Soy Extract Is More Potent than Genistein on Tumor Growth Inhibition

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Abstract. *Soybean and soy products have received much attention for their potential heath benefits. Recently it has been reported that the bioactivity of soy products is influenced by the degree of soy processing. This study was conducted to evaluate and compare the influence of diets containing genistein and soy extract on the growth of the estrogen-independent human breast cancer cells, MDA-MB-231, implanted into female Balb/c mice. Four-week-old female athymic nude mice (Balb/c) were acclimatized to an AIN-93G control diet for one week prior to initiating the experimental diets. The animals were placed into three treatment groups, each of which was provided with containing DMSO, genistein (750 μg/g AIN-93G diet) or 0.6% soy extract (containing genistein at 750 μg/g AIN-93G diet) for three weeks from one week prior to the injection of MDA-MB-231 cells (1×10^6/site) and subsequently fed on the AIN-93G control diet until sacrifice. The tumor volumes increased steeply in the control group and the genistein-treated group. However, tumor growth was significantly reduced in the soy extract-treated group compared to the control and genistein-treated groups. Immunohistochemistry of proliferating cell nuclear antigen (PCNA) also revealed that the soy extract treatment effectively reduced cell proliferation of the implanted tumors. In conclusion, soy extract is more potent than genistein in the inhibition of tumor growth, presumably resulting from the synergistic effect of the various bioactive components in the soy extract.*

Epidemiological studies have indicated that diet might be one factor that explains the lower incidence of breast cancer in Asian countries compared with Western countries (1, 2). The effects appeared to be dietary and not genetic since the protection against breast cancer conferred on Asian women was lost upon emigration and exposure to Western lifestyles within a few generations (3). The major difference between Asian and Western diets lies in soy consumption. The Asian diet is characterized by a high content of soy and soy products (2, 4). Much effort has been made to identify the bioactive components in soybeans which have the potential to prevent breast cancer.

Soybeans contain a number of biologically active compounds that may be associated with chemoprevention including isoflavones, protease inhibitors, inositol hexaphosphate, lignans, phytosterols and saponins. Isoflavones have been of interest and thoroughly studied due to their putative activities in preventing and reducing breast cancer risk. Isoflavones are a group of flavonoids which are similar to endogenous 17β-estradiol in structure and biological activity (5, 6). Soybeans are the most common source of isoflavones in human food. The major isoflavones found in soy are genistein, daidgein and glycitein which exist naturally in several glycoside forms and are transformed to the active aglycone form by enzymatic reaction. Among them, genistein, the predominant isoflavone of soybeans, has been the focus of attention associated with its chemopreventive activity against breast cancer (5-7).

Several mechanisms have been proposed for the anticarcinogenic activity of isoflavones. Isoflavones have been reported to inhibit the growth of various cancer cells through the modulation of genes related to cell growth and programmed cell death (6, 7). Genistein has been found to inhibit the activation of the nuclear transcription factor, NF-kappaB and the Akt signaling pathway, both of which are known to maintain a balance between cell survival and apoptosis (8, 9). Genistein also targets the signaling pathway mediated by estrogen and androgen in the processes of carcinogenesis. Moreover, genistein is known to be an inhibitor of angiogenesis and metastasis (10, 11). However, genistein treatment has led to paradoxical actions on breast cancer cells depending on the concentration to which the

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cells are exposed. Physiological concentrations (1 nM to 10 μM) of genistein stimulated the growth of estrogen-dependent human breast cancer (MCF-7) cells *in vitro* and *in vivo* through the estrogen receptor-α (12-16), whereas a high concentration of genistein (>10 μM) inhibited the growth and survival of human breast cancer cells *via* a route independent of estrogen receptors (12, 13, 16). Animal studies have demonstrated that the timing of exposure to genistein is also important in the development of breast cancer (17-22). Exposure to dietary genistein from the neonatal through prepubertal periods protected against chemically induced mammary cancer in rats. The protective effects included increased latency, reduced tumor incidence and multiplicity, and more rapid maturation of undifferentiated end buds to differentiated lobules. In contrast, limiting exposure to dietary genistein to the prenatal or adult periods did not predispose or protect against mammary cancer (22). In studies using xenograft animal models, treatment with genistein before tumor formation was effective for tumor suppression, however genistein treatment after tumor formation stimulated tumor development (17, 18, 20).

