoflavone daily intake (25 to 50 mg) consumed in Asian countries (Messina and others 2006). The experimental solutions or the vehicle (sterile water) were administered to the rats by gavage daily. The experiment was terminated 20 wk after carcinogen exposure. Blood was taken via cardiac puncture and centrifuged at 1000 × *g* for 10 min, followed by collection of the plasma. Plasma samples were stored at −80 °C. Tumors were weighed and fixed immediately in buffered formalin and paraffin-embedded within 24 h of excision. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical Univ.

In situ apoptotic cell detection

Detection of DNA fragmentation was done according to the manufacturer's instructions using an *in situ* cell death detection kit measuring terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Roche, Beijing, China). The apoptotic index was calculated as the percentage of positive cells over total cells counted. From each tumor, more than 1000 cells were evaluated for the presence of apoptotic cells.

Tumor cell proliferation measurement

Cellular proliferation in tumors was determined using BrdU immunohistochemistry. Each rat was injected intraperitoneally with 50 mg BrdU/kg body weight 2 h before killing the animals. The nuclei labeled with BrdU were identified using an anti-BrdU monoclonal antibody (Sigma, Shanghai, China). More than 1000 cells were randomly scored from each tumor and the percent of BrdU-labeled cells was evaluated.

Immunohistochemical determination of angiogenesis

Immunohistochemical quantitation of microvascular density (MVD) was carried out using the Streptavidin Peroxidase kit (Zhongshan Biotechnology Co., Beijing, China). CD34 monoclonal antibody was used as the primary antibody. Detection was carried out using 3,3′-diaminobenzidine chromogen, which resulted in a positive brown staining. Negative control slides were obtained by omitting the primary antibody. The quantification of MVD was assessed according to the method of Weidner (1995). The sections were initially screened at low magnifications (×40) to identify the most vascular area of the tumor (hot spot). Within the hot spot area, the stained microvessels were counted in at least 3 high-power (×200) fields. MVD was expressed as the number of microvessels/field. Slides of the immunohistochemical studies were quantified in a blinded fashion by 2 independent reviewers at 2 different times.

ELISA assays of plasma angiogenic and antiangiogenic factors

Concentrations of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and endostatin in plasma samples were measured with enzyme immunoassay kits for rat VEGF, bFGF, and endostatin (R&D Systems and Shanghai Transhold Tech, Shanghai, China). ELISAs were performed according to the manufacturer's instructions. Inter- and intra-assay coefficients of variance were 5% to 8.5% and 3.5% to 6.5%, respectively.

Statistical analysis

Data from cell culture studies, apoptotic index, proliferation index, tumor weight, microvessel density, and plasma angiogenic factors were evaluated by analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. All statistical analyses were done using the SPSS 16.0 program (SPSS Inc., Beijing, China). Differences were considered significant at a P value of < 0.05.

Results

Antiproliferative effects of genistein, daidzein, and soy phytochemical extraction on MCF-7 cells, MD-MBA-231 cells, and endothelial cells

At a dose of 5 mg/L, genistein slightly promoted MCF-7 cell growth by 12%, but inhibited the growth of MD-MBA-231 cells and HUVECs (92% and 84% of control, respectively). The inhibitory ratio of HUVECs was higher than those of MCF-7 and MD-MBA-231 cells by genistein at 5 and 10 mg/L ($P < 0.05$). As the dose of genistein was increased to 15 and 20 mg/L, MCF-7 cell growth was dramatically suppressed, and the inhibitory ratio exceeded those of MD-MBA-231 and HUVEC ($P < 0.05$). Daidzein showed less antiproliferative activity in comparison to genistein in MCF-7,

MD-MBA-231, and HUVEC cells. The dose-dependent antiproliferative effect was also observed when cells were treated with SPE. HUVEC growth was inhibited 64% by 15 mg/L SPE, 70% growth was inhibited by 20 mg/L SPE, and they were higher than those of genistein ($P < 0.05$). SPE also demonstrated a better antiproliferative effect on MD-MBA-231 cells (50% compared with 42% for genistein at 20 mg/L, $P < 0.05$; Figure 1 and 2).

