

Antitumor and antiangiogenic activity of soy isoflavone genistein in mouse models of melanoma and breast cancer

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Abstract. Tumor invasion, angiogenesis and metastasis involve secretion of proteolytic enzymes and cell migration into blood vessels. Tumor cells are capable of degrading the extracellular matrix via a proteolytic cascade that includes urokinase-type plasminogen activator (uPA) and matrix metalloproteases (MMPs). We have investigated the antitumor and antiangiogenic properties of soy isoflavone genistein in B16 melanoma and F3II mammary carcinoma mouse models. At non-cytotoxic concentrations (0.1-50 μ M) genistein induced dose-dependent spindle-cell morphology and significantly reduced motility in both cell lines. Genistein inhibited uPA secreted by F3II cell monolayers, while inducing an increase in the proteolytic activity of B16 cells. On the contrary, the compound did not modify the MMP-9 and -2 produced by tumor cells. *In vivo*, i.p. administration of genistein at a dose of 10 mg/kg/day reduced tumor-induced angiogenesis in syngeneic mice implanted with B16 or F3II cells. Similar antiangiogenic effects were obtained with a soybean-based diet. This data suggest that tumor cell migration and proteolysis may be associated with the antitumor and antiangiogenic activity of soy isoflavone genistein.

Introduction

Tumor angiogenesis plays a pivotal role in the complex, multi-step nature of cancer growth and spread. Angiogenesis is intimately involved in metastasis at the site of entry of tumor cells into the vasculature, as well as at the site of eventual metastasis (1,2). In this regard, a relationship between tumor cell invasion and tumor-induced angiogenesis was described, with cooperative functions of both processes during tissue breakdown and cell migration (3).

Degradation of tissue barriers during tumor invasion is associated with proteolytic activity, mediated by tumor cells and also the surrounding host cells (4-6). Cancer cells are capable of digesting the extracellular matrix (ECM) via a proteolytic cascade that includes the urokinase-type plasminogen activator (uPA) and the matrix metalloproteases (MMPs). uPA is a serine protease that activates plasmin, a broad-specificity protease which degrades ECM components such as fibronectin and laminin. In addition, uPA can trigger activation of MMPs, which are responsible for collagen degradation. Increased pericellular proteolysis facilitates cell migration, and may promote the release of angiogenic and growth factors from the ECM (5).

Genistein (4',5,7-trihydroxyisoflavone) occurs in the plant family Leguminosae, including soybean (*Glycine max*). Epidemiologic evidence suggests that soybean-based diets, and particularly genistein, may reduce the risk of tumor formation. Other evidence is based on the molecular and cellular mechanisms of action of genistein on both normal and cancer cells (7). Hewitt and Singletary have studied the effect of genistein alone or as a part of a commercial soy extract on the growth of the highly metastatic mouse mammary carcinoma model F3II (8). Genistein significantly inhibited F3II cell proliferation *in vitro* at micromolar concentrations, and caused G2/M arrest associated with increased expression of phosphorylated p34 and cyclin B1. *In vivo*, diets supplemented with either a soy extract or genistein induced a significant reduction of F3II subcutaneous tumors. Similarly, genistein blocked G1/S transition in B16 melanoma cells and induced the cyclin-dependent kinase-2 inhibitor p21 (9). The isoflavone also reduced subcutaneous growth of B16 tumors (10), and exerted antiangiogenic effects in combination with cyclophosphamide treatment in the same model (11).

In the present study, we have investigated the antitumor properties of genistein in F3II mammary carcinoma and B16 melanoma cell cultures, with particular interest in the effects on cell motility and protease secretion. *In vivo*, antitumor activity was explored using a tumor-induced angiogenesis assay in mice implanted with tumor cells and treated with genistein or fed with a soybean-based diet.

Materials and methods

Compound. Genistein was purchased from Sigma (St. Louis, MO). For cell culture experiments, genistein was dissolved in dimethyl sulfoxide (DMSO) and then diluted into culture

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medium at a final concentration of 0.5% DMSO, a level which had no effect on tumor cells. For *in vivo* studies genistein was sonicated into suspension in 0.9% saline prior to injection. The dose administered *in vivo* was selected based upon previous preclinical and clinical studies (12,13).

