Genistein Inhibits Growth of Estrogen-Independent Human Breast Cancer Cells in Culture but Not in Athymic Mice\textsuperscript{1}

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ABSTRACT The studies presented were conducted to assess the effect of the soy isoflavone genistein on proliferation of estrogen-independent human breast cancer cells (MDA-MB-231) in vitro and in vivo. Genistein (20 \(\mu\)mol/L) inhibited cell proliferation in vitro by \(\approx50\%\). Cell cycle progression was blocked in G\textsubscript{2}/M with 40 and 80 \(\mu\)mol/L genistein. To evaluate the effect of dietary genistein on tumor growth in vivo, genistein was fed to female athymic mice inoculated with MDA-MB-231 cells. After solid tumor masses had formed, mice were fed genistein at a dose (750 \(\mu\)g/g AIN-93G diet), shown to produce a total plasma genistein concentration of \(\approx1\) \(\mu\)mol/L. This dose of genistein did not significantly (\(P > 0.05\)) alter tumor growth. Studies were then conducted to assess the effect of dietary genistein on initial tumor development and growth. Genistein (750 \(\mu\)g/g AIN-93G diet), fed 3 d before cells were inoculated into mice, did not significantly (\(P > 0.05\)) inhibit tumor formation or growth. The plasma concentration of genistein in mice fed this dose of genistein (750 \(\mu\)g/g AIN-93G diet) does not appear sufficient to inhibit tumor formation or growth. Dietary genistein at 750 \(\mu\)g/g AIN-93G diet does not inhibit tumor formation or growth. Additional studies were conducted to determine the effect of dietary dosages ranging from 0 to 6000 \(\mu\)g/g AIN-93G diet on plasma genistein concentration. Plasma genistein concentration increased in a dose-dependent manner up to 7 \(\mu\)mol/L at 6000 \(\mu\)g/g AIN-93G diet. These data suggest that although genistein inhibits cancer cell growth in vitro, it is unlikely that the plasma concentration required to inhibit cancer cell growth in vivo can be achieved from a dietary dosage of genistein. J. Nutr. 130: 1665–1669, 2000.

KEY WORDS: • MDA-MB-231 • athymic mice • genistein • breast cancer

Epidemiologic studies have shown wide geographical variations in the occurrence of breast cancer in women (Gray et al. 1979, Miller 1977, Wynder and Hirayama 1977). Incidence rates are as high as 1 in 8 in the United States and as low as 1 in 30 in Japan. Furthermore, the incidence of breast cancer in migrant Japanese women to the U.S. (Buell 1973, Hirayama 1978). These data suggest environmental factors, such as diet, may play a primary role in the onset and development of breast cancer.

Soy products contain a number of compounds with the potential to inhibit carcinogenesis, including protease inhibitors (Kennedy and Manzone 1995), phytates (Shamsuddin 1995) and isoflavones (Barnes et al. 1990). Studies presented in this report focus on genistein, which is present in soy products at concentrations as high as 1.5 mg/g (Coward et al. 1993, Eldridge and Kwolek 1995, Murphy and Wang 1993).

Genistein is chemopreventive in the carcinogen-induced rat breast cancer model. When, genistein is administered prepubertally to rats, a reduction in dimethylbenzanthracene-initiated tumor number is observed. Additionally, this reduction in tumor number has been observed when genistein was injected (Lamartiniere et al. 1995, Murrill et al. 1996) or fed at 250 \(\mu\)g/g in the diet (Fritz et al. 1998). We have demonstrated (Hsieh et al. 1998) that dietary genistein can stimulate growth of existing estrogen-dependent tumors in ovariectomized athymic mice. Thus it is possible that genistein can be preventative when administered early in an animal’s life (before a chemical carcinogen) but can stimulate growth of an existing estrogen-dependent tumor.

Genistein inhibits the proliferation of a number of transformed cell lines in cell culture. Inhibition of cell growth by genistein may be due to inhibition of topoisomerase II (Markovits et al. 1989, Okura et al. 1988) and protein tyrosine kinases (Akiyama et al. 1987, Geissler et al. 1990). Genistein concentrations as low as 12 \(\mu\)mol/L can inhibit topoisomerase II activity, whereas concentrations as low as 3 \(\mu\)mol/L inhibit tyrosine kinase activity. Both of these enzymes are involved in cell proliferation. Concentrations of genistein within this range have been shown to inhibit proliferation of a number of tumor cell lines, e.g., genistein inhibited MCF-7 adriamycin resistant, MCF-7/WT and MDA-MB-231 human breast cancer cells with concentrations of genistein that inhibit 50% of cell proliferation (IC\textsubscript{50}) of between 7 and 37 \(\mu\)mol/L (Monti

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and Sinha 1994), MCF-7 and MDA-468 human breast cancer cells with IC50 of 24–44 μmol/L (Peterson and Barnes 1993), stomach and colon cancer cell lines with IC50 of ~25 μmol/L (Yanagihara et al. 1993) and AGS human gastric cells with an IC50 of between 7 and 23 μmol/L (Piontek et al. 1993).