Tumor apoptosis and proliferation after genistein, daidzein, and soy phytochemical extraction treatment

In rats receiving genistein and SPE50, the tumor apoptotic index (AI) was raised by 40.2% and 24.5%, respectively, of that of the control group. SPE100 treatment showed a remarkably higher increase of apoptosis in breast tumors (increased by 72.5% of that in the control group). Genistein was observed to increase the proliferative index (PI), while daidzein and SPE50 did not significantly affect the proliferative index and SPE100 decreased tumor proliferation. When the AI/PI ratio was calculated, SPE100 produced a significant increase among these groups (1.64 times compared with control) (Table 1).

Final tumor weight and tumor microvascular density after treatment

The final tumor weight in rats treated with genistein or daidzein was 418.5 ± 53.2 and 410.2 ± 24.9 mg, which was not significantly different compared to the controls ($P > 0.05$). SPE50 and SPE100 reduced tumor weights to 329.8 ± 28.1 and 318.5 ± 33.9 mg, respectively ($P < 0.05$; Figure 3). Among these 5 groups, genistein, SPE50, and SPE100 were found to decrease microvascular counts

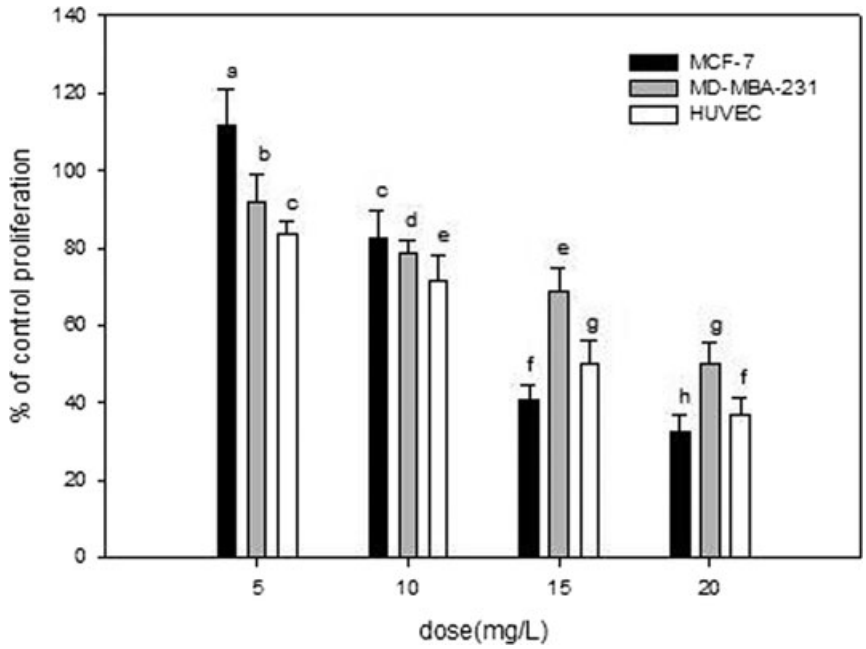

Figure 1—Effects of different doses of genistein on *in vitro* cell proliferation. The antiproliferative effects of genistein were detected in MCF-7, MD-MBA-231, and HUVEC cell lines. Columns and bars represent mean values ± SD. The columns labeled with no letters in common are significantly different ($P < 0.05$).

