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Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis

(diet/endothelial cells/invasion/plasminogen activator/plasminogen-activator inhibitor)

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ABSTRACT **Consumption of a plant-based diet can prevent the development and progression of chronic diseases that are associated with extensive neovascularization; however, little is known about the mechanisms. To determine whether prevention might be associated with dietary-derived angiogenesis inhibitors, we have fractionated urine of healthy human subjects consuming a plant-based diet and examined the fractions for their abilities to inhibit the proliferation of vascular endothelial cells. Using gas chromatography-mass spectrometry, we showed that one of the most potent fractions contained several isoflavonoids, which we subsequently synthesized. Of all synthetic compounds, the isoflavonoid genistein was the most potent and inhibited endothelial cell proliferation and *in vitro* angiogenesis at concentrations giving half-maximal inhibition of 5 and 150 μM, respectively. As we have previously demonstrated, genistein concentrations in urine of subjects consuming a plant-based diet are in the micromolar range, while those of subjects consuming a traditional Western diet are lower by a factor of >30. The high excretion of genistein in urine of vegetarians and our present results suggest that genistein may contribute to the preventive effect of a plant-based diet on chronic diseases, including solid tumors, by inhibiting neovascularization. Thus, genistein may represent a member of a new class of dietary-derived anti-angiogenic compounds.**

Angiogenesis, the generation of new capillaries, is virtually absent in the healthy adult organism and is restricted to a few conditions including wound healing and the formation of corpus luteum, endometrium, and placenta. These conditions of normal angiogenesis represent ordered, tightly regulated, and self-limited processes (1) and are probably the result of a well-balanced activity of angiogenesis inhibitors and stimulators. Otherwise, angiogenesis is virtually nonexistent, and of all vascular endothelial cells present in the organism, <0.1 percent proliferate (2).

In contrast, in certain pathological conditions, angiogenesis is dramatically enhanced and is no longer self-limited: 10% or more of the vascular endothelial cells in affected tissues do actively proliferate (2). Pathological angiogenesis is seen during the development and progression of many diseases, for example in rheumatoid arthritis, psoriasis, retrolental fibroplasia, diabetic retinopathy, and hemangiomas and during the rejection of organ transplants. Probably the most clinically important manifestation of pathological angiogenesis is that induced by solid tumors (3). Well-vascularized tumors expand both locally and by metastasis, while avascular tumors do not grow beyond a diameter of 1 to 2 mm (1, 4). Though the mechanisms leading to pathological angiogenesis are still unclear, recent evidence indicates that it is the result of an imbalance between angiogenic factors (5) and inhibitors (6–8), with the activity of angiogenic factors being too high and/or that of the inhibitors being reduced or lost (9).

That pathological angiogenesis could be also modulated by factors of exogenous origin, for example from diet, appeared to us an important possibility meriting further investigation. Indeed, dietary factors contribute to about a third of potentially preventable cancers (10). If dietary compounds were to inhibit angiogenesis, this could explain, at least in part, the long-known preventive effect of plant-based diets on tumorigenesis and other chronic diseases like inflammation (11). To examine this possibility, we screened urine of human subjects consuming a diet rich in plant products for the presence of anti-angiogenic compounds, using a purification procedure developed by us previously to separate lipophilic compounds of steroid- or steroid-like structure (12–14).

MATERIALS AND METHODS

Materials. Unless otherwise indicated, dishes and medium used for tissue culture (15), human recombinant basic fibroblast growth factor (bFGF) (15), and chemicals and reagents used for urine purification (12, 16) were from sources mentioned earlier. Human recombinant bFGF used in the *in vitro* angiogenesis assay was provided by P. Sarmientos (Farmitalia Carlo Erba, Milan). Genistein, daidzein, equol, and enterolactone were synthesized as described (14, 17) and, because of their limited solubility in aqueous solvent, were dissolved in 100 mM sodium carbonate. When stored at 4°C, stock solutions of the substances remained bioactive for more than 1 month.

