[CANCER RESEARCH 61, 5045–5050, July 1, 2001]

Soy Diets Containing Varying Amounts of Genistein Stimulate Growth of Estrogen-dependent (MCF-7) Tumors in a Dose-dependent Manner[1]

Clinton D. Allred,[2] Kimberly F. Allred,[2] Young H. Ju, Suzanne M. Virant, and William G. Helferich[3]

Department of Food Science and Human Nutrition [C. D. A., K. F. A., Y. H. J., S. M. V., W. G. H.], and Division of Nutritional Sciences [C. D. A., W. G. H.], University of Illinois, Urbana, Illinois 61801

ABSTRACT

We have demonstrated that the isoflavone, genistein, stimulates growth of estrogen-dependent human breast cancer (MCF-7) cells *in vivo* (C. Y. Hsieh *et al.,* Cancer Res., *58:* 3833–3838, 1998). The isoflavones are a group of phytoestrogens that are present in high concentrations in soy. Whether consumption of genistein from soy protein will have similar effects on estrogen-dependent tumor growth as pure genistein has not been investigated in the athymic mouse tumor implant model. Depending on processing, soy protein isolates vary widely in concentrations of genistein. We hypothesize that soy isolates containing different concentrations of genistein will stimulate the growth of estrogen-dependent cells *in vivo* in a dose-dependent manner. To test this hypothesis we conducted experiments in which these soy protein isolates were fed to athymic mice implanted s.c. with estrogen-dependent tumors. Genistein content (aglycone equivalent) of the soy isolate diets were 15, 150, or 300 ppm. Positive (with 17β-estradiol pellet implant) and negative (no 17β-estradiol) control groups received casein-based (isoflavone-free) diets. Tumor size was measured weekly. At completion of the study animals were killed and tumors collected for evaluation of cellular proliferation and estrogen-dependent gene expression. Incorporation of bromodeoxyuridine into cellular DNA was used as an indicator of cell proliferation, and pS2 mRNA was used as an estrogen-responsive gene. Soy protein diets containing varying amounts of genistein increased estrogen-dependent tumor growth in a dose-dependent manner. Cell proliferation was greatest in tumors of animals given estrogen or dietary genistein (150 and 300 ppm). Expression of pS2 was increased in tumors from animals consuming dietary genistein (150 and 300 ppm). Here we present new information that soy protein isolates containing increasing concentrations of genistein stimulate the growth of estrogen-dependent breast cancer cells *in vivo* in a dose-dependent manner.

INTRODUCTION

The Food and Drug Administration recently approved a health claim for soy protein. The claim states "25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (1). As a result, the consumption of soy protein by Americans has increased. Soy and soy-derived supplements are not being used solely to lower serum cholesterol and reduce risk of heart disease. Soy contains a complex mixture of a variety of phytochemicals; our focus is on the estrogenic isoflavones referred to as phytoestrogens. Isoflavones have been demonstrated to act as estrogen agonists by binding to the estrogen receptor and generating estrogen-induced responses (2–4). As a result, postmenopausal women may consume soy for the estrogenic effects of these compounds to relieve menopausal symptoms. Soy products are marketed as a "natural" alternative to hormone replacement therapy with the perception that these phytoestrogens are without the risks associated with hormone replacement therapy. Isoflavone-containing products are produced in various forms, including supplement capsules, which contain mixtures of isoflavones derived from soy. Isoflavones are also present in bioactive concentrations in food products such as soy protein isolates, which can contain varying isoflavone content. It is generally accepted that consuming the phytochemical components of soy, particularly the isoflavones, in pure form, as in supplements, may pose some health concerns but that consumption of more whole foods containing these are natural and, as such, safe. It is important to note that the concentration of these compounds in soy protein isolates is dependent on the method of processing, and specific processing methods can be used to enrich the isoflavone content in soy protein isolates.

Clinical and preclinical laboratory animal and *in vitro* studies have demonstrated the hormonal activity of dietary isoflavones. Our laboratory has examined the estrogenic activity of genistein. *In vitro*, when human estrogen-dependent breast cancer (MCF-7) cells were treated with increasing concentrations of genistein (1–10 μM), the cells expressed increased mRNA levels of pS2, an estrogen-responsive gene, in a dose-dependent manner. *In vivo*, 25-day-old ovariectomized, athymic mice consuming dietary genistein for 5 days had increased number and size of terminal end buds in the mammary gland when compared with control animals (5). Not only do the isoflavones have estrogenic activity on various tissues when given in pure form but also when consumed in products such as soy protein isolate. In humans, Petrakis *et al.* (6) demonstrated that consumption of soy protein isolate had stimulatory effects on the breast tissue of premenopausal women. They found that duct fluid aspirates contained greater numbers of hyperplastic epithelial cells in women consuming soy protein isolate. Nipple aspirate apolipoprotein D and pS2 expression has also been shown to be elevated in women consuming 60 g of soy in the form of ground, textured vegetable protein for as little as 2 weeks (7). These data collected from both laboratory animals and humans demonstrate the estrogenic activity of the isoflavones whether they are taken as a pure compound or consumed in soy protein isolate containing isoflavones.

The role that isoflavones play in breast cancer is unclear. Some reports indicate that exposure to genistein is preventative in the development of breast cancer (8–10), whereas others show that genistein stimulates the growth of existing estrogen-dependent tumors (5). Studies published over the last 5 years have demonstrated that exposure to dietary genistein before puberty reduces the number of chemically induced mammary tumors formed in female Sprague Dawley rats. The authors suggest protection against the development of breast cancer tumors is attributable to the estrogenic effects of genistein that causes increased cellular differentiation in mammary gland cells of prepubertal animals. A differentiated cell undergoes less proliferation and therefore is less likely to progress through the cancer process (8–10). If prepubertal exposure to genistein results in earlier differentiation of the mammary gland, then the assumption can be made that earlier differentiation would be protective against chemically induced mammary tumors. Cohen *et al.* (11) found that continual feeding of soy protein isolate (containing 1.67 mg total isoflavones per g isolate) postpubertally for 18 weeks after chemical induction of mammary tumors in rats had no detectable effect on the development of these tumors. Our research has focused on the effect dietary

Received 8/9/00; accepted 4/27/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grant CA77355 (to W. G. H.).