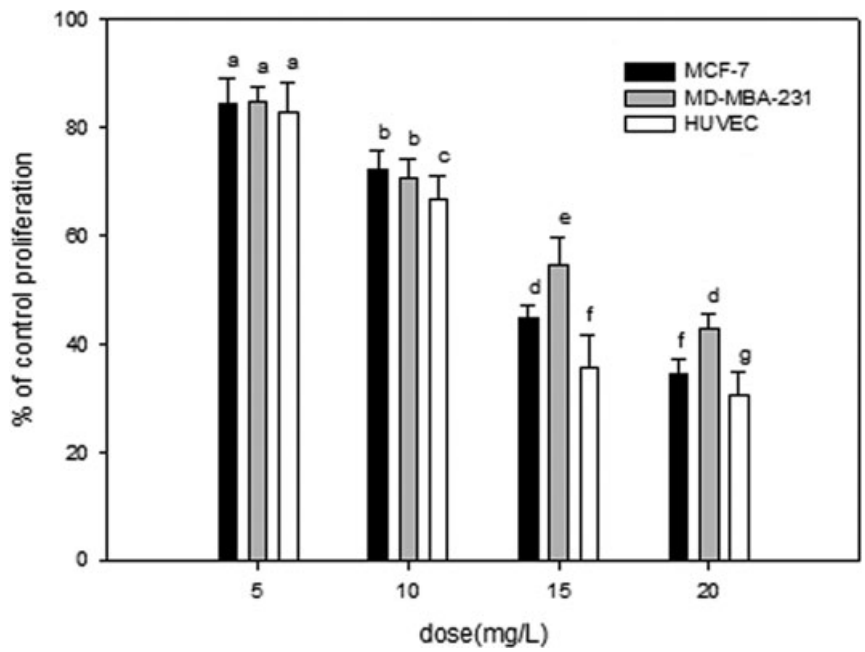

Figure 2—Effects of different doses of soy phytochemical extraction on *in vitro* cell proliferation. The antiproliferative effects of soy phytochemical extraction were detected in MCF-7, MD-MBA-231, and HUVEC cell lines. Columns and bars represent mean values ± SD. The columns labeled with no letters in common are significantly different ($P < 0.05$).

(9.20 ± 1.25, 11.7 ± 1.37, and 7.5 ± 1.46/×200 field, respectively) compared with control (14.3 ± 2.14/×200 field; $P < 0.05$), and SPE100 produced lower counts compared with genistein ($P < 0.05$; Figure 4).

Plasma levels of VEGF, bFGF, and endostatin after treatment

The mean plasma VEGF levels in rats receiving genistein or daidzein were 20.8% and 17.6% lower than controls ($P < 0.05$). They did not affect plasma bFGF levels, and plasma endostatin levels were increased by 26% after daidzein treatment. SPE50 and SPE100 reduced plasma VEGF levels by 17.6% and 24.6% (compared with control, $P < 0.05$), and they decreased plasma bFGF levels by 3.9% and 3%, which made no significant difference compared with the control. SPE50 and SPE100 demonstrated higher data in plasma endostatin levels (increased by 73.2% and 55% compared with control) than those receiving genistein or daidzein ($P < 0.05$; Table 2).

Table 1 — Effects of agents on apoptosis, proliferation, and their ratio.

Group	Apoptotic index (%) (mean ± SD)	Proliferative index (%) (mean ± SD)	AI/PI ratio (mean ± SD)
Control	10.2 ± 0.8[a]	74.7 ± 6.2[a]	0.14 ± 0.03[a,b]
Genistein	14.3 ± 1.2[b]	86.5 ± 7.8[b]	0.16 ± 0.01[a]
Daidzein	9.5 ± 0.9[a]	78.5 ± 8.3[a]	0.12 ± 0.01[b]
SPE50	12.7 ± 1.5[b]	79.3 ± 5.4[a]	0.16 ± 0.02[a]
SPE100	17.6 ± 2.3[c]	68.4 ± 6.1[c]	0.23 ± 0.02[c]

Data in the same column labeled with no letters in common are significantly different ($P < 0.05$).

Table 2 — Effects of agents on plasma angiogenic and antiangiogenic factors.

Group	VEGF (pg/mL) (mean ± SD)	bFGF (pg/mL) (mean ± SD)	Endostatin (ng/mL) (mean ± SD)
Control	115.7 ± 22.5[a]	10.1 ± 4.2[a]	13.1 ± 4.3[a]
Genistein	91.6 ± 16.3[b]	11.4 ± 5.5[a]	12.3 ± 5.9[a]
Daidzein	95.3 ± 15.1[b]	10.2 ± 6.1[a]	16.5 ± 4.3[b]
SPE50	87.2 ± 18.2[c]	9.7 ± 2.8[a]	22.7 ± 5.9[c]
SPE100	79.3 ± 15.4[d]	9.8 ± 3.0[a]	20.3 ± 9.6[c]

Data in the same column labeled with no letters in common are significantly different ($P < 0.05$).