Urine Purification. Twenty-four-hour urine samples from individuals consuming a soy-rich vegetarian diet were collected in bottles containing ascorbic acid (1–2 g/liter). Sodium azide was added to a final concentration of 0.1%, and aliquots were stored at −20°C. Urine purification (Fig. 1*A*) was carried out essentially as described (12, 13) with minor modifications (14).

Gas Chromatography/Mass Spectrometry (GC/MS). GC/MS analysis of the trimethylsilyl ether derivatives of the compounds was carried out with a Hewlett–Packard 5995B quadrupole instrument equipped with a 0.2 mm × 12.5 m bonded-phase BP 1 capillary vitreous-silica column and with helium as a carrier gas. Temperatures of the transfer line, ion source, and analyzer were 310°C, 250°C, and 250°C, respectively. The ionization energy was 70 eV. Temperature pro-

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Abbreviations: bFGF, basic fibroblast growth factor; PA, plasminogen activator; PAI, PA inhibitor; BBCE, bovine brain-derived capillary endothelial cells; BME, bovine microvascular endothelial cells.

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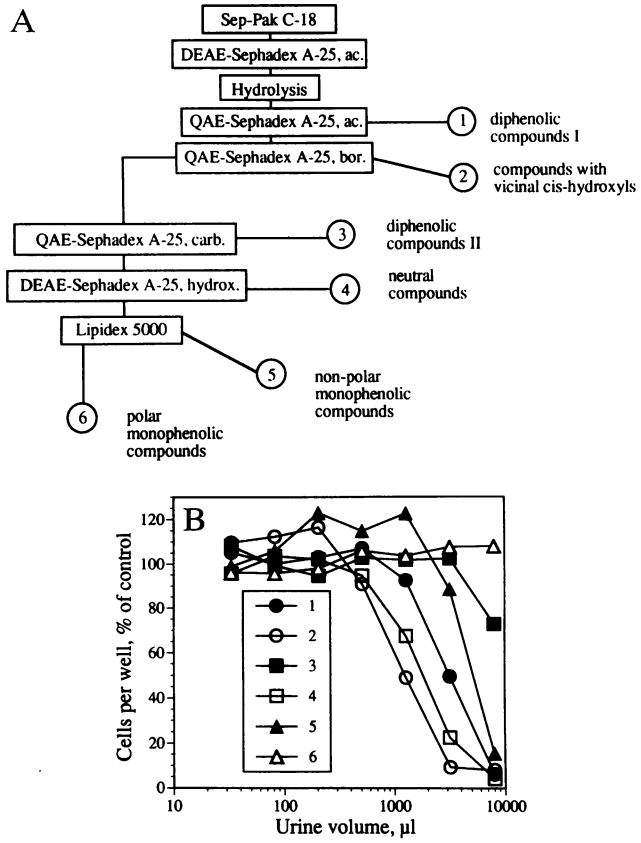

FIG. 1. Purification of human urine and the effect of various semipure urine preparations on the proliferation of vascular endothelial cells. (*A*) Urine was purified as described in *Materials and Methods*. The flow diagram shows the chromatographic steps, the fractions obtained (1–6), and the compound classes contained in each fraction. (*B*) BBCE cells were seeded at a density of 5000 cells per ml into each well and received every other day 10-μl aliquots containing bFGF (2.5 ng) plus 10 μl aliquots containing various concentrations of the purified urine fractions (1–6) diluted in buffer or buffer only. The urine volume to which each dilution corresponds is indicated. Cells were counted after 5 days. Values are expressed as percent of controls—i.e., cells receiving bFGF and buffer only (≈100,000 cells per well).

grams, internal standard, and ion-monitoring were as described (12, 14).

Cell Culture. Endothelial cells derived from bovine brain capillaries (BBCE), bovine adrenal cortex, bovine aorta, and from human umbilical vein were provided by D. Gospodarowicz (University of California, San Francisco) and maintained as described (18, 19). These cell culture stocks received bFGF (2.5 ng/ml) every other day until confluent.

Cloned bovine microvascular endothelial (BME) cells from adrenal cortex (20) were provided by M. B. Furie and S. C. Silverstein (Columbia University, New York) and maintained as described (21).