Soy isoflavones have received much attention for their potential health benefits. In Asia, soybeans are consumed as minimally processed foods and not as processed and purified ingredients, whereas in Western countries soy products are produced and consumed in highly processed forms such as soy protein isolates (80-90% protein) and isoflavone-enriched products (40-70% isoflavones). These products may not have the same health benefits as the soy foods consumed in Asian countries because they may have lost some of their bioactive components. In fact, previous research has shown that a highly refined isoflavone-rich diet may have detrimental effects. Allred *et al*. (23, 24) reported that isoflavone-supplemented diets containing an equal amount of genistein differentially stimulated mammary tumor growth in athymic mice based on the degree of processing. The diets containing the more processed products showed more stimulation of estrogen-dependent breast tumor growth.

In the present study, the effects of dietary genistein and soy extract containing equivalent genistein contents on mammary tumor growth in an estrogen-independent animal model were investigated.

Materials and Methods

Chemicals. The chemicals and enzymes were purchased from Sigma, Inc. (St. Louis, MO, USA) except where otherwise indicated. Genistein was obtained from Indofine Chemical Company (Somerville, NJ, USA). Novasoy® was obtained from Archer Daniels Midland (ADM, Decatur, IL, USA) and consisted (w/w) of 40% isoflavones (1.3:1.0:0.3 ratio of the glycosides genistin, daidzin, and glycitin), 40% natural soy components (mostly saponins), 7-12% protein, 1% fat, and 4% ash. All isoflavone forms, such as malonyl and acetyl, were included in the isoflavone determinations. The concentration of aglycones was less than 1%.

Cell culture. The estrogen-independent human breast cancer cells, MDA-MB-231, were routinely cultured in RPMI-1640 medium (MEM; Sigma) supplemented with 15% fetal bovine serum (FBS; Atlanta Biologics, Norcross, GA, USA), 2 mM L-glutamine and 80 μg/ml penicillin and grown at 37˚C with 5% CO_2. The cells were quantified by hemocytometer counting and were more than 90% viable, as determined by trypan blue exclusion.

Animal studies. Four-week-old female athymic nude mice (Balb/c) were purchased from Harlan Laboratories (Indianapolis, IN, USA). The animals were housed in a climate-controlled room (22±2˚C, 50±10% relative humidity) with a 12-hour light/dark cycle and provided with diet and water *ad libitum*. The mice were acclimatized to an AIN-93G control diet for one week prior to initiating the experimental diets. The animals were then randomly assigned to one of three treatment groups. The treatment groups were: DMSO (G1); genistein (750 μg/g AIN-93G diet) (G2) and 0.6% Novasoy® (containing genistein at 750 μg/g AIN-93G diet) (G3). The animals were provided with one of the experimental diets for three weeks from one week prior to the injection of MDA-MB-231 cells (1×10^6/site) and then fed on AIN-93G control diet until sacrifice. The MDA-MB-231 cells (1×10^6/site) were injected into both the right and left flank on the back of each animal. During the study, tumor growth and body weight were monitored weekly and dietary intakes were also measured every week. The animals were sacrificed 13 weeks after cell inoculation and the tumors were dissected for further examination.

Immunohistochemistry. Tumor cell proliferation was measured by immunohistochemical analysis using proliferating cell nuclear antigen (PCNA). The tumors were removed and fixed in 10% neutral-buffered formalin and embedded in paraffin. Four-μm serial sections were cut from the paraffin blocks and placed on slides. The slide sections were incubated in xylene to remove the paraffin and rehydrated using a series of graded alcohol. Visual staining was achieved using a mouse monoclonal PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by streptividine-horseradish peroxidase (HRP) conjugate and diaminobenzidine (DAB) substrate. Both the PCNA-positive proliferating cells and the total tumor cells were counted in three non-necreotic areas of each section using light microscopy at 400-fold magnification.