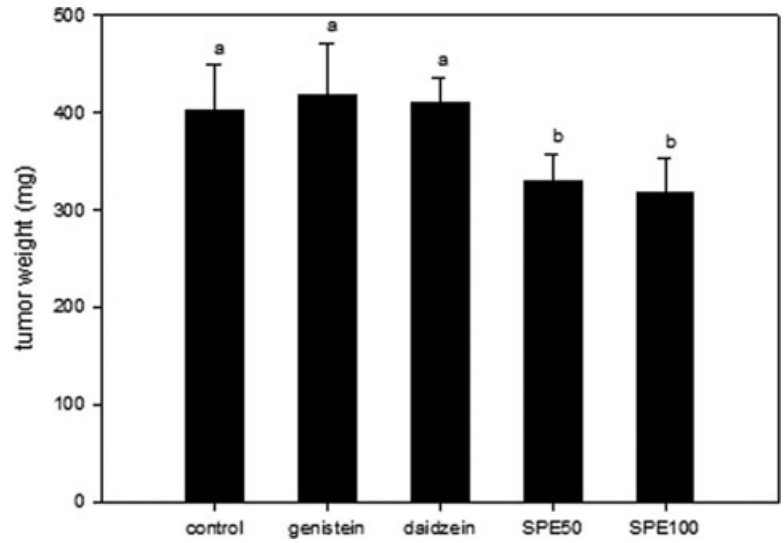

Figure 3 — Effects of genistein, daidzein, and soy phytochemical extraction on final tumor weight. The final tumors were weighed in ovariectomized rats that were fed with genistein, daidzein, and soy phytochemical extraction. Columns and bars represent mean values ± SD. The columns labeled with no letters in common are significantly different ($P < 0.05$).

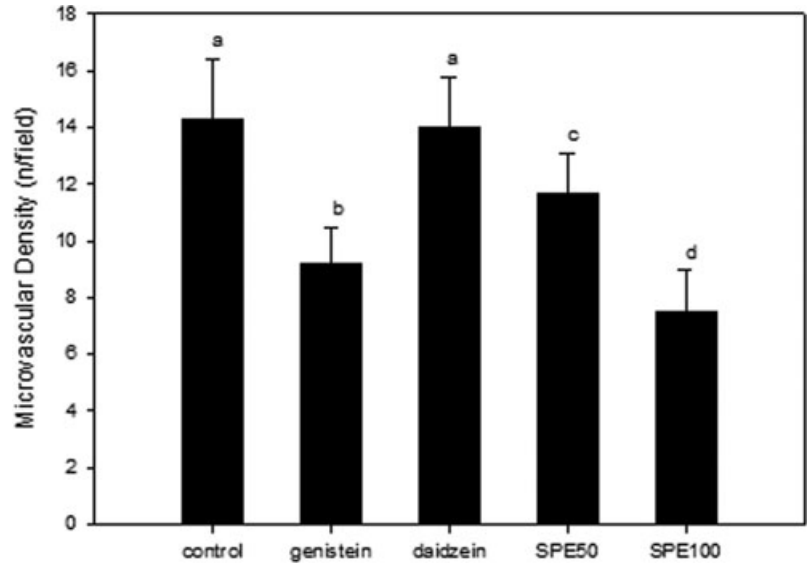

Figure 4 — Effects of genistein, daidzein, and soy phytochemical extraction on tumor microvascular density. The microvascular density was calculated in rats treated with genistein, daidzein, and soy phytochemical extraction. Columns and bars represent mean values ± SD. The columns labeled with no letters in common are significantly different ($P < 0.05$).

Discussion

Due to the estrogenic properties of soy-derived isoflavones, many postmenopausal women are using these compounds as a natural alternative to hormone replacement therapy (Ma and others 2008). Experimental and clinical evidence shows that estrogen has the ability to stimulate both breast epithelial cell growth and angiogenesis (Groothuis 2005). In this study, we attempted to determine whether these soy phytochemicals stimulate or inhibit breast tumor growth and angiogenesis in postmenopausal animal models.