[2] C. D. A. and K. F. A. contributed equally to this paper.

[3] To whom requests for reprints should be addressed, at 580 Bevier Hall, Department of Food Science and Human Nutrition, University of Illinois, Urbana, IL 61801. Phone: (217) 244-5414; Fax: (217) 244-7877; E-mail: helferic@uiuc.edu.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/2485826/5045.pdf by guest on 06 October 2022

genistein has on the growth of existing estrogen-dependent (MCF-7) tumors in animals with low circulating E_2[4] concentrations. We have demonstrated that genistein fed at 750 ppm stimulates the growth of MCF-7 cells implanted into athymic mice. Mice consuming 750 ppm dose of genistein have a total plasma genistein concentration of ~2 μM (5). This is a relevant dietary dosage because women who consume varying amounts of isoflavones from soy milk have plasma genistein levels of 0.8-2.2 μM (12). *In vitro* genistein has been shown to stimulate the growth of MCF-7 cells at concentrations as low as 200 nM (2, 5). Whereas these data demonstrate genistein can stimulate the growth of estrogen-dependent breast cancer tumors, it has not yet been determined if soy protein isolate, which contains a complex mixture of phytochemicals, can exhibit similar effects on growth of estrogen-dependent tumors.

The study presented here was designed to evaluate the effects of dietary soy protein isolates containing varying concentrations of genistein: (*a*) on the growth of MCF-7 tumors in athymic mice; (*b*) on cellular proliferation of estrogen-dependent tumors in ovariectomized athymic mice; and (*c*) on expression of the estrogen-responsive gene, pS2, in response to dietary exposure to soy protein isolates with varying concentrations of genistein.

MATERIALS AND METHODS

Effects of Consumption of Soy Protein Isolate and Casein-based Diets Containing Genistein on the Growth of Estrogen-dependent Tumors

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 and allowed a week to recover before 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 were then injected into the four flanks on the back of each animal. Within the first 4 weeks, tumors that subsequently formed were allowed to develop to an average cross-sectional area of ~40 mm^2. At this point, animals were placed into eight treatment groups (seven animals/group). The treatment groups were: (*a*) positive control; (*b*) negative control; (*c*) dietary soy protein isolate providing 15 ppm genistein; (*d*) dietary soy protein isolate providing 150 ppm genistein; (*e*) dietary soy protein isolate providing 300 ppm genistein; (*f*) dietary genistein in casein-based diet at 15 ppm; (*g*) 150 ppm; and (*h*) 300 ppm. The E_2 pellet was removed from the negative control animals and from all of the mice on the various dietary treatments. The E_2 pellet was also removed from the positive control group, but these animals were again implanted with an identical 2-mg E_2 pellet. Negative and positive controls were given AIN 93G as a control diet. This diet provided protein from casein. The remaining animals were put on one of the six treatment diets. Tumor area and body weight were measured weekly. At the end of the study, tumors and plasma samples were collected for tissue analysis. We felt that weekly tumor area was the most critical outcome evaluated. Measurements were made over a total of 29 weeks after removal of E_2 pellets. However, at the conclusion of the study, tumors from the negative control, low soy isolate, and low genistein groups were small, making it impossible to collect representative samples for mRNA and cell proliferation data. Therefore, to obtain these measurements for the negative control group, a second set of negative control animals were used. These animals were killed 11 weeks after removal of the E_2 pellet when their tumors were at an average cross-sectional area of 25 mm^2. We feel this was the most accurate method to obtain tissues from the negative control group that were regressing but still large enough to obtain data for mRNA and immunohistochemical analysis.

Diet Formulation. AIN-93G semipurified diet was selected as a base diet for control animals as it has been established as meeting all of the nutritional requirements of mice (13). Soy oil was removed from all of the diets and corn oil added to eliminate any additional components of soy being added to the diets. Treatment animals were fed either soy isolate-containing diets or AIN-93G diet plus genistein. Three soy protein isolates with varying concentrations of genistein were used in this study. Each isolate was added at 20% of the diet as the sole source of protein in the AIN-93G diet. Both the casein-based diet and the soy-based diet provided ~18% protein, but 2.2 g of methionine was added to each kilogram of soy isolate diet to meet the sulfur-containing amino acid requirements and as a result, all of the essential amino acid requirements of the mouse were met. The three soy protein isolates (Protein Technologies International, St. Louis, MO) were processed to contain varying amounts of genistein. There were no significant differences in amino acid content or proximate analysis among the isolates. Additionally, the isolates were very low in fiber and as such the lignin content was negligible. The majority of the isoflavones in the protein isolates were present as the aglycone form (~60%). The genistein contents were 0.075, 0.75, and 1.5 mg genistein (aglycone equivalents)/g of product. This resulted in the final concentrations of genistein in the three soy isolate diets to be 15, 150, and 300 mg genistein/kg diet respectively. To compare the genistein content from soy to pure genistein, it was added to the AIN-93G diet at equal concentrations to that found in the soy isolate diets and provided 15, 150, and 300 ppm genistein in a casein-based diet. The eight final treatment groups were: positive controls (2 mg E_2 pellet); negative controls; low soy isolate [LSI (15 ppm genistein)]; medium soy isolate [MSI (150 ppm genistein)]; high soy isolate [HSI (300 ppm genistein)]; low genistein [LG (AIN-93G + 15 ppm genistein)]; medium genistein [MG (AIN-93G + 150 ppm genistein)]; and high genistein [HG (AIN- 93G + 300 ppm genistein)].