Tumor cells and culture conditions. We used the sarcomatoid mammary carcinoma cell line F3II, established from a clone of a spontaneous, hormone-independent Balb/c mouse mammary tumor (14) and the parental B16F0 melanoma cell line, syngeneic for the C57BL/6 mouse strain. Stock F3II and B16 cells were maintained in monolayer culture in minimal essential medium (MEM) and Dulbecco's modified Eagle's medium (DMEM) from Gibco (Grand Island, NY), respectively, supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM glutamine and 80 μ g/ml gentamicin. For harvesting, cells were trypsinized using standard procedures and incubated in serum-free culture medium for 1 h at 37°C for recovery. Quantification of cell number was made by hemacytometer counting. In all cases, viability was >90%, as assayed by trypan blue exclusion test.

In vitro growth and cytotoxicity assays. The effect of genistein on the *in vitro* growth of F3II and B16 cells was determined by colorimetric assay. Tumor cells suspensions were seeded on 96-well plates at a density of 200 cells/well in culture medium plus 5% FBS. After 24 h, a range of concentrations from 0.5 to 100 μ M of genistein was added and incubation continued for 96 h. Plates were fixed with 10% formalin, stained with toluidine blue and the cellular mass was estimated from the absorbance at 595 nm. In addition, to assess the cytotoxicity of genistein against slowly-growing tumor cells, semi-confluent F3II and B16 monolayers were exposed to genistein for 24 h. Cytotoxicity was evaluated using a commercial kit from Promega (Madison, WI).

Tumor cell morphology. To examine the effects of genistein on cell morphology, F3II and B16 monolayers were cultured in 6-well plates for 24 h in the presence of genistein and photographed in a phase contrast microscope at x200 magnification (Olympus, Tokyo, Japan).

Adhesion assay. Semi-confluent F3II and B16 monolayers were cultured for 24 h in the presence or absence of genistein. Control and pretreated tumor cell suspensions were prepared from these monolayers and seeded at 4×10^4 cells/well in culture medium plus 5% FBS on wells precoated with the ECM protein fibronectin (2 μ g/cm²). After 60-min incubation at 37°C, wells were washed with PBS and adherent cells were fixed with methanol, stained with crystal violet, solubilized in acetic acid-methanol (10-5%) and quantified at 595 nm.

Migration assay. Tumor cell migration was measured by an *in vitro* wound assay (15). Confluent F3II and B16 monolayers were wounded by scratching 0.5-mm lines with a plastic tip. After washing with PBS, tumor cells were incubated for 15 h in culture medium containing 10% FBS, with or without genistein, to allow the cells to migrate into the wound. Monolayers were fixed, stained with methylene blue and the number of cells that had advanced into the cell-free space was counted with a 0.36-mm² reticule at x100 magnification.

Preparation of conditioned media. Secreted tumor-derived proteases were investigated in conditioned media, as previously described (16). Semi-confluent F3II and B16F0 monolayers were extensively washed in PBS to eliminate serum traces and incubated in serum-free culture medium for 24 h with the appropriate concentrations of genistein. Samples were individually harvested and stored at -20°C until assayed. The remaining monolayers were used to estimate the cell number.

Casein zymography and radial caseinolytic assay. SDS-polyacrylamide gels were performed using 4% stacking and 7.5% separating gels. The separating gel was copolymerized with 12 μ g/ml purified plasminogen (Chromogenix, Mölndal, Sweden) and 5 mg/ml non-fat dried milk as casein source. After electrophoresis, gels were washed with 2% Triton X-100 and incubated at 37°C for 72 h in 20 mM Tris buffer (pH 8.3) containing 15 mM EDTA. Upon staining with Coomassie blue and destaining, the final gel had a uniform blue background except in those regions to which plasminogen activators had migrated and activated plasmin degradation. Molecular weights were determined by pre-stained standards (Bio-Rad, Hercules, CA). Plasminogen-free gels were used to test plasminogen-independent protease activity. A complementary radial caseinolytic assay using plasminogen-rich casein-agarose plates was employed to quantify uPA and tissue-type plasminogen activator (tPA) activities contained in conditioned media, as described (17). To assess tPA-dependent activity, amiloride was added to the substrate at a concentration of 0.2 mM. This drug selectively blocks uPA activity, having no inhibitory activity against tPA or plasmin (18).