Phytoestrogen can bind to estrogen receptors in our bodies and have either proestrogenic effects or antiestrogenic effects on the target tissues, which may depend on tissue-type, status of the receptor, and the structure or characteristics of certain phytochemicals (Setchell 2001). For this reason, they are sometimes classified as selective estrogen receptor modulators. Our study shows that genistein slightly stimulates MCF-7 breast cancer cell proliferation at low doses, but not daidzein and SPE. The inhibitory ratio of cell proliferation increased dose dependently with higher doses of genistein, daidzein, and SPE. The biphasic effect of genistein is attributed to estrogen-like effects at lower doses and nonestrogen receptor-mediated antitumor effects at higher doses, some of which include potent inhibition of the activity of several enzymes and growth factors that control the cell cycle and apoptosis (Sarkar and Li 2002; Vantyghem and others 2005). Several researchers have found that isoflavones tend to bind more strongly to estrogen receptor beta (Kuiper and others 1998; Kostelac and others 2003), while it is estrogen receptor alpha that estrogen binds to in producing several estrogenic effects. This difference may help to explain the different effects of phytoestrogen and estrogen on breast cancer cells. SPE also showed a better antiproliferative effect on MD-MBA-231 breast cancer cells and HUVECs compared with genistein and daidzein. Their antiproliferative effects on estrogen receptor negative breast cancer cells indicated that soy phytochemicals may exert anticancer effects through other mechanisms, independent of their interactions with estrogen receptors. SPE also contains B-group saponins and bioactive proteins, which may play an important role in the enhanced antiproliferative effects on endothelial cells (Kim and others 2006).

Angiogenesis is an essential event in breast cancer that provides the nourishment necessary for tumor growth and the passageway for metastases (Schneider and Miller 2005). Among soy isoflavones, genistein has been shown to inhibit endothelial cell proliferation and angiogenesis (Su and others 2005), but the relationship between soy intake and angiogenesis in breast cancer is not well understood. The process of angiogenesis is regulated by a balance between proangiogenic and antiangiogenic factors. A variety of factors secreted by breast tumors have been identified to promote tumor angiogenesis, with VEGF and bFGF being two of the most important factors. High levels of circulating VEGF and bFGF indicated poor prognosis in breast cancer (Sliutz and others 1995; Adams and others 2000). Endostatin is a proteolytic cleavage product of type XVIII collagen and is one of the most potent angiogenesis inhibitors known. Raised plasma endostatin levels have correlated inversely with breast cancer angiogenesis in several studies (Teh and others 2004; Alba and others 2006). In the present study, we look into these angiogenesis-related factors, which might be affected by dietary soy phytoestrogen. We found that SPE produced surprisingly good antiangiogenic effects, which were demonstrated by lower microvascular density, reduced plasma VEGF, and increased plasma endostatin. Additionally, doses of SPE100 exerted more powerful effects than SPE50. Recent reports have shown that increased levels of VEGF are associated with a poor response of breast cancer to antihormone treatment (Liang and others 2006). We suspect that soy phytochemicals might help to increase the sensitivity of breast tumors to antihormone treatment through their antiangiogenic activities.

The appeal of these more general biomarkers such as alterations in cell proliferation (proliferation index [PI]) and apoptosis (apoptotic index [AI]) is that they seem to be end points that are modulated by multiple agents. Both cell proliferation and apoptosis have been used clinically for assessment of tumor prognosis because there is a relationship between AI/PI and malignancy in many tumors, including breast cancer, and for analysis of response of cancer cells to clinical interventions (Beresford and others 2006). In this study, SPE showed the potential to inhibit PI and increase AI in mammary tumors. The significantly higher AI/PI ratio with SPE indicated its improved antitumor effects, and those rats receiving SPE also had lower tumor weight compared with control. Genistein increased both AI and PI in mammary tumors, and its AI/PI ratio and tumor weight had no significant difference with control. We propose that the additive and synergistic effects of phytochemicals in soybeans are responsible for these potent antiangiogenic and anticancer activities of SPE. Evidence from Liu's research suggests that bioactive compounds are best acquired through whole-food consumption and not from individual phytochemicals. The thousands of phytochemicals that are present in whole food may affect the bioavailability and distribution of each phytochemical in different cells and organs (Liu 2003, 2004). This explains why no single phytochemical can replace the combination of natural phytochemicals in foods to achieve their health benefits.

Conclusions

Our study suggests that soy phytochemical extraction exerts antitumor effects in an animal model of postmenopausal breast cancer, and its antitumor effect is partly attributed to its marked antiangiogenic activity. However, we do not feel comfortable using significant amounts of the purified soy phytochemicals in patients, because it is unknown if these soy isoflavones are safe at such high concentrations. More studies on women using phytoestrogens need to be conducted to establish both the benefits and risks.

Acknowledgments

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