Statistical analysis. All the data are presented as the mean±standard error. Statistically significant differences ($p<0.05$) among the means were determined by one-way ANOVA using a PC-based version of SPSS (Version SPSS/PC 11.5, Chicago, IL, USA).

Results

Body weight and dietary intake. The mice fed the experimental diets gained weight at similar rates and no significant difference was observed among the control and experimental groups (Figure 1). Food intake was also measured periodically throughout the study and no significant difference was observed in any of the groups (Figure 2).

Tumor growth. The development of palpable tumors was first observed in the mice seven weeks after injecting the MDA-MB-231 cells. The tumor volumes increased steeply

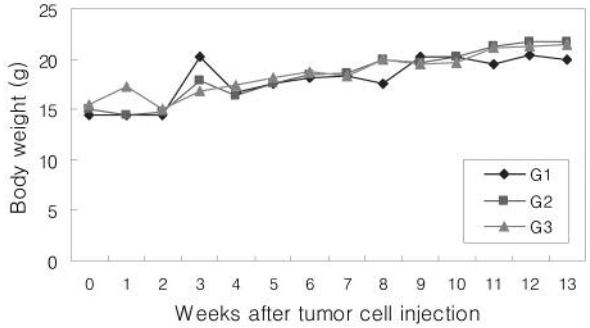

Figure 1. *Changes of Body weight in mice injected with MDA-MB-231 cell (1×10^6/site). Values are mean±SE (n=8). G1: DMSO, G2: genistein (750 μg/g AIN-93G diet), G3: 0.6% Novasoy (750 μg genistein/g AIN-93G diet).*

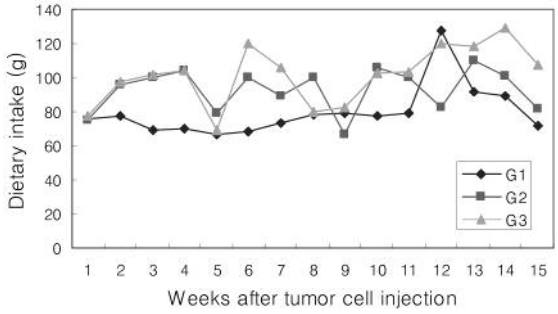

Figure 2. *Dietary intake per week in mice injected with MDA-MB-231 cell (1×10^6/site). Values are mean±SE (n=8). G1: DMSO, G2: genistein (750 μg/g AIN-93G diet), G3: 0.6% Novasoy (750 μg genistein/g AIN-93G diet).*