Cell Proliferation Assay. Stock cultures were trypsinized (19), and cells were adjusted to a density of 5000 per ml (or 20,000 per ml in the case of human umbilical vein endothelial cells) in their respective medium and were seeded in 1-ml aliquots per well into 12-well cluster dishes. After 16 h, wells received every other day either 10-μl aliquots of buffer only or buffer containing various concentrations of samples to be tested or bFGF (2.5 ng/ml) or both. Cells were counted at the times indicated with a Coulter Counter (19). Unless otherwise indicated, values of cell densities represent the means of duplicate determinations, which varied by <10% of the mean.

***In Vitro* Angiogenesis Assay.** Three-dimensional collagen gels were prepared in 18-mm tissue culture wells as described (21). BME cells (5×10^4 in a volume of 0.5 ml) were seeded into each well, and medium was changed every 2–3 days until the cells were confluent. Recombinant human bFGF (30 ng/ml) or various concentrations of genistein were then added; when the effect of genistein on bFGF-induced invasion was tested, genistein was added 2 h prior to bFGF. After 2 days of incubation, medium was changed and cells were exposed to the same conditions again. Cultures were photographed after another 3 days. Invasion was quantified from three randomly selected fields per well measuring 1 × 1.4 mm, each at a single level beneath the surface monolayer. Quantification was done by measuring the total length of all cells that penetrated the underlying gel either as single cells or in the form of cell cords.

Zymographic and Reverse Zymographic Assays for Plasminogen Activator (PA) and PA Inhibitor 1 (PAI-1). BME cells (2×10^5 per dish) were seeded into gelatinized 35-mm culture dishes containing 1.5 ml of medium and were grown to confluence. Medium was changed every 2–3 days and 24 h before adding the compounds to be tested. Confluent cultures received bFGF (30 ng/ml) with or without genistein (200 μM) and were incubated for 15 h at 37°C. The cultures were then washed three times with phosphate-buffered saline, and 1.5 ml of serum-free medium containing Trasylol (200 kallikrein units per ml) and compounds to be tested were added. After a further 3 h of incubation, cell extracts were prepared and subjected to zymographic and reverse-zymographic analyses as described (22); these involve the detection of plasminogen-dependent caseinolytic and inhibitory activities in electrophoresed samples by placing the electrophoresis gel onto an agar underlay containing plasminogen and casein (and urokinase for reverse zymography). A second series of dishes treated in the same manner in parallel was used to determine cell numbers for normalization of samples prior to electrophoresis. Zymograms and reverse zymograms were photographed under dark-field illumination after incubation at 37°C (22).

RESULTS

Human Urine Contains Inhibitors of Endothelial Cell Proliferation. Fig. 1*A* outlines the various fractions (1–6) obtained from the purification of urine and the compound classes present therein. All fractions were tested for their effect on the basal or bFGF-stimulated proliferation of BBCE cells. Fractions 1, 2, 4, and 5 were able to inhibit the bFGF-stimulated proliferation (Fig. 1*B*) and to a weaker extent basal proliferation of BBCE cells (not shown). Fractions 2 and 4 were most potent in inhibiting BBCE cell proliferation but were not further examined at this stage because (*i*) fraction 4 contains neutral steroids (12), which are known angiogenesis inhibitors (23), and (*ii*) substances present in fraction 2 are being examined in a separate study (T.F., H.A., and L.S., unpublished data).

Instead, we decided to further investigate the diphenolic compounds present in fractions 1 and 3. Initial identification by GC/MS was carried out by comparing the mass spectra of the different peaks with those of reference mass spectra collections. Synthetic standards of potential candidate compounds were then prepared, and definite identification was established by comparing their mass spectra with those of the unknown compounds. In this way, we were able to demonstrate the presence of the isoflavonoids genistein, daidzein, and O-desmethylangolensin in fraction 1 (Fig. 2*A*) and the lignans enterodiol, enterolactone, and matairesinol as well as the isoflavonoid equol in fraction 3 (Fig. 2*B*).