Genistein (60 μmol/L) has been shown to reversibly arrest cell cycle progression of human gastric cancer (HGC-27) cells at G2/M (Matsukawa et al. 1993) and of Jurkat T-leukemia cells at G2/M (18.5–37.0 μmol/L), whereas higher doses (74–110 μmol/L) blocked cell cycle progression through the S phase (Spinozzi et al. 1994). Blocking progression of the cell cycle is likely due to the inhibition of tyrosine kinase activity by genistein at key regulatory points in the cell cycle, thus preventing progression through mitosis, resulting in an inhibition of cell proliferation.

In this study, the effect of genistein on the cell cycle and proliferation of estrogen receptor–negative human breast cancer (MDA-MB-231) cells was investigated in culture. It is important to note that the concentration of genistein required to inhibit cancer cell growth is high, usually >10 μmol/L. Whether dietary genistein can produce plasma levels of genistein equivalent to those required to inhibit tumor cell growth in vitro was also examined. Studies are also presented on the effect of dietary genistein on growth of MDA-MB-231 cells in female athymic mice.

**MATERIALS AND METHODS**

**Chemicals.** Genistein was synthesized from organic precursors as described by Chang et al. (1994). Chemical identity was assessed by nuclear magnetic resonance and purity assessed at ≥98%. Analytical grade reagents were used for HPLC analysis. All other chemicals, unless otherwise specified, were purchased from Sigma Chemical (St. Louis, MO).

**Animals and diets.** Athymic female mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). In all experiments, mice were housed three or four to a cage. Athymic mice were kept under aseptic conditions (enclosed laminar flow hood, sterilized cages, bedding, and water). All mice were kept in a temperature-controlled (22 ± 2°C), relative humidity–controlled (40–70%) and light-controlled (12-h light:dark cycle) animal facility. All mice were fed the AIN-93G semipurified diet (corn oil replaced soybean oil) containing genistein at the levels specified below (Reeves et al. 1993). Tetracycline (500 mg/L) was added to the drinking water.

**Cell proliferation.** MDA-MB-231 human breast cancer cells were obtained from Dr. Clifford Welsch at Michigan State University. Cells were maintained in minimal essential medium (MEM) with the following: Na-bicarbonate, 2.2 g/L; l-glutamine, 0.292 g/L; fetal bovine serum (FBS), 100 mL/L (10%); Na-pyruvate, 8.8 mg/L; bovine insulin, 10 mg/L; penicillin, 1000 u/L; and streptomycin 10 mg/L. Cells were collected at 100 mm × 20 mm tissue culture plates at 80% confluence by washing twice with 1X PBS followed by trypsinization. Cells were counted using a hemocytometer and the cell suspension diluted to ~5 × 106 cells/L.

The cells were plated in 24-well plates at 15,000 cells per well and incubated for 16 h in maintenance medium (described above). After 16 h, the medium was removed and replaced with similar maintenance medium but with only 1 mL/L FBS. Cells were incubated for 48 h after which the medium was replaced with fresh maintenance medium (MEM + 10% FBS) containing genistein (in ethanol) at 0, 10, 20, and 40 μmol/L. Ethanol vehicle for the treatments did not amount to >0.1% total volume of the medium in the well.

Cells were collected at 1, 3, 5 and 7 d for DNA analysis as described by West et al. (1985) with minor modifications. For the DNA assay, the medium was removed, cells washed twice with 1X PBS and then lysed with 2.7 mL of 10 mmol/L EDTA, pH 12.3. After a 30-min incubation at 37°C, the pH was adjusted to 7.0 with KH2PO4 (~150 μL) and 20 μL (1000 ng dye) of a stock solution of Hoechst-33258 dye (200 mg/dl diluted 1:4) was added to each well. Aliquots of 200 μL were transferred to a black 96-well plate for reading on a fluorescent plate reader (Cytofluor II, Perspective Biosystems, Framingham, MA.). The fluorophore was excited at 350 nm and emission measured at 455 nm. Fluorescence values were converted to DNA values on the basis of data from a DNA standard curve made from salmon testes DNA.