Gelatin zymography. MMP activity secreted by F3II and B16F0 cells was determined in SDS-polyacrylamide gels copolymerized with gelatin (1 mg/ml), using 4% stacking and 7.5% separating gels. After washing, gels were incubated at 37°C for 72 h in 0.2 M NaCl, 5 mM CaCl₂ and 50 mM Tris-HCl buffer (pH 7.4) for MMP detection or in the same buffer containing 15 mM EDTA to detect metal-independent protease activity. Gels were stained with Coomassie blue, and gelatinolytic enzymes were detected as gelatin degraded bands in a dark background.

Preparation of soybean-based diet. We used soy grains from the 'Don Mario' 4800RR variety. An amount of 500 g of grains was soaked overnight with 1350 ml of tap water to obtain a paste by liquefaction. The paste was microwaved for 1 min to inactivate trypsin inhibitors, manipulated to generate a soybean-based rodent chow and then dried at 40°C for 48 h. A standard rodent chow with regular fat content was used as a control.

In vivo assays. Specific pathogen-free female Balb/c and C57BL/6 inbred mice from UNLP (Buenos Aires, Argentina), with an age of 6-8 weeks and an average weight of 20-25 g, were used. They were housed in plastic cages under standard conditions and had access to rodent chow and water *ad libitum*. On the designated day 0 of the experiment, F3II or B16F0 cells, at concentrations of 2×10^5 or 1×10^5 viable cells, respectively, were injected intradermally in the flanks of unanesthetized mice. At day 5, animals were sacrificed after anesthesia with sulfuric ether. The sites of tumor implantation were photo-

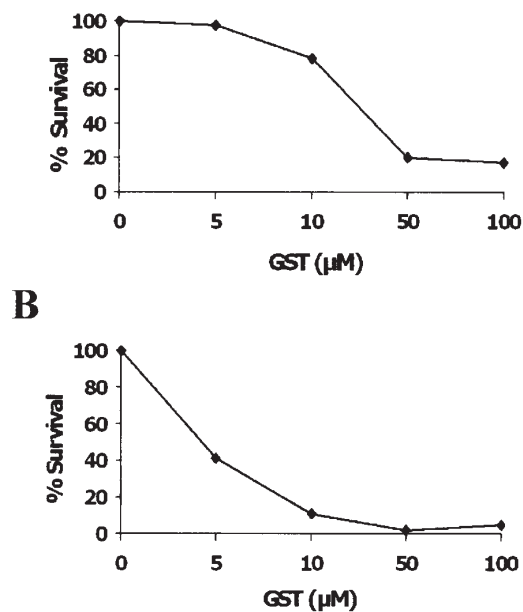


Figure 1. Effect of genistein on the growth of (A) F3II mammary tumor cells and (B) B16F0 melanoma cells. Survival relative to untreated control cells was determined by a colorimetric assay after incubation for 96 h with genistein (0.5-100 μM). Results are representative of three independent experiments. In all cases, standard errors of means average <10%.

graphed and the vascularization was quantified as reported previously (19). To study the effect of genistein on tumor implants mice were injected i.p. at daily doses of 15 mg/kg body weight from days 1-5 or received a soybean-based diet. Animals were monitored for food and water consumption, weight, and general behavioral status.

Results

Effects of genistein on *in vitro* growth. The IC₅₀ value for genistein, evaluated by a 96-h exposure to the compound, was 26.3 and 6.6 μM for F3II and B16F0 cells, respectively (Fig. 1). However, genistein had no cytotoxic effects when tumor cells were treated at a dose of up to 100 μM for 24 h followed by incubation for 3 days without the compound, as indicated by approximately 90% survival at these doses (data not shown). Similarly, this range of concentrations (1-100 μM) had no cytotoxic effects when assayed for 24 h on semi-confluent, slowly-growing F3II and B16F0 monolayers. Since further *in vitro* experiments were all performed treating tumor cells for up to 24 h, these concentrations of genistein were employed to achieve maximum levels of drug action consistent with an absence of cytotoxicity.