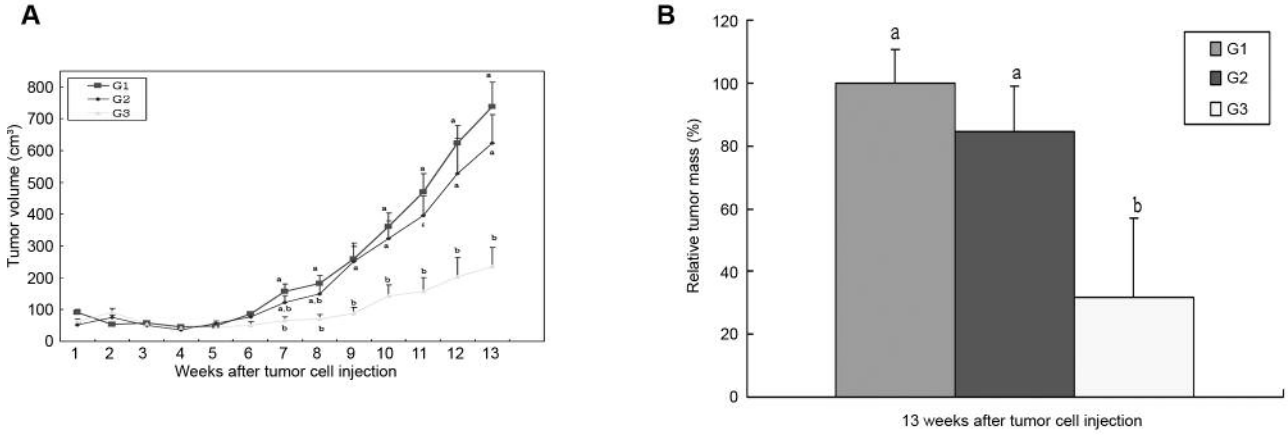

Figure 3. *Tumor growth (A) and tumor mass relative to controls at 13 weeks (B) in mice injected with MDA-MB-231 cell (1×10^6/site). Values are mean±SE (n=8). G1: DMSO, G2: genistein (750 μg/g AIN-93G diet), G3: 0.6% Novasoy (750μg genistein/g AIN-93G diet). *a, b are significantly different groups.*

in the control group (G1). The tumor volumes increased in the genistein-treated group (G2), but did not outgrow that of the control group. In contrast, soy extract (G3) significantly reduced tumor growth compared to the control and genistein-treated groups (Figure 3A). The tumor volume of the soy extract-treated group 13 weeks after cell injection was reduced to 31.9% of that in the control group (Figure 3B). The results of PCNA also revealed that the soy extract remarkably reduced cell proliferation and was more efficient than genistein in inhibiting proliferation of the implanted tumor cells in the athymic nude mice (Figure 4), which was very consistent with the results of tumor growth.

Discussion

Conflicting data exist on the effects of soy-based products and pure phytoestrogens on breast tumor progression in animal studies. Stimulatory effects of genistein on estrogen-dependent MCF-7 tumors have been reported in many studies. Helferich's group (20, 25) observed that dietary treatment with genistein/genistin (the glycoside form of genistein) dose-dependently enhanced the growth of MCF-7 tumors transplanted in ovariectomized athymic mice as did soy protein isolates containing increasing concentrations of isoflavones (26). On the other hand, using an estrogen-maintained animal model, genistein and soy isoflavones inhibited the growth of MCF-7 tumors (27). In the present study, the tumor growth of the genistein (750 μg/g)-treated group did not exceed that of the control group and moreover the dietary soy extract significantly inhibited tumor growth in mice bearing MDA-MB-231 xenografts. Shao *et al*. (28) also showed an inhibitory effect of genistein in MDA-MB-231 cells implanted in athymic mice. They treated tumor-bearing mice with 500 μg of genistein by subcutaneous administration. In the study of Hewitt and Singletary (29), mice were fed on diets supplemented with soy extract (Novasoy® containing genistein at 750 ppm) and with 750 ppm genistein for 5 days prior to injection with F3II murine mammary carcinoma cells. The results showed that soy extract

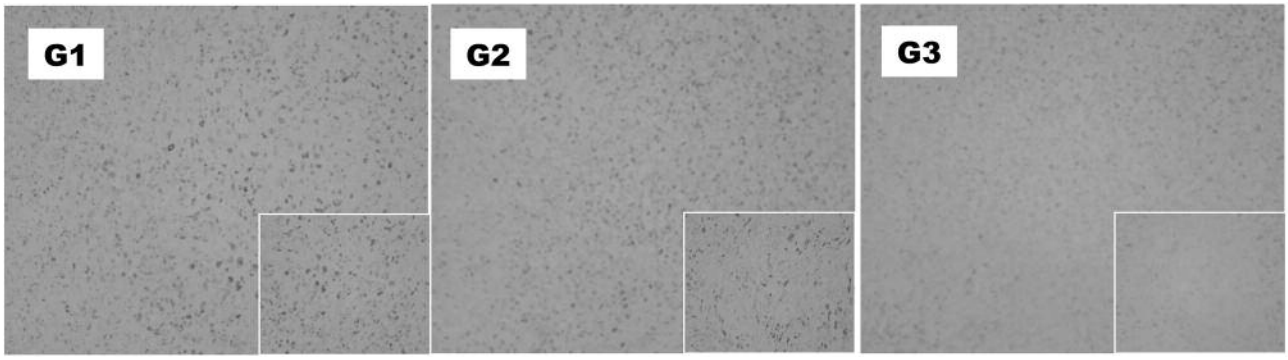

Figure 4. *The proliferation of MDA-MB-231 cells implanted in athymic mice (n=8 in each group) measured by immunohistochemical analysis using proliferating cell nuclear antigen (PCNA). G1: DMSO, G2: genistein (750 μg/g AIN-93G diet), G3: 0.6% Novasoy® (750 μg genistein/g AIN-93G diet).*