**Cell cycle and cell viability analysis.** MDA-MB-231 cells were collected from 100 mm × 20 mm tissue culture plates at 80% confluence by washing twice with 1X PBS followed by trypsinization. Cell number was determined using a hemocytometer and the cell suspension diluted to ~5 × 106 cells/L. Maintenance media (3 mL; described above) was added to each 60 mm × 15 mm tissue culture plate and 1 mL of the cell suspension added (300,000 cells/plate). The cells were incubated for 16 h and the medium removed and replaced with similar maintenance medium but with only 1 mL/L FBS. Cells were incubated for 48 h; the medium was then replaced with fresh maintenance medium (MEM + 10% FBS) containing genistein at 0, 10, 20, 40 and 80 μmol/L. The ethanol vehicle for the treatments did not amount to >0.1% total volume of the medium in the well.

Cells were collected at 12, 24, 48 and 72 h for cell cycle and viability analysis. Cells were collected by removing the medium from the tissue culture plates into flow cytometry tubes (13 mm × 75 mm) and trypsinizing the cells, collecting them into the same tubes and centrifuging at 350 × g for 5 min. The supernatant fraction was aspirated and the pellets washed with MEM containing 10% FBS. For analysis of cell viability, two 90-μL aliquots were collected into 0.5-mL microfuge tubes, 10 μL of 4% trypsin blue added, the samples incubated at room temperature for 5 min and cell viability determined by examining the ability of the cells to exclude trypsin blue.

For flow cytometry, the remaining samples were centrifuged at 350 × g for 5 min and the supernatant removed. The cellular pellet was resuspended in 1.5 mL of 70% ethanol and then placed at 4°C for 96 h. The cells were removed from 4°C, centrifuged at 350 × g, the ethanol removed and the cellular pellet washed with 1X PBS. The following day, the flow cytometry staining reagents were then added: 945 μL reagent A (stock solution of reagent A containing 100 μL of 100 mmol/L EDTA, pH 7.4, 100 μL of Triton X-100 in 99.8 mL 1X PBS), 50 μL reagent B (stock solution of reagent B containing propidium iodide, 1 g/L in H2O) and 10 μL of RNase that had been boiled for 6 min. The tubes were gently vortexed and placed in the dark at 4°C until reading on the flow-activated cell sorter. Fluorescence was measured by excitation with an Argon laser at 488 nm and the emission measured at 525–550 nm. The percentage of cells in each phase of the cell cycle was calculated using the MPLUS software (Phoenix, AZ).

**Genistein fed to mice inoculated with MDA-MB-231 cells.** To evaluate the effect of dietary genistein on the growth of solid tumor masses, the following study was conducted. Female athymic mice (n = 30) were received at 3–4 wk of age and allowed unrestricted access to water and the AIN-93G diet for 7 d before subcutaneous injection of MDA-MB-231 cells. These cells had been collected from 80% confluent 100 mm × 20 mm plates by washing two times with 1X PBS, trypsinizing, and then collecting and pooling the cells in MEM supplemented with 10% FBS. Cell counts were determined using a hemocytometer and the cells diluted to 5 × 106 cells/L. The mice (n = 30) were anesthetized and injected subcutaneously with 200 μL of the cell suspension (106 cells/site), using a 1-mL syringe with a 25-gauge needle (1.6 cm), in each of their four flanks. After 5 wk, to equalize tumor number and cross-sectional area, the mice were grouped into a 750 μg genistein/g AIN-93G diet group (tumors = 27) and an AIN-93G diet control group (tumors = 33) and treatment began. Only mice with tumors >3 mm × 3 mm (measured by caliper) at the time of grouping were used in the study. Tumor length (L) and width (W) were measured weekly for 5 wk and cross-sectional area determined by the following formula (L/2)2 × (W/2) × π. Food intake was measured during the last 3 d of the study. At the end of the study, mice were anesthetized, weighed, bled via cardiac puncture and killed by overexposure to anesthesia.

To evaluate the effect of dietary genistein on initial tumor formation and growth, the following study was conducted. Female athymic mice were received at 3–4 wk of age and allowed unrestricted access to water and the AIN-93G diet for 7 d. One group of mice (n = 9) were then fed 750 μg genistein/g diet 3 d before injection of the...
MDA-MB-231 cells; the control group (n = 9) was fed the AIN-93G diet. The genistein-treated mice developed 25 tumors and the control group 32 tumors. Injection of tumor cells and tumor measurements were performed as described above.