Effects of genistein on tumor cell morphology, adhesion and motility. At non-cytotoxic concentrations, genistein induced a

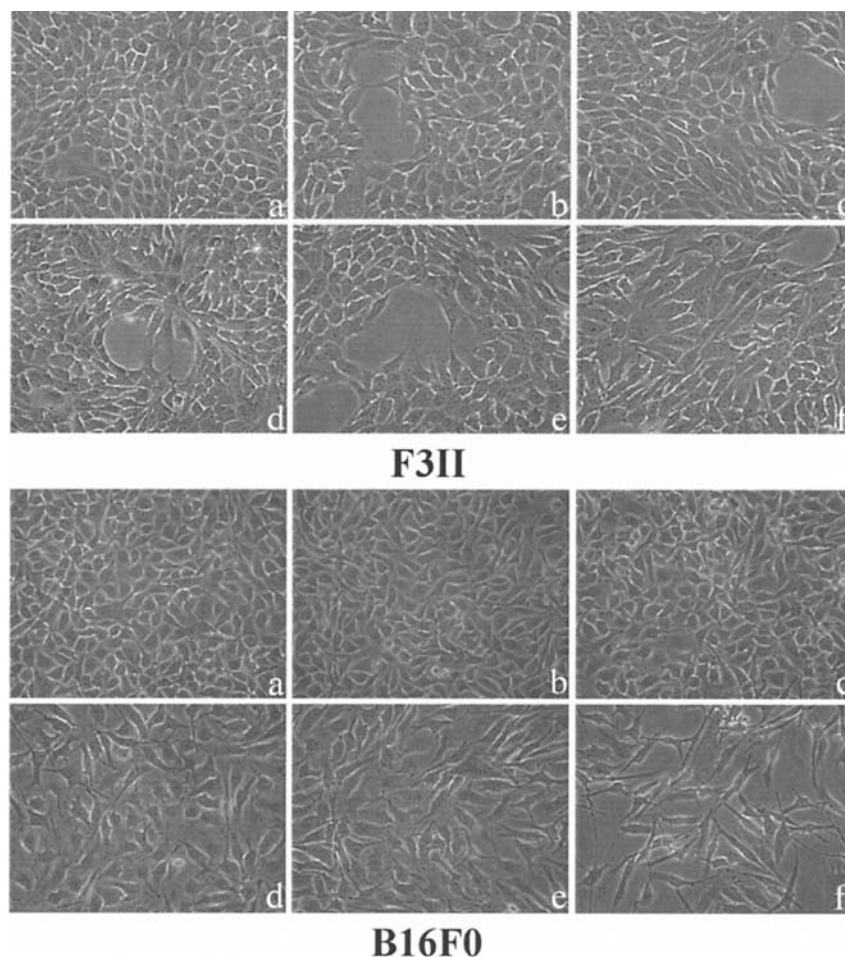


Figure 2. Effect of genistein on F3II and B16F0 tumor cell morphology. Monolayers were cultured for 24 h with or without genistein and photographed in a phase contrast microscope at x200 magnification. (a) Control, (b) 0.5 μM genistein, (c) 1 μM genistein, (d) 10 μM genistein, (e) 20 μM genistein, (f) 50 μM genistein.

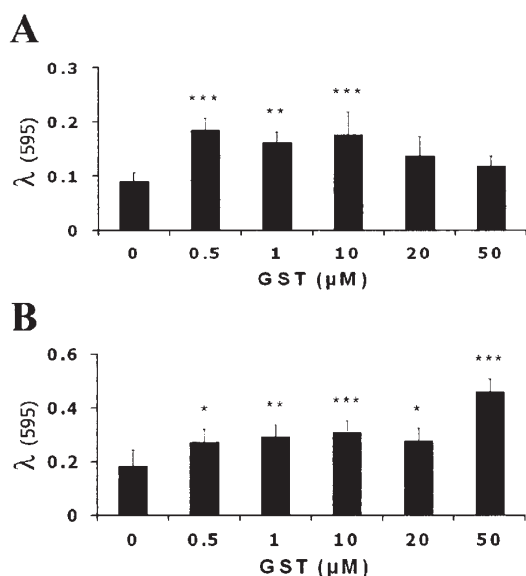


Figure 3. Effect of genistein on F3II and B16F0 cell adhesion. Tumor cells were pretreated in monolayer culture with different doses of genistein for 24 h, harvested and seeded on wells precoated with fibronectin ($2 \mu\text{g}/\text{cm}^2$) in the presence of serum. GST, genistein. Data are expressed as mean \pm SD of triplicate determinations, and are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control (ANOVA test).