Synthetic Genistein Inhibits bFGF-Stimulated Endothelial Cell Proliferation. The synthetic analogs of the identified

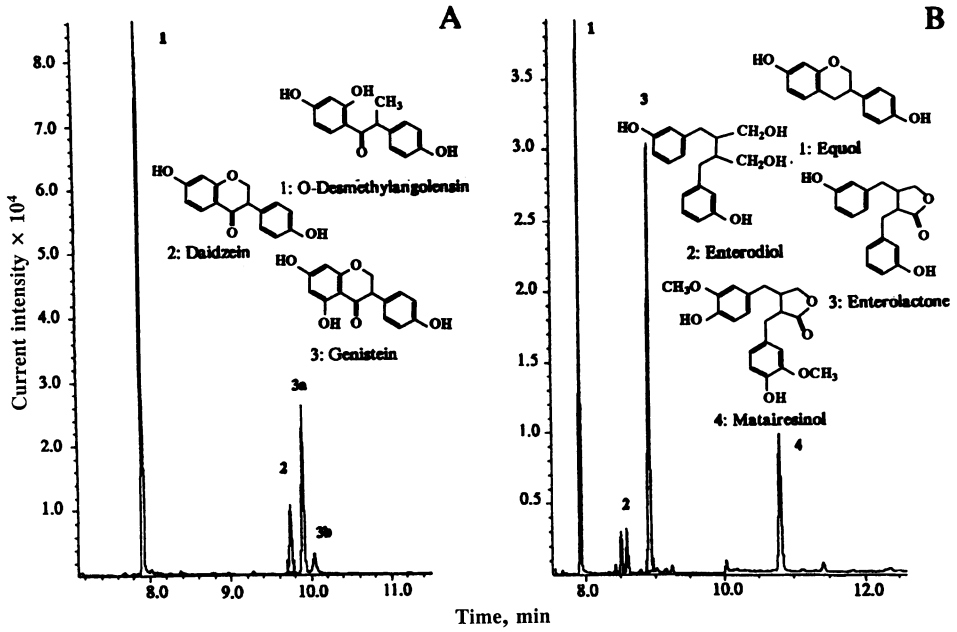

FIG. 2. Identification of compounds present in human urine preparations by GC/MS. Total ion-current chromatogram of fractions 1 (*A*) and 3 (*B*) shows the structures of the compounds identified therein. The total ion current was reconstructed from the total sum of the two most characteristic mass fragments of each compound by using the selected ion-monitoring technique. Genistein forms a bistrimethyl ether and a trimethylsilyl ether derivative, both of which give two well-separated peaks (3a and 3b in *A*) during GC/MS analysis.

compounds were tested with regard to their effect on the bFGF-stimulated proliferation of BBCE cells. Genistein had a potent and dose-dependent inhibitory effect on BBCE cell proliferation with half-maximal and maximal effects at 5 and 50 μM concentrations, respectively. In contrast, the remaining isoflavonoids, some of which are closely related to genistein (Fig. 2), were considerably less potent (Fig. 3*A*). Genistein also inhibited the proliferation of vascular endothelial cells derived from bovine adrenal cortex or aorta and from human umbilical vein (Fig. 3*B*).

In low-density cultures of proliferating endothelial cells (Fig. 4*A*), genistein induced marked morphological changes: at concentrations up to 25 μM, genistein induced a highly spread cell morphology compatible with growth arrest (Fig. 4*B*); cell densities always exceeded those determined at seeding (data not shown), indicating that cell death was not involved. When exposed to genistein concentrations above 25 μM, the cells acquired an elongated shape (Fig. 4*C*) and eventually died (not shown). These results indicate that genistein is cytostatic up to concentrations of ≈25 μM and that it becomes cytotoxic above this level. Reversibility experiments, in which low-density cultures of endothelial cells were exposed to bFGF (2.5 ng/ml) and increasing concentrations of genistein and then were exposed to medium without genistein, confirmed this assumption (data not shown). The result was the same on uncoated and gelatin-coated substrata. In contrast, confluent endothelial cells did not exhibit toxicity signs even at genistein concentrations up to 200 μM (data not shown; Fig. 5*C*).