E_2 Pellet Preparation. MCF-7 cells will not produce tumors in ovariectomized mice unless they are supplemented with estrogen. Therefore, 1 week after delivery, animals were implanted with E_2 pellets. E_2 pellets were made containing 2 mg of E_2 mixed with 18 mg of cholesterol as a carrier. A 20-mg mixture containing E_2 and cholesterol was placed into a pellet mold and pressed into a compact pellet ~4.5 mm in diameter and ~2.5 mm in depth. Pellets were then placed s.c. in the interscapular region of mice (14).

Tumor Implantation. MCF-7 cells were maintained in 100 mm × 20 mm plastic tissue culture plates in IMEM media (Biofluids) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 units/ml). E_2 (1.0 nM) was added in the media to keep the cell line estrogen-dependent. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown to confluence, collected using trypsin-EDTA, and counted. Cells were suspended in matrigel matrix (Becton Dickinson) before being injected (15) and then 40 μl of the cell suspension 1.5×10^5 cells were injected 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 × π] (5, 16). When tumors reached an average cross-sectional area of 40 mm^2, animals were divided into treatment groups with each group normalized for tumor number, tumor size, and animal number. E_2 pellets were removed from all of the animals, and the mice were then placed on the treatment diets. Positive control mice were reimplanted with a fresh pellet containing 2 mg of E. We then resumed measuring tumor areas weekly as described above.

Tissue/Tumor Collection. At the completion of the study, mice were killed by cervical dislocation, and tumors were harvested. Tumors from each mouse were fixed in 10% formalin and embedded for immunohistochemical staining. Additional tumors from each mouse were immediately frozen in liquid nitrogen for subsequent RNA isolation and analysis.

Analysis of Estrogen-responsive pS2 mRNA

RNA Preparation. The mRNA was isolated using procedures routinely used in our laboratory (17). Briefly, frozen tumors (≤200 mg) from liquid nitrogen were smashed and the coarse tumor powder was transferred into TRIZOL (Life Technologies, Inc., Grand Island, NY) in a 15-ml tube and was homogenized using a Polytron-Aggregate (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 removed and 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 7,500 × g for 5 min at 4°C. The RNA pellet was air-dried then dissolved

[4] The abbreviations used are: E_2, 17β-estradiol; BrdUrd, 5-bromo-2′-deoxyuridine; LSI, low soy isolate; MSI, medium soy isolate; HSI, high soy isolate; LG, low genistein; MG, medium genistein; HG, high genistein; AIN 93G, American Institute of Nutrition 93 growth diet.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/2485826/5045.pdf by guest on 06 October 2022

with RNase-free dH_2O. RNA was stored at −80°C. RNA concentration was measured at 260 and 280 nm (1 $A_{260\ nm}$ = 40 μg of single-stranded RNA/ml).

Northern Blot Analysis. Expression of pS2 was used as a biomarker of estrogenic activity (18). For the detection of pS2 expression, 10 μg of RNA were separated on 1.2% formaldehyde denaturing agarose gels and transferred to a Magnacharge, Nylon, Transfer membrane (Osmonics, Westboro, MA). The RNA was UV cross-linked onto the membrane. The membrane was prehybridized in a formamide prehybridization solution containing denatured salmon sperm DNA for 3 h at 42°C. After 3-h prehybridization, the DNA probe was labeled using Random Primers DNA Labeling System (Life Technologies, Inc.). For the estrogen-responsive pS2 gene, a 25 ng of *pS2* cDNA, or for the control, a 25 ng of glyceraldehyde-3-phosphate dehydrogenase cDNA probe was labeled with 50 μCi α-[^{32}P]dCTP. The membrane was incubated with ^{32}P-labeled probe overnight at 42°C. The membrane was washed twice with 5 ml of 0.2% SSC/0.1% SDS at 24°C for 5 min each, three times with 5 ml of 0.1% SSC/0.1% SDS at 68°C for 15 min each, and rinsed with 2% SSC. The membrane was exposed to X-ray film for 6 h, and hybridizing RNA molecules were detected by performing autoradiography. Film was analyzed using Collage (version 4.0) software (Image Dynamics Corp.) with Foto Analyst (Futodyne).

Tumor Cell Proliferation Immunohistochemistry

BrdUrd Analysis. BrdUrd incorporation into cellular DNA was used as an indicator of cells that were actively proliferating (19). Four h before killing the animals each mouse was injected i.p. with 50 mg BrdUrd/kg body weight. Tumors were excised, skin and fat removed, and processed for tissue staining. Prepared sections were then stained for the presence of BrdUrd using a modified immunohistochemistry protocol (20). Briefly, slides were deparaffinized and hydrated by immersing in xylene twice for 12 min and immersing in a series of alcohol/water solutions for 5 min each. To block endogenous peroxidase, slides were immersed in 0.3% H_2O_2 for 20 min then washed with distilled water. Slides were then microwaved in a Pyrex dish in 18 mM citrate buffer at pH 6.0 for 20 min and cooled. Then slides were washed in PBS (pH 7.1–7.4) for 5 min, and tissue sections were rimmed with wax. 50-μl anti-BrdUrd primary antibody (Amersham) was added to slides and incubated for 1 h at room temperature in a humidity chamber. Slides were washed in PBS and 50 μl of diluted secondary antibody (Sigma Chemical Co.) was added to slides and incubated for 30 min at room temperature. Slides were then washed in PBS. One drop of 3,3′-diaminobenzidine + Ni enhancer solution, freshly prepared, was added to each slide with a Pasteur pipette. Slides were then washed in water twice, in PBS, and counterstained with 20% hematoxylin for 1 min. The slides were then dehydrated by placing them in 80% alcohol for 5 min, 95% ethanol for 5 min, and 100% ethanol for 5 min followed by xylene four times for 5 min each. Slides were then coverslipped and analyzed by light microscope. Both positive and background stained cells were counted in a given area of tissue. The data were then presented as percentage of cells proliferating in a given area.