**Plasma genistein concentration in response to dietary genistein.**

To assess plasma genistein concentration in mice fed different amounts of genistein, the following study was conducted. Female Balb/C mice (n = 36) were received at 4 wk of age and allowed unrestricted access to water and the AIN-93G diet. After 2 wk, mice were weighed, sorted three mice to a cage; two cages per group, and provided 6 g AIN-93G diet/mouse ∙ d. Cages and food cups were collected each day for 4 d, the bedding sifted for food and baseline control food intake determined. Genistein was then mixed into the AIN-93G diet in the following amounts: 0, 375, 750, 1500, 3000 and 6000 μg/g and fed to each group for 4 d. Food intake was determined each day as described above. After 5 d of genistein consumption, mice were anesthetized, weighed and plasma collected by cardiac puncture for genistein analysis.

**Plasma genistein analysis.** Mice were anesthetized and bled by cardiac puncture. Blood (~800 μL/mouse) was placed in microtube containers containing 10 μL of 15% EDTA, centrifuged at 12,000 × g and the plasma removed and stored at −20°C. To determine conjugated and unconjugated genistein, 50 μL of plasma was divided into duplicate aliquots; one set received 5 μL (515 U) β-glucoronidase Type H-1 (Sigma Chemical). All aliquots were incubated in 0.5-mL microtube tubes at 37°C for 24 h. After the incubation, 50 μL of absolute methanol was added to each tube, the tubes vortexed and then centrifuged at 15,000 × g for 10 min. Approximately 75 μL was removed and placed at −20°C until analysis. For analysis of genistein, the microtube tubes were centrifuged at 15,000 × g for 10 min and 20 μL of the content injected onto a C-18 column Rainin Instrument, Woburn, MA) with a flow rate of 1.0 mL/min of 50:50 methanol/water with 1 mL/m of acetic acid. Sample absorbance was monitored at 260 nm. Recovery was determined by adding a known amount of genistein (28 μL of 21.1 mg/L in ethanol) to 600 μL plasma from control mice and diluting this with plasma to a final concentration of 1.82 μmol/L. Mean recoveries were determined to be 96 ± 5.87%. Genistein was not detected in the plasma of control mice fed the AIN-93G diet. Plasma genistein concentrations are reported only for samples in which genistein was detected. The level of detection for plasma genistein was ~130 nmol/L.

**Statistical analyses.** All statistical tests were performed using a PC-based version of SPSS (Version SPSS/PC 9.0, Chicago, IL). Significant differences in tumor area, food intake per cage and mouse weight were assessed by Student’s t test at the end of each experiment. Significant differences in cell culture studies were assessed by ANOVA. When a significant (P < 0.05) treatment effect was detected, treatment means were compared using Tukey’s post-hoc comparisons. Significant differences in the genistein feeding study were assessed by ANOVA. Variance in plasma genistein concentration were nonhomogeneous with respect to treatment; thus, these data (excluding the 0 μg genistein/g diet group) were log transformed before ANOVA. When a significant (P < 0.05) treatment effect was detected, treatment means were compared using Tukey’s post-hoc comparisons. The 0 μg genistein/g diet group was compared with the 375 μg genistein/g diet by Student’s t test. Values in the text and figures are means ± SEM.

**RESULTS**

**Effect of genistein on MDA-MB-231 cell proliferation, cell viability and cell cycle in culture.** Genistein inhibited cell proliferation by ~50% at a concentration of ~20 μmol/L (Fig. 1). Genistein at 10 μmol/L had no apparent effect on cell proliferation, whereas 20, 40 and 80 μmol/L resulted in a dose-dependent decline in cell proliferation.

Genistein at 40 and 80 μmol/L, produced a block in the G2/M phase of the cell cycle (Table 1). Cell viability, as assessed by the cellular exclusion of trypan blue dye, was >86% at all concentrations of genistein.

### Table 1

<table>
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<tr>
<th>Genistein, μmol/L</th>
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<tr>
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<td>S</td>
<td>33</td>
<td>32</td>
<td>27</td>
<td>35</td>
</tr>
<tr>
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<td>21</td>
<td>40</td>
<td>43</td>
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<tr>
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<td>31</td>
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<td>S</td>
<td>30</td>
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<td>34</td>
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<tr>
<td>72 h</td>
<td>G2/M</td>
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<td>11</td>
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<table>
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<tr>
<th>Genistein, μmol/L</th>
<th>% cells</th>
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<tr>
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1 Cells were treated with genistein at 0, 20, 40 and 80 μmol/L for 48 and 72 h. Data are expressed as the percentage of cells in each phase of the cell cycle. Cells were stained with propidium iodide and analyzed by flow cytometry.
We have demonstrated that genistein inhibits the proliferation of MDA-MB-231 human breast cancer cells in cell culture (Fig. 1) and that the probable mechanism is inhibition of the cell cycle at G2/M (Table 1). We have also shown that 40 and 80 μmol/L genistein blocked cultured cells in the G2/M phase of the cell cycle. These data are consistent with the published work of others using different transformed cell lines (Hunakova et al. 1994, Matsukawa et al. 1993, Monti and Sinha 1994, Peterson and Barnes 1991, Piontek et al. 1993, Spinozzi et al. 1994, Traganos et al. 1992, Yanagihara et al. 1993). In the studies presented here, we did not see any evidence of a G1 block in cells treated with genistein at concentrations up to 80 μmol/L. It is likely that genistein is inhibiting one or more tyrosine kinases required for transition through the different phases of the cell cycle and that the specific perturbation in the cell cycle produced by genistein depends on the cell line utilized.