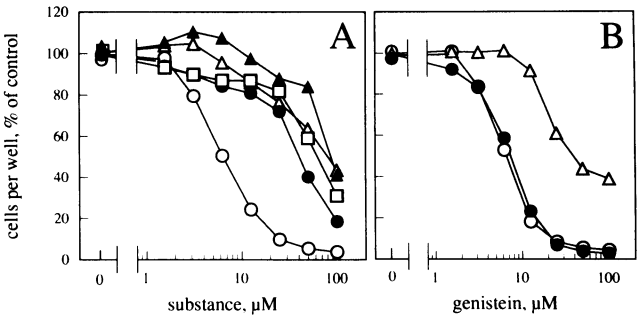

FIG. 3. Effect of increasing concentrations of synthetic isoflavonoids and lignans on the proliferation of bovine and human vascular endothelial cells. (*A*) BBCE cells were seeded at a density of 5000 cells per well and received every other day bFGF (2.5 ng/ml). In addition, they received every other day 10-μl aliquots of buffer only or of buffer containing the indicated concentrations of synthetic genistein (○), equol (●), *O*-desmethylangolensin (□), enterolactone (△), or daidzein (▲). (*B*) Endothelial cells derived from bovine adrenal cortex (○), bovine aorta (●), or human umbilical vein (△) were seeded at densities of 5000, 5000, or 20,000 cells per well, respectively, and received every other day 10-μl aliquots of bFGF (2.5 ng/ml) and of buffer with or without the indicated concentrations of genistein. Cells in *A* and *B* were counted after 5 days. Values are expressed as the percent of controls—i.e., cells receiving buffer only (usually in the range of 100,000 cells per well).

Genistein Inhibits Angiogenesis *in Vitro*. To examine whether genistein could inhibit angiogenesis, we used an experimental *in vitro* system that mimics angiogenesis *in vivo*. As shown (24), BME cells seeded on the surface of collagen gels (Fig. 5*A*) invade the gels when exposed to bFGF and form capillary-like tubes beneath the gel surface (Fig. 5*B*). Genistein alone at 200 μM concentrations had no effect on confluent BME cultures (Fig. 5*C*). However, when added together with bFGF, it inhibited their ability to invade the gels and generate capillary-like structures (Fig. 5*D*). A quantitative analysis (Fig. 5*E*) revealed that genistein inhibited bFGF-induced invasion of BME cells with a half-maximal effect at ≈150 μM concentration.

Genistein Inhibits Production of PA and PAI-1 in Vascular Endothelial Cells. We next set out to examine the mechanism by which genistein might inhibit angiogenesis. Normal an-

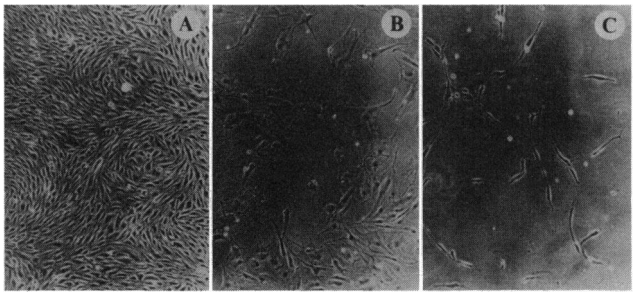

FIG. 4. Effect of various concentrations of genistein on the morphology of vascular endothelial cells. Bovine adrenal cortex endothelial cells were seeded at a density of 5000 cells per well and received every other day bFGF (2.5 ng/ml) and either only buffer (*A*) or buffer containing 12.5 μM (*B*) or 100 μM (*C*) genistein. Cells were photographed after 5 days. (×150.)