Statistical Analysis. Tumor area data were analyzed using one-way or repeated-measures ANOVA according to the characteristics of the data set using the SAS program. pS2 gene expression data were analyzed using one-way treatment ANOVA. If the overall treatment *F*-ratio was significant ($P < 0.05$), the differences between treatment means were tested with Fisher's least significant difference test.

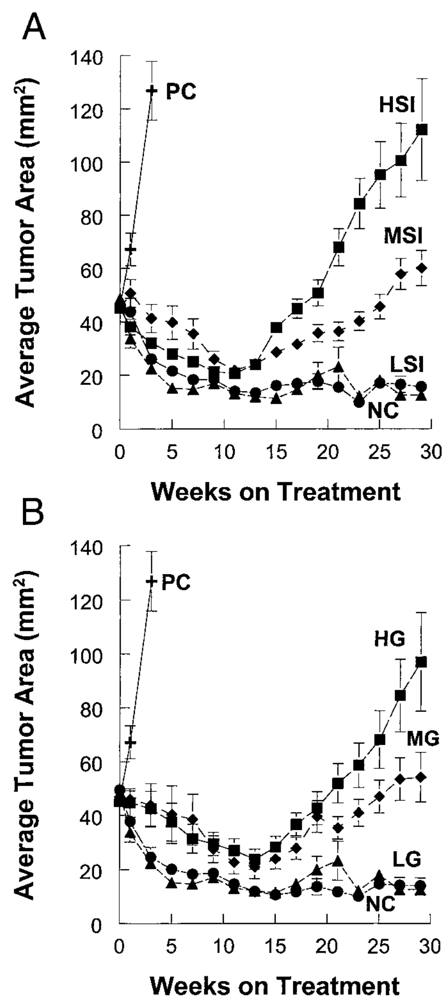

Fig. 1. *A* and *B*, effects of soy protein isolates and genistein on MCF-7 tumor growth in athymic mice. Female ovariectomized athymic mice were implanted with a 2-mg E_2 pellet. The animals were then injected with 1.5×10^5 MCF-7 cells/site in four locations. Subsequently, tumors developed and were allowed to grow to an average cross-sectional area of 40 mm^2. At this time, E_2 pellets were removed from all of the mice, and they were assigned to one of eight treatment groups: positive controls that were reimplanted with a new 2-mg E_2 pellet (8 mice; $n = 32$ tumors), negative controls that were fed AIN 93G rodent diet alone (6 mice; $n = 24$ tumors), LSI containing 15 ppm genistein (7 mice; $n = 27$ tumors), MSI containing 150 ppm genistein (8 mice; $n = 31$ tumors), HSI containing 300 ppm genistein (6 mice; $n = 23$ tumors), LG AIN 93G + 15 ppm genistein (7 mice; $n = 27$ tumors), MG AIN 93G + 150 ppm genistein (6 mice; $n = 23$ tumors), and HG AIN 93G + 300 ppm genistein (7 mice; $n = 27$ tumors). *A*, the effects of soy protein isolate on the growth of MCF-7 tumors. *B*, the effect of consumption genistein in AIN 93G on the growth of MCF-7 tumors. In soy-containing diets, the soy protein isolates were the sole source of protein whereas casein was the protein source in AIN93G diets supplemented with genistein. Both soy- and casein-based diets were formulated to meet all of the nutritional requirements of the mice. The day animals were started on experimental diets and were designated as measurement 0. Tumors were then measured weekly. Data are expressed as average cross-sectional tumor area for all of the tumors in each treatment. *Bars,* SE.

RESULTS

Effect of Soy Isolates and Genistein on MCF-7 Tumor Growth in Athymic Mice. By week 3 after retreatment with a new 2-mg E_2 pellet, the average cross sectional area of the tumors in the positive control group was 126 mm^2 (Fig. 1, *A* and *B*). At this point, these mice were killed. By week 12 after the E_2 pellets were removed, the negative control tumors regressed to an average area of 12 mm^2. They then maintained this size until termination of the study. Soy protein isolates stimulated growth of MCF-7 tumors in a dose-dependent fashion (Fig. 1*A*). Also, casein-based diets containing equivalent levels of genistein stimulated tumor growth in a dose-dependent manner (Fig. 1*B*). By week 29 after E_2 pellet removal, both the LSI and LG groups were similar to the negative control group resulting in average tumor areas of 16 mm^2 and 14 mm^2, respectively. The average tumor area in the MSI and MG groups were significantly higher than the negative control group with final cross sectional areas of 60 mm^2 and 54 mm^2 ($P < 0.01$) respectively. The MSI and MG groups were not significantly different from one another. The tumors from both the HSI and HG groups were significantly larger than the negative control and other dietary treatment groups ($P < 0.01$). The HSI average area was 112 mm^2 whereas the HG group had a final average area of 97 mm^2. These (HSI and HG) were not significantly different from one another and were similar to that obtained from the positive control group 3 weeks after retreating them with E_2 pellets

Downloaded from http://aacrjournals.org/cancerres/article-pdf/2485826/5045.pdf by guest on 06 October 2022

(Fig. 2). Body weight was monitored weekly, and no significant difference was observed among the treated and control groups (data not shown). These data indicate that dietary soy isolates containing increasing concentrations of genistein and casein-based diets containing equalized concentrations of genistein act in a dose-dependent manner to stimulate growth of human estrogen-dependent breast cancer cells transplanted into athymic mice. Also, it is important to note that there were no significant differences in growth of tumors in the negative control, low soy isolate, and low genistein groups. These data suggest that there is a threshold level of dietary genistein below which no increase in estrogen-dependent tumor growth is observed.