dose-dependent spindle-cell morphology on both cell lines (Fig. 2). The adhesion rate of F3II and B16F0 cells to uncoated plastic surfaces was time-dependent and the plateau was reached after 30-60 min. Similar results were obtained using wells precoated with fibronectin. Pretreatment with genistein at concentrations from 0.5 to $50 \mu\text{M}$ increased the adhesion of F3II and B16F0 tumor cells (Fig. 3). However, the presence of genistein during the adhesion assay, but without pretreatment of tumor cells, did not reduce tumor cell attachment (data not shown). Genistein significantly reduced tumor cell migration, in doses from 1 to $50 \mu\text{M}$ for F3II cells and from 20 to $50 \mu\text{M}$ for B16F0 cells (Fig. 4).

Effects of genistein on tumor-derived proteolytic activity. Zymographic analysis of plasminogen activators contained in conditioned media revealed a main band of 45 kDa and a less prominent band of 67 kDa. These forms were identified as uPA and tPA, respectively, by preincubation with anti-catalytic antibodies and Western blotting, as reported previously (19). Genistein was able to disrupt the secretion of plasminogen activators by F3II and B16 cells. Genistein decreased uPA secreted by F3II cells in doses ranging between 10 and $50 \mu\text{M}$ (Fig. 5A). Likewise at 0.5-1 μM the drug increased tPA, an enzyme related with good prognosis in mammary tumors. On the contrary, genistein induced an increase on uPA and tPA secretion in B16 cells (Fig. 5B). These results were confirmed using a radial caseinolytic assay (data not shown). F3II and B16F0 cultures secreted a main metalloproteinase activity of 105 kDa and a minor band of 70 kDa, corresponding to MMP-9 and -2, respectively. Genistein did not modify this gelatinolytic pattern (data not shown).

In vivo effects of genistein and soybean-based diet on tumor-induced angiogenesis. Since adhesion, proteolysis and

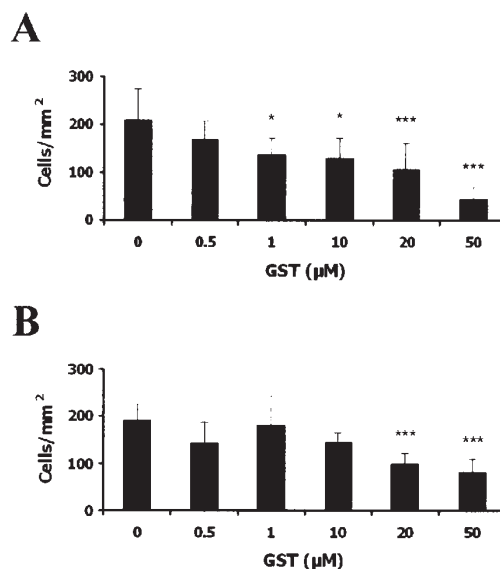


Figure 4. Effects of genistein on F3II and B16F0 cell migration. The *in vitro* wound assay was performed as described in Materials and methods. (A) F3II, (B) B16F0 cells. GST, genistein. Value represents means \pm SD of at least 12 determinations from three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control (ANOVA test).

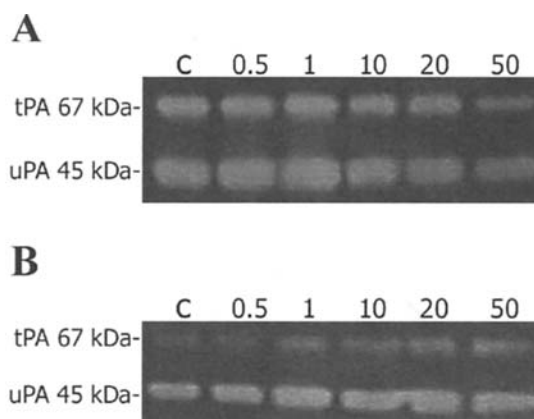


Figure 5. Zymographic analysis of plasminogen activators secreted by tumor cell monolayers. (A) F3II, (B) B16F0 cells. C, control; 0.5, 1, 10, 20 and $50 \mu\text{M}$ concentrations of genistein.

migration are steps strongly involved in tumor progression, we studied the capacity of genistein (10 mg/kg/day) to inhibit the generation of new blood vessels by tumor cell implants *in vivo*. Likewise, we evaluated the same effect with the administration of a soybean-based diet. Both treatments were able to reduce the number of blood vessels induced by tumor implants of F3II mammary carcinoma and B16F0 melanoma cells in syngeneic mice (Fig. 6). Treatments were not associated with over-toxic effects.