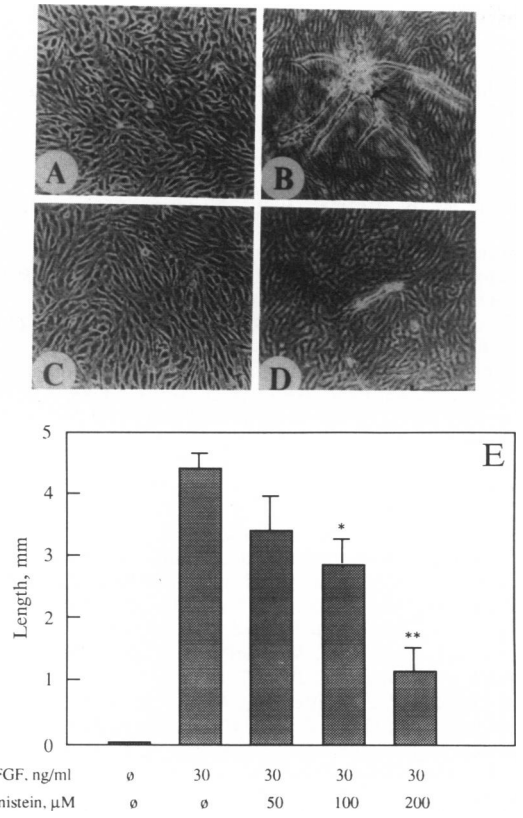

FIG. 5. Effect of genistein on *in vitro* angiogenesis. (*A–D*) BME cells were grown to confluence on three-dimensional collagen gels as described in text. After confluence, they received either no additions (*A*), bFGF (30 ng/ml) only (*B*), genistein (200 μM) only (*C*), or genistein (200 μM) 2 h prior to bFGF (30 ng/ml) (*D*). The additions were repeated as described under *Materials and Methods*, and representative pictures were taken after 5 days. In *B*, the arrow shows the lumen of a capillary-like tube; in *D*, genistein inhibits the formation of such tubes. (Bar = 150 μM.) (*E*) Quantitative analysis of the results demonstrated in *A–D*. Confluent cultures of BME cells were treated for 5 days as described in *A–D*. Fields measuring 1 × 1.4 mm were then randomly selected and photographed, and BME cell invasion was quantified as described in text. Addition of genistein 2 h prior to addition of bFGF significantly (*, $P < 0.025$; **, $P < 0.0001$) inhibited bFGF-induced invasion. Results are expressed as the mean length in mm + SEM per field from nine photographic fields—i.e., three fields from each of three separate experiments.

giogenesis appears to depend on tightly controlled proteolytic degradation of the extracellular matrix (25). For example, bFGF, an important and ubiquitous angiogenic factor, induces the production of urokinase-type PA (24) and its physiological inhibitor, PAI-1, in vascular endothelial cells (Fig. 6, lanes C and F; ref. 25). As shown in Fig. 6, genistein markedly reduced both bFGF-stimulated levels (lanes F and G) and basal levels (lanes G) of both PA (*A*) and PAI-1 (*B*) activity in BME cells.

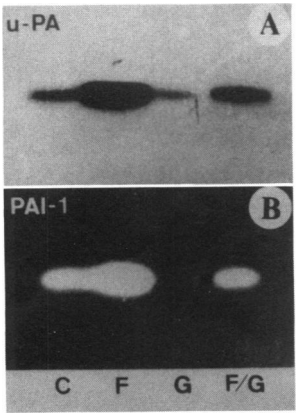

FIG. 6. Effect of genistein on the activity of PA and PAI-1 in vascular endothelial cells. Extracts of BME cells exposed to medium only (controls; lanes C) or bFGF (30 ng/ml) only (lanes F) or genistein (200 μM) only (lane G) or to bFGF (30 ng/ml) and genistein (200 μM) (lanes F/G) were subjected to zymographic analysis (*A*) or reverse zymographic analysis (*B*), which demonstrate the activity of urokinase-type PA (u-PA) (*A*) or of its inhibitor, PAI-1 (*B*).