Effect of Soy Isolates and Genistein on Estrogen-responsive pS2 mRNA Expression in MCF-7 Tumors. To evaluate the ability of soy isolates and genistein in the diet to enhance the expression of an estrogen-responsive gene, pS2, we conducted Northern blot analysis using mRNA isolated from tumors excised from animals in each treatment group. At the conclusion of the study, tumors from the negative control, low soy isolate, and low genistein groups were too small to obtain sufficient RNA for Northern blot analysis. Negative control tumors were collected from a separate set of tumor-bearing athymic mice in which the tumors were regressed for 11 weeks after E_2 pellets were removed. This allowed us tumors that were regressing (attributable to low E_2) but were large enough for RNA isolation. Expression of pS2 was not detectable in the RNA isolated from negative control animals. Expression of pS2 was very low in the isoflavone-treated animals, ~one-tenth that of the E_2-treated mice. However, pS2 expression was significantly ($P < 0.05$) higher in animals consuming the medium and high soy protein isolates when compared with the negative control group. Levels of pS2 expression in tumors from animals that were consuming medium and high concentrations of genistein in the AIN93G diet were also significantly ($P < 0.05$) higher than the negative control group (Fig. 3). However, there was no significant difference between the MSI and HSI groups or the MG and HG groups.

Cellular Proliferation in MCF-7 Tumors Excised from Animals Consuming Soy Protein Isolates and Genistein. Cellular incorporation of BrdUrd was used as an indicator of cellular proliferation in MCF-7 tumors. Cells that stained positive after immunohistochemical analysis were considered actively proliferating cells. Both proliferating and nonproliferating cells in a given field of view were counted, and final values were expressed as percentage of proliferating cells. The percentage of proliferating cells value for the negative control group was 1.4%. Both the MSI and MG groups had a significantly higher percentage of cellular proliferation when compared with the negative control group with values of 9.4% and 8.2% ($P < 0.001$). The animals consuming the high soy isolate with the highest concentration of genistein and the animals consuming the highest concentration of genistein in control diet had proliferation of 11.9% and 11.8% respectively. These values were significantly higher than all of the other groups ($P < 0.05$; Fig. 4).

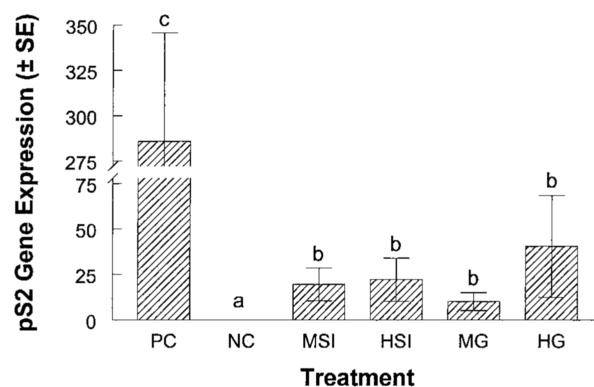

Fig. 3. Effect of soy and genistein treatments on pS2 gene expression in MCF-7 tumors. At the end of the study, tumors were collected for analysis. For the detection of pS2 expression, mRNA was isolated from each tumor. Northern blot analysis was used to quantify the amount of mRNA produced as a result of the treatments. Three tumors from each treatment group were used for pS2 analysis. pS2 expression is presented as the relative pS2 mRNA level. Glyceraldehyde-3-phosphate dehydrogenase was used as a standard. *Bars,* SE.

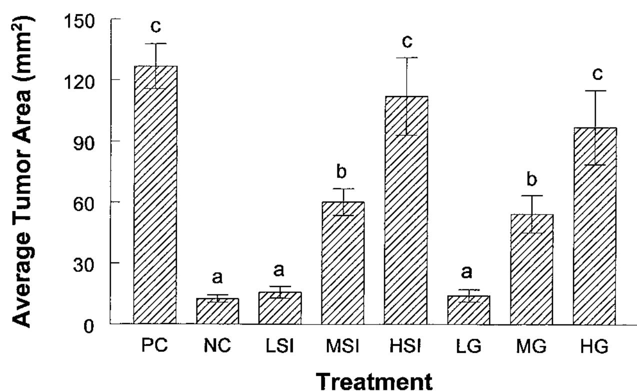

Fig. 2. Average tumor area for week 29. Female ovariectomized athymic mice were implanted with a 2-mg E_2 pellet. The animals were then injected with 1.5×10^5 MCF-7 cells/site in four locations. Subsequently, tumors developed and were allowed to grow to an average cross-sectional area of 40 mm^2. At this time, E_2 pellets were removed from all of the mice and they were assigned to one of eight treatment groups: positive controls that were reimplanted with a new 2-mg E_2 pellet, negative controls that were fed AIN 93G rodent diet alone, LSI containing 15 ppm genistein, MSI containing 150 ppm genistein, HSI containing 300 ppm genistein, LG AIN 93G + 15 ppm genistein, MG AIN 93G + 150 ppm genistein, and HG AIN 93G + 300 ppm genistein. Tumors from each treatment group were evaluated by measuring tumor area. Measurements from all of the tumors in each treatment group were combined and averaged. *Bars,* SE.