Discussion

The present studies provide evidence that daily *in vivo* administration of genistein reduced tumor-induced angiogenesis in syngeneic mice implanted with B16 or F3II cells.

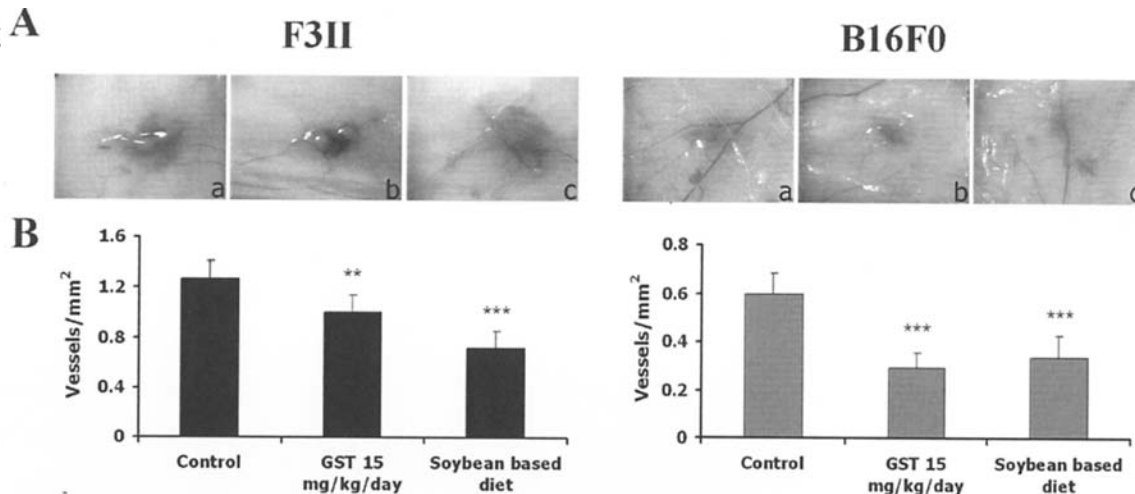


Figure 6. Effect of genistein on tumor-induced angiogenesis *in vivo*. Mice were injected i.p. with daily doses of genistein or received a soybean-based diet. (A) Representative images of data in B (a, control mice; b, genistein treatment; c, soybean-based diet). (B) Quantification of vessels around tumor implants, as described in Materials and methods. GST, genistein. Values represent means \pm SD of at least 8 determinations. ** $p < 0.01$, *** $p < 0.001$ versus control (ANOVA test).

Similar antiangiogenic effects were obtained with a soybean-based diet and are consistent with numerous other studies that have reported this inhibitory effect toward different tumor types (20). Our findings also provide additional experimental support suggesting that cell migration and proteolysis may be associated with the antitumor and antiangiogenic activity of genistein.

Genistein has been shown to inhibit the development of both hormone-related and non-hormone-related cancers, including mouse models of breast, prostate, and skin cancer. Several mechanisms have been proposed for this effect. Genistein and other isoflavones have demonstrated weak estrogenic activity at lower concentrations but are estrogen receptor antagonists at higher concentrations. At 40 μ M, genistein inhibited proliferation of LNCaP prostate cancer cells and enhanced apoptosis. Treatment of human bladder cancer cells with genistein resulted in inhibition of cdc2 kinase activity and G₂/M phase cell cycle arrest (21). Genistein has been shown to inhibit 20S proteasomal activity in LNCaP and MCF7 cells, resulting in accumulation of p27^{Kip-1}, I κ B and Bax (22). Genistein has also been shown to inhibit tyrosine kinase and topoisomerase activity (23). The relative importance of each of these mechanisms remains to be determined *in vivo*.