Previous studies have shown that 750 μg genistein/g AIN-93G diet exerts biological effects in estrogen-responsive tissues in rats (Santell et al. 1997). In addition, 750 μg genistein/g AIN-93G diet produces a plasma genistein concentration of ~1 μmol/L in mice, which is similar to that seen in humans consuming soymilk containing a total of 36 mg of genistein (Xu et al. 1994). Tumor growth in mice fed this amount of genistein (750 μg genistein/g AIN-93G) did not differ significantly from tumor growth in control mice (Fig. 2).

In the first study, 750 μg genistein/g AIN-93G diet was administered to the mice after the formation of solid tumor masses. Solid tumors >2–3 mm in diameter require a vasculature for nourishment. This vasculature is formed (angiogenesis) through an intricate balance of protease activity, resulting in degradation of the basement membrane in a controlled fashion, thus permitting the generation and infiltration of blood vessels. Genistein has been shown to inhibit this process, although the concentration required in vitro was ~150 μmol/L (Fotsis et al. 1993). This concentration is much greater than that observed in the plasma of mice fed genistein; however, the effect of lower concentrations of genistein on the initial stages of solid tumor development in vivo has not been investigated. In addition, in vitro endothelial cell proliferation was inhibited with IC50 of 5 (Fotsis et al. 1993) and 12 μmol/L genistein (Koroma and de Juan 1994). In the studies presented here, when genistein was fed at an amount that would produce a plasma concentration of 1 μmol/L (750 μg genistein/g AIN-93G diet) either before or after inoculating tumor cells, the growth of tumors was not altered.

Shao et al. (1998) administered genistein by injection (500 μg/kg body weight) to athymic mice implanted with MDA-231 tumors and demonstrated a significant reduction in tumor growth with genistein. Although the authors did not determine plasma genistein concentration, it is possible that the injection of genistein produced plasma concentrations of genistein high enough to inhibit tumor growth.

In mice fed different doses of genistein (0–6000 μg/g AIN-93G), we observed a dose-dependent increase in plasma concentration from 0 to 7 μmol/L (free + conjugated forms) with ~2 μmol/L free genistein at the highest dose. Even if we assume that all forms of genistein are biologically active, it is unlikely, based on the IC50 from our in vitro data (Fig. 1), that we will see any reduction in cancer cell growth in vivo. We have fed dietary genistein to athymic mice with MDA-231 tumors, and found no difference in tumor growth compared to control mice (Fig. 2).
tumors at a concentration of 3000 µg/g AIN-93G diet (data not shown). It is important to note that at this high level of dietary genistein, we observed reduced food intake along with a reduction in tumor growth rate. Mice fed 3000 µg genistein/g AIN-93G consumed ~11% less food. Thus it was not possible to determine whether the reduction in tumor growth rate was due to the dietary genistein or energy restriction (Welsch et al. 1994).

The long-term physiologic effects of dietary genistein are unknown. Genistein is a known phytoestrogen and at the doses used in these studies, does produce estrogenic responses in various organs, including the mammary gland, in ovariectomized rodents (Santell et al. 1997). Furthermore, genistein has been shown to induce estrogen receptor positive human breast cancer cell (MCF-7) proliferation in vitro and (MCF-7) tumor growth in vivo (Hsieh et al. 1998). The effect of administering genistein to humans, particularly those susceptible to estrogen-dependent cancers, is unknown.

LITERATURE CITED


Barnes, S., Grubbs, C., Setchell, K.D.R. & Carlson, J. (1990) Soybeans inhibit breast cancer cell (MCF-7) proliferation in vitro and (MCF-7) has been shown to induce estrogen receptor positive human breast cancer cell (MCF-7) proliferation in vitro and (MCF-7) tumor growth in vivo (Hsieh et al. 1998). The effect of administering genistein to humans, particularly those susceptible to estrogen-dependent cancers, is unknown.