DISCUSSION

The purpose of this study was to determine the influence of dietary soy protein isolates containing increasing concentrations of genistein on the growth of estrogen-dependent human breast cancer cells transplanted into athymic mice. The results presented here demonstrate that soy protein isolates containing varying concentrations of genistein can stimulate growth of estrogen-dependent tumors similar to that seen with pure dietary genistein. Soy protein isolates stimulated the growth of MCF-7 tumors in a dose-dependent manner as the concentration of genistein increased in the isolates. Tumor growth was significantly increased in animals consuming genistein at concentrations of 150 and 300 ppm in both the casein- and soy-based diets. Dietary concentrations in this study are lower than previous studies in which MCF-7 tumor growth was observed in athymic mice consuming dietary genistein at 750 ppm (5). In the study presented here, tumors took longer to reach a maximum size when compared with the previous study, which was likely attributable to the lower concentrations of genistein in the diet. Cellular proliferation was increased in a dose-dependent manner as genistein concentrations increased in both the soy protein and casein-based diets consistent with the tumor growth data. The increase in MCF-7 cell proliferation was likely attributable to an estrogenic effect as indicated by the modest increase in pS2 expression. These findings suggest that genistein, when fed to athymic mice in pure form or in a food source rich in the isoflavone such as soy protein isolate, can stimulate estrogen-dependent tumor growth in a dose-dependent manner.

The estrogenic activity of isoflavones has been well documented in *in vitro*, *in vivo*, and clinical studies. Genistein can bind to the estrogen receptor α with an affinity 100-1000 times lower than E_2 (21). Additionally, in ovariectomized Sprague Dawley rats, dietary

Downloaded from http://aacrjournals.org/cancerres/article-pdf/2485826/5045.pdf by guest on 06 October 2022

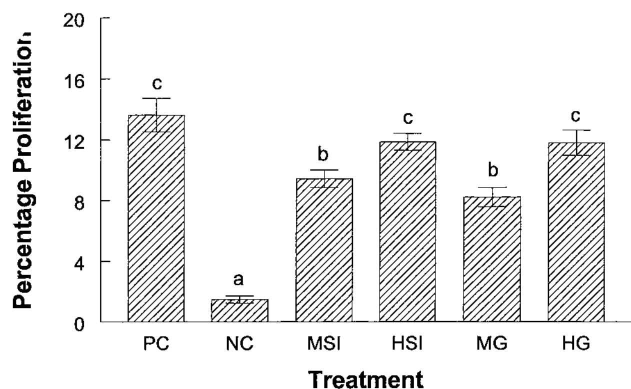

Fig. 4. Effect of soy and genistein on the cellular proliferation within MCF-7 tumors. Tumors were removed from the mice for immunohistochemical analysis. Incorporation of BrdUrd into cellular DNA was used as a marker of cellular proliferation. Immunohistochemistry was used to stain for cells containing BrdUrd. Positively staining as well as background cells were counted to give a final count on both proliferating and total cells in a given area of tissue. Cell counts from each treatment group were then combined and averaged (n = 25 fields/five tumors/treatment group). The data are presented as the percentage of cells actively proliferating in a given area of tissue. *Bars,* SE.

genistein (750 ppm) enhanced lobular-alveolar mammary gland development, increased uterine weight, and at 750 ppm, increased pituitary prolactin secretion and serum prolactin levels. In the uterus, dietary genistein increased *c-fos* mRNA expression (21). *In vitro* studies showing an increase in the levels of the estrogen-responsive genes pS2 and *c-fos* when cells are treated with genistein, provide additional evidence of the estrogenic property of genistein (5, 22). Clinical studies have also confirmed the preclinical studies evaluating the estrogenicity of genistein. Premenopausal women consuming textured vegetable protein containing 45 mg of isoflavones had prolonged menstrual cycles. Additionally, the follicular phase was extended by suppression of the normal surge of follicle-stimulating hormone and luteinizing hormone (23, 24). These data in conjunction with the observations that dietary soy increases cell proliferation in human breast tissue (6) and increases pS2 expression (7) demonstrates biological (estrogenic) activity in humans.

Isoflavones are being marketed to postmenopausal women in both supplement form and in soy food products for the relief of the symptoms of menopause. It is, therefore, critical to understand what activities genistein may have in these women. Consideration must be given to how genistein may influence the endogenous levels of serum E_2 in women consuming the compound. There is evidence suggesting diets high in soy have the ability to lower serum E_2 concentrations (25, 26). A study supporting this finding showed that normally cycling women consuming a soy diet containing ~154 mg total isoflavones/day had a 25% reduction in circulating E_2 (27). Contrarily, other studies in which premenopausal women have consumed soy have found no change in serum E_2 concentrations (6, 28), and one study showed increased serum E_2 in women consuming soy (23). It is unclear how isoflavones in soy effect normal circulating E_2 concentrations in premenopausal women. Postmenopausal women consuming a soy diet have been shown to have no change in serum circulating E_2 levels (28). This finding in conjunction with reports that genistein acts estrogenically, and postmenopausal women naturally have low plasma concentrations of E_2, suggest that in a postmenopausal woman, these weak estrogens may have significant estrogenic activity.

At concentrations above 10 μM genistein has been demonstrated to inhibit *in vitro* cell proliferation in a variety of cell types including estrogen-dependent (MCF-7) and estrogen-independent (MDA-468) human breast cancer cells (29). Genistein has also been shown to inhibit activities of protein tyrosine kinase at concentrations >20 μM (30). However, at levels as low as 200 nM, genistein stimulates the growth of MCF-7 cells *in vitro* (2, 5) and *in vivo* at 1 μM (5). In MCF-7 cells, at concentrations from 20–90 μM genistein inhibited DNA synthesis, but stimulated DNA synthesis at concentrations of 0.1–10 μM (31). In this study we demonstrated that dietary genistein consumed at 15 ppm, whether from soy protein or as a pure compound, does not stimulate growth that is significantly different from the negative control group lacking any form of estrogen exposure in regard to stimulation of MCF-7 tumor growth. These results suggest that there is a level of dietary genistein that will not lead to an increase in tumor growth. Additional investigation will be necessary to determine the concentration of this threshold dose.