Previous reports also indicated that genistein exhibits strong antiangiogenic activity. The precise underlying mechanism of inhibition, however, remains unclear. We found that at non-cytotoxic concentrations genistein induced a dose-dependent spindle-cell morphology in both cell lines. In parallel, we observed that F3II and B16 tumor cells exposed to genistein (0.5–50 μ M) increased the adhesion to coated or uncoated plastic surfaces. Bergan *et al* (24) carried out a morphogenic analysis revealing that genistein caused cell flattening in a variety of cell lines: PC3-M, PC3 and DU-145 prostate carcinoma cells, as well as MCF-7 breast carcinoma cells. Mechanistic studies focused on the highly metastatic PC3-M cell line, revealed that cell flattening was accompanied by an increase in cell adhesion. The authors suggest that

binding of focal adhesion kinase to β 1-integrin may be responsible for this biological behavior.

Proteolytic profiles were modified by genistein. In F3II cells genistein (10–50 μ M) decreased uPA and increased tPA. Interestingly, in B16 cells both uPA and tPA levels were increased. A decrease in uPA after genistein treatment was also reported by Valachovicova in breast cancer cells (25). The same results were found in prostatic cancer cells (26), in neuroblastoma cell lines (27) and endothelial cells (28). We report for the first time that genistein induced an increase in tPA, an enzyme related with good prognosis in mammary tumors, in both cell types. Furthermore, this is the first time where the effect of genistein on plasminogen activators is analyzed in a melanoma model.

No modification in the secretion pattern of metalloproteases was observed at non-cytotoxic doses tested. The same results were found by Sugiura *et al* in MDA-MB-231 (29). On the contrary, Kousidou *et al* showed that the addition of genistein resulted in down-regulation of the transcription of all MMP genes in MDA-MB-231 and most MMPs in MCF-7 cells (30).

Using an *in vitro* wound assay, we were able to assess the effects of genistein on tumor cell migration. Genistein significantly decreased F3II cell migration in doses ranging from 1 to 50 μ M for F3II, and from 20 to 50 μ M for B16 cells. Accordingly, Valachovicova *et al* demonstrated that genistein suppresses cell adhesion and migration by inhibiting the constitutively active transcription factors NF- κ B and AP-1, resulting in the suppression of secretion of uPA in breast cancer cells MDA-MB-231 (25).

Cooperative functions of tumor invasion and tumor-induced angiogenesis were described during cell adhesion, proteolysis and cell migration (3). There are remarkable similarities in the molecular mechanism which enables tumor cells to invade into surrounding tissues and that which activates endothelial cells to generate new capillaries, which facilitate the growth and dissemination of cancer (31). In our experiments, both genistein and a soybean-based diet were able to reduce tumor-

induced angiogenesis in breast and melanoma models in syngeneic mice. The same results using genistein *in vivo* were found by Wietrzyk *et al* in melanoma (11) and Shao *et al* in breast cancer (32). Among others, down-regulation of the vascular endothelium growth factor could be the main mechanism involved in the antiangiogenic effect of genistein (7). Hewitt and Singletary (8) showed an enhanced suppression of *in vivo* F3II cell invasiveness for animals fed a 0.6% soy extract-supplemented diet, suggesting that the inhibition of invasiveness by diets containing a mixture of soy constituents warrants further evaluation.

In addition, Connolly *et al* (33) demonstrated that dietary soy at 10% and 20% (w/w) significantly reduced the lung invasiveness of MDA-MB-435 human breast cancer cells.

In 1991 Lee *et al* (34) reported that Asian women who consumed a traditional diet (high in soy products) had a low incidence of breast cancer. Yuan *et al* (35), however, reported no protection against breast cancer from soy consumption. In a third investigation, Wu *et al* (36) reported a correlation between tofu intake and a reduced rate of mammary cancer in a population-based case-control study of breast cancer among Chinese American, Japanese American, and Filipino American women.

There is a strong body of evidence relating soy consumption with a lower cancer incidence in humans. However, recent contrasting data have been reported on the potential of phytoestrogens to prevent hormone-dependent cancers (37). Our studies clearly demonstrated that tumor cell migration and proteolysis may be associated with the antitumor and anti-angiogenic activity of soy isoflavone genistein. Its usefulness in humans should be studied carefully, and significant matters such as bioavailability and drug concentration should be clearly analyzed.

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