and genistein induced a significant reduction in F3II tumor growth compared to controls. In contrast, Santell *et al*. (30) obtained a negative result when they treated estrogen-independent tumor-bearing mice with 750 μg of genistein/g diet. Yuan *et al*. (31) showed that the treatment of MDA-MB-231 bearing mice with a relatively high dose of genistein (about 4 mg per mouse per day) resulted in statistically significant tumor regression. In another study, Allred *et al*. (23) evaluated the ability of various soy products, including soy flour, two crude extracts of soy (soy molasses and Novasoy®), a mixture of isoflavones and genistin, to affect the growth of MCF-7 cells transplanted into ovariectomized athymic mice. Each of the soy flour-processed products was added to the diet after tumor formation to provide the equivalent of 750 ppm genistein. Tumors in the soy flour-fed animals remained basically the same size. In the animals consuming soy molasses, Novasoy®, mixed isoflavones or genistin alone, the tumor growth was stimulated when compared with the animals consuming a control diet devoid of soy. In another study (24), the degree of soy processing was found to affect isoflavone bioavailability by the loss of bioactive components. These results show that the effect of genistein and soy extract on breast tumors may be affected by the ovarian status in the animal models.

Previous research demonstrated that the timing of exposure to genistein was also a critical factor for inhibiting breast cancer development (17-22). Genistein administration during the early period of life protected against chemically induced mammary cancer in rats, however, limiting exposure to dietary genistein in adults did not protect against mammary cancer. Similarly, dietary genistein has been found to both reduce and promote mammary cancer development in different animal models depending on the treatment time. Hewitt and Singletary (29) provided 750 ppm genistein in the diet before F3II cell injection which resulted in a significant reduction in F3II tumor growth. Zhou *et al*. (27) also reported that pretreatment with genistein before breast cancer cell injection significantly reduced MCF-7 tumor growth. Mammary tumor latency was significantly delayed in mammary tumor virus (MMTV)-neu mice fed genistein (29). Once tumors formed, however, genistein did not reduce the number or size of tumors. On the other hand, Gallo *et al*. (32) reported that the administration of soy extract (16.8% isoflavone glycosides, 22.3% saponine) from the beginning of breast cancer cell injection did not stimulate or inhibit estrogen-dependent and estrogen-independent cells implanted in athymic mice. In the present study, the genistein and soy extract were provided one week before cell injection and continued for three weeks, leading to tumor growth inhibition in the soy extract group.

The present finding that soy extract (40% isoflavone glycosides, 40% saponins) fed in the diet was much more effective in suppressing MDA-MB-231 tumor growth than the equivalent amount of pure genistein is of particular interest. Hewitt and Singletary (29) also reported that soy extract (Novasoy® containing genistein at 750 ppm) induced a significant 90% reduction in F3II tumor growth compared to controls, whereas 750 ppm genistein alone induced a significant 40% reduction in F3II tumor growth compared to controls. This suggested that the mixture of isoflavones or other soybean components present in the soy extract in combination with genistein may be contributing to a tumor inhibitory action, which presumably resulted from a synergistic effect of the components in the soy extract. The degree of soy processing has also been reported to affect the estrogenicity of products containing a constant amount of genistein. Therefore, the consumption of a less processed form of soy food is more advisable than consuming genistein in a purer form.

Additionally, the data from the PCNA assay demonstrated that the soy extract was much more effective than genistein in reducing proliferation of the tumor cells.

In conclusion, soy extract in combination with genistein, other isoflavones and other soybean components may be more effective in tumor inhibition than genistein alone. Therefore, the consumption of soy food in a more complex form is more advisable than consuming pure genistein.

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