Numerous studies have focused on the chemopreventative effects of genistein and demonstrate that if exposed to isoflavones before puberty a rat has a reduced risk of developing breast cancer when exposed to a chemical carcinogen (8–10). It is reasonable then to assume a similar protective effect might be seen in women if they are exposed to isoflavones before puberty. Early exposure of women in Asia to isoflavones may explain in part why Asian women have a lower rate of breast cancer incidence when compared with American women. It is hypothesized that a high soy diet throughout life protects against development of breast cancer. This idea is supported by the fact that upon moving to the United States, Asian women have a similar breast cancer rate as that of American women after two generations, indicating early exposure is critical (32–34). In evaluating the correlation between urinary phytoestrogen levels and breast cancer incidence in postmenopausal women, a recent report showed high urinary genistein excretion was weakly and nonsignificantly associated with a reduced breast cancer risk (35). Another study shows no effect of soy consumption on the progression of chemically induced mammary tumors in the rat (11). However, it is important to note that in this study animals were intact with normal circulating E_2. Paradoxically, we have shown that, in ovariectomized mice transplanted with human estrogen-dependent breast cancer cells, genistein and now soy protein isolates containing genistein stimulated tumor growth (5). Ovariectomized athymic mice implanted with MCF-7 cells are an appropriate model of postmenopausal women with estrogen-dependent breast cancer. Plasma levels of E_2 in ovariectomized athymic mice are 27–38 pg/ml compared with postmenopausal women 10–40 pg/ml (36). We believe the low endogenous estrogen environment created in this model by ovariectomizing the mice affords genistein the opportunity to be a significant source of estrogenicity in these animals and, hence, allows for the compound to stimulate the growth of these tumors. Therefore, if a postmenopausal woman with low endogenous E_2 levels has an existing estrogen-dependent breast tumor it is also possible that consumption of dietary genistein from various food sources including soy protein isolate may produce sufficient plasma levels of genistein to result in enhanced estrogen-dependent tumor growth as observed in athymic mice. These results collectively suggest that the time of exposure to genistein is critical to the overall effect genistein will have on estrogen-dependent breast cancer tumor growth.

From the discussion presented here it is clear that the isoflavones possess diverse biological activities and potency. These activities are often dependent upon the concentration and timing of administration of the isoflavones. As a result, it is important to focus on what activities occur at plasma concentrations that are relevant to what is observed in humans consuming isoflavone containing diets. In regard to breast cancer, the isoflavones, specifically genistein, have paradoxical effects that can be resolved when you consider dosage and timing of administration. For example, prepubertal exposure to genistein appears to be protective against the development of breast cancer, but consumption of the phytoestrogen in either pure form or in soy protein isolate, after development of an estrogen-dependent breast cancer may

Downloaded from http://aacrjournals.org/cancerres/article-pdf/2485826/5045.pdf by guest on 06 October 2022

enhance the growth of that tumor as determined by this study. Additional investigation into the biological activities of the isoflavones and soy itself is needed. The use of soy protein isolate as a dietary supplement may have numerous health benefits such as reducing risk of coronary heart disease by lowering serum cholesterol. However, for the subgroup of postmenopausal women who have or are at high risk of developing breast cancer there is need for additional consideration into the possibility of enhancing estrogen-dependent tumor growth by consumption of isoflavone containing products.