DISCUSSION

We have demonstrated here that dietary ingested compounds present in human urine modulate the proliferation of endothelial cells *in vitro*. One of them, the isoflavonoid genistein, inhibited the growth of low-density cultures of proliferating endothelial cells with a half-maximal effect at 5 μM (Fig. 3), while it had no effect on confluent, quiescent cells irrespective of the substratum used (Fig. 5*C* and data not shown). This is consistent with the results obtained by the *in vitro* angiogenesis assay, in which confluent endothelial cell cultures on collagen gels were used. Genistein inhibited *in vitro* angiogenesis with a half-maximal effect at ≈150 μM concentration (Fig. 6*E*). The effect of genistein on endothelial cells is clearly cell density dependent, and this probably accounts for the difference in the half-maximal inhibitory concentrations of genistein in the endothelial cell proliferation assays and the *in vitro* angiogenesis model.

Genistein could inhibit basal or bFGF-stimulated endothelial cell proliferation and *in vitro* angiogenesis through attenuation of the activity of tyrosine kinases. Indeed, genistein has been shown to be a competitive inhibitor of ATP binding to the catalytic domain of tyrosine kinases (26) and was found to inhibit the tyrosine kinase activities of epidermal growth factor receptor (26) and platelet-derived growth factor receptor (27) both in intact cells and *in vitro*. This appears as an attractive hypothesis, since high-affinity FGF receptors are tyrosine kinases (28) and since vanadate, an inhibitor of phosphotyrosine phosphatases, induces angiogenesis in the collagen gel assay (29). Alternatively, genistein might elicit its action on endothelial cells by attenuating the activity of S6 kinase (30), an enzyme that is also activated by bFGF (31, 32). As a third possibility, genistein might exert its effects by modulating the activity of topoisomerases I and II (33, 34), enzymes that are involved in nuclear events like transcription, replication, recombination, and mitotic chromosome segregation. In the present paper, we have demonstrated that genistein decreases the activity of PA and PAI-1. Both molecules play a crucial role during angiogenesis, and their activities are modulated by bFGF and other angiogenic stimuli (25). Thus, genistein could inhibit angiogenesis by attenuating the activities of PAs and their inhibitors.

It appears that endothelial cells are not the only target of genistein. The mitogenic effect of epidermal growth factor on NIH 3T3 cells was inhibited by genistein with a half-maximal concentration of 12 μM (30), and we have reported that genistein inhibits the *in vitro* proliferation of a number of neuroblastoma, rhabdomyosarcoma, and Ewing's sarcoma cells lines with half-maximal concentrations of about 20–45 μM (35). Though endothelial cells are more sensitive (IC_{50} = 5 μM) to the inhibitory effect of genistein, it appears that genistein has a broader inhibitory effect on proliferating cells. Therefore, it may affect tumor progression both indirectly by inhibiting the tumor-induced angiogenesis and directly by modulating the growth of the tumor cells themselves.

It is possible that genistein has a physiological role in the organism. Genistein precursors are present in soy products (36), and genistein itself is present at a high concentration in urine of individuals consuming a traditional soy-rich Japanese diet (about 6 μmol/24 h) (37) but at a concentration ⅓₀th or less in urine of omnivores (0.18 μmol/24 h) (14). Therefore, it is tempting to speculate that genistein might contribute to the long-known preventive effect of plant-based diet on chronic neovascular diseases, including solid tumor growth. In this context it is interesting that Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the

original traditional Japanese diet have low breast cancer incidence and mortality (38). Also, men of Japanese origin in Hawaii have low mortality from prostate cancer, although the incidence of *in situ* prostate cancer in autopsy studies is similar to that of men in Western societies (39). Finally, soy products, which are important constituents of Japanese diet, inhibit mammary tumorigenesis in rat models (40–42).

By assuming a physiological systemic role of dietary-derived genistein, it will be important to evaluate the levels of genistein in the serum of subjects consuming various diets. Nevertheless, it seems possible that urinary genistein may have a local effect in the urinary tract, where it could inhibit the formation of solid tumors which depend on neovascularization induced by bFGF or other angiogenic factors (43, 44). Further studies should focus on the *in vivo* effects of genistein on angiogenesis and tumorigenesis, and further investigation of dietary ingested compounds is merited as it might provide additional molecules contributing to the protective effect of diet on the incidence of solid tumors in humans.

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