REFERENCES

1. Soy protein and coronary heart disease. Federal Register., *64:* 57699–57733, 1999.
2. Martin, P. M., Horwitz, K. B., Ruyan, D. S., and McGuire, W. L. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology, *103:* 1860–1867, 1978.
3. Setchell, K., Borriello, S. P., Hulme, P., Kirk, D. N., and Axelson, M. Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am. J. Clin. Nutr., *40:* 569–578, 1984.
4. Farmakalikis, E., Hathcock, J. N., and Murphy, P. A. Oestrogenic potency of genistin and daidzin in mice. Food Chem. Toxic., *23:* 741–745, 1985.
5. Hsieh, C. Y., Santell, R. C., Haslam, S. Z., and Helferich, W. G. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. Cancer Res., *58:* 3833–3838, 1998.
6. Petrakis, N. L., Barnes, S., King, E. B., Lowenstein, J., Wiencke, J., Lee, M. N., Miike, R., Kirk, M., and Coward, L. Stimulatory influence of soy protein isolate on breast fluid secretion in pre- and postmenopausal women. Cancer Epidemiol. Biomark. Prev., *5:* 785–794, 1996.
7. Hargreaves, D. F., Potten, C. S., Harding, C., Shaw, L. E., Morton, M. S., Roberts, S. A., Howell, A., and Bundred, N. J. Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. J. Clin. Endocrinol. Metab., *84:* 4017–4024, 1999.
8. Fritz, W. A., Coward, L., Wang, J., and Lamartiniere, C. A. Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. Carcinogenesis (Lond.), *19:* 2151–2158, 1998.
9. Lamartiniere, C. A., Moore, J. B., Brown, N. M., Thompson, R., Hardin, J. J., and Barnes, S. Genistein suppresses mammary cancer in rats. Carcinogenesis (Lond.), *16:* 2833–2840, 1995.
10. Murrill, W. B., Brown, N. M., Zhang, J. X., Manzolillo, P. A., Barnes, S., and Lamartiniere, C. A. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. Carcinogenesis (Lond.), *17:* 1451–1457, 1996.
11. Cohen, L. A., Zhao, Z., Pittman, B., and Scimeca, J. A. Effect of intact and isoflavone-depleted soy protein on NMU-induced rat mammary tumorigenesis. Carcinogenesis (Lond.), *21:* 929–935, 2000.
12. Xu, X., Wang, H. J., Murphy, P. A., Cook, L., and Hendrich, S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J. Nutr., *124:* 825–832, 1994.
13. Reeves, P. G., Nielsen, F. H., and Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of AIN -76A rodent diet. J. Nutr., *123:* 1939–1951, 1993.
14. McManus, M. J., and Welsch, C. W. Hormone-induced ductal DNA synthesis of human breast tissues maintained in the athymic nude mouse. Cancer Res., *41:* 3300–3305, 1981.
15. Noel, A., Simon, N., Raus, J., and Foidart, J. M. Basement membrane components (Matrigel) promote the tumorigenicity of human breast adenocarcinoma MCF7 cells and provide an *in vivo* model to assess the responsiveness of cells to estrogen. Biochem. Pharmacol., *43:* 1263–1267, 1992.
16. Gottardis, M. M., Jiang, S. Y., Jeng, M. H., and Jordan, V. C. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. Cancer Res., *49:* 4090–4093, 1989.
17. Helferich, W. G., Jump, D. B., Anderson, D. B., Skjaerlund, D. M., Merkel, R. A., and Bergen, W. G. Skeletal muscle α-actin synthesis is increased pretransitionally in pigs fed the phenethanolamine ractopamine. Endocrinology, *126:* 3096–3100, 1990.
18. Brown, A. M. C., Jeltsch, J. M., Roberts, M., and Chambon, P. Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. Proc. Natl. Acad. Sci. USA, *81:* 6344–6348, 1984.
19. De Fazio, A., Leary, J. A., Hedley, D. W., and Tattersall, M. H. Immunohistochemical detection of proliferating cells *in vivo*. J. Histochem. Cytochem., *35:* 571–577, 1987.
20. Spady, T. J., Harwell, D. M. E., Snyder, M. C., Pennington, K. L., Mcomb, R. D., and Shull, J. D. Estrogen-induced tumorigenesis in the Copenhagen rat: disparate susceptibilities to development of prolactin producing pituitary tumors and mammary carcinomas. Cancer Lett., *124:* 95–103, 1998.
21. Santell, R. C., Chang, Y. C., Nair, M. G., and Helferich, W. G. Dietary genistein exerts estrogenic effects on the uterus, mammary gland, and the hypothalamic/pituitary axis in rats. J. Nutr., *127:* 263–269, 1997.
22. Sathyamoorthy, N., Wang, T. T. Y., and Phang, J. M. Stimulation of pS2 expression by diet-derived compounds. Cancer Res., *54:* 957–961, 1994.
23. Cassidy, A., Bingham, S., and Setchell, K. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am. J. Clin. Nutr., *60:* 333–340, 1994.
24. Cassidy, A., Bingham, S., and Setchell, K. Biological effects of isoflavones in young women: importance of the chemical composition of soyabean products. Br. J. Nutr., *74:* 587–601, 1995.
25. Nagata, C., Takatsuka, N., Inaba, S., Kawakami, N., and Shimizu, H. Effect of soy milk consumption on serum estrogen concentrations in premenopausal Japanese women. J. Natl. Cancer Inst., *90:* 1830–1835, 1998.
26. Xu, X., Duncan, A. M., Merz, B. E., and Kurzer, M. S. Effects of soy isoflavones on estrogen and phytoestrogen metabolism in premenopausal women. Cancer Epidemiol. Biomark. Prev., *12:* 1101–1108, 1998.
27. Lu, L. W., Anderson, K. E., Grady, J. J., Kohen, F., and Nagamani, M. Decreased ovarian hormones during a soya diet: implications for breast cancer prevention. Cancer Res., *60:* 4112–4121, 2000.
28. Baird, D. D., Umbach, D. M., Lansdell, L., Hughes, C. L., Setchell, K. D. R., Weinberg, C. R., Haney, A. F., Wilcox, A. J., and McLachlan, J. A. Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. J. Clin. Endocrinol. Metab., *80:* 1685–1690, 1995.
29. Peterson, G., and Barnes, S. Genistein inhibition of the growth of human breast cancer cells: independence from the estrogen receptor and the multi-drug resistance gene. Biochem. Biophys. Res. Comm., *179:* 661–667, 1991.
30. Peterson, G. Evaluation of the biochemical targets of genistein in tumor cells. J. Nutr., *125:* 784S–789S, 1995.
31. Wang, C., and Kurzer, M. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr. Cancer, *28:* 236–247, 1997.
32. Buell, P. Changing incidence of breast cancer in Japanese-American women. J. Natl. Cancer Inst., *51:* 1479–1483, 1973.
33. Hirayama, T. Epidemiology of breast cancer with special reference to the role of the diet. Prev. Med., *7:* 173–195, 1978.
34. Kodama, M., Kodama, T., Miura, S., and Yoshida, M. Nutrition and breast cancer risk in Japan. Anticancer Res., *11:* 745–754, 1991.
35. Tonkelaar, I., Keinan-Boker, L., Van't Veer, P., Arts, C. J. M., Adlercreutz, H., Thijssen, H. H., and Peeters, H. M. Urinary phytoestrogens and postmenopausal breast cancer risk. Cancer Epidemiol. Biomarkers Prev., *10:* 223–228, 2001.
36. Seibert, K., Shafie, S. M., Triche, T. J., Whang-Peng, J. J., O'Brien, S. J., Toney, J. H., Huff, K. K., and Lippman, M. E. Clonal variation of MCF-7 breast cancer cells *in vitro* and in athymic nude mice. Cancer Res., *43:* 2223–2239, 1983.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/2485826/5045.pdf by guest on 06 October 2022