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The novel targets for anti-angiogenesis of genistein on human cancer cells

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Abstract

Genistein has been reported to be a natural chemopreventive in several types of human cancer. In our prior study, soy isoflavones were shown to induce cell cycle arrest and apoptosis of bladder cancer cells in the range of human urine excretion. This study was designed to identify the novel molecular basis underlying anti-angiogenic activities of soy isoflavones. An immortalized E6 and five human bladder cancer cell lines were studied by immunoassay, flow cytometry, functional activity, reverse transcription-polymerase chain reaction, immunoblotting, and transwell co-culture in vitro. The efficacy of soy isoflavones on angiogenesis inhibition in vivo was examined by nude mice xenograft and chick chorioallantoic membrane bioassay. Factors analyzed included angiogenic factors, matrix-degrading enzymes, and angiogenesis inhibitors. Genistein was the most potent inhibitor of angiogenesis in vitro and in vivo among the isoflavone compounds tested. It may also account for most of the reduced microvessel density of xenografts observed and the suppressed endothelial migration by soy isoflavones. Genistein exhibited a dose-dependent inhibition of expression/excretion of vascular endothelial growth factor$_{165}$, platelet-derived growth factor, tissue factor, urokinase plasminogen activator, and matrix metalloprotease-2 and 9, respectively. On the other hand, there was an up-regulation of angiogenesis inhibitors—plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1. In addition, a differential inhibitory effect between immortalized uroepithelial cells and most cancer cell lines was also observed. Altogether, we discovered that tissue factor, endostatin, and angiostatin are novel molecular targets of genistein. The current investigation provides further evidence in support of soy-based foods as natural dietary inhibitors of tumor angiogenesis.

Keywords: Bladder cancer; Genistein; Anti-angiognesis; Angiogenic factors; Angiogenesis inhibitors; Matrix-degrading enzymes

1. Introduction

Angiogenesis, the generation of new blood vessels, is required for the growth as well as expansion of solid tumors, especially those at 1–2 mm in diameter [1]. The initiation of this vascular phase marks a period of accelerated growth, local invasion, and, ultimately, metastasis of epithelial neoplasm. The prognostic importance of estimating the degree of angiogenesis in patients with carcinoma, including bladder cancer, supports the hypothesis that tumor angiogenesis is imperative in the development

Abbreviations: CAM, chick chorioallantoic membrane; COX-2, cyclooxygenase-2; MMPs, matrix metalloproteinases; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-polymerase chain reaction; TSP-1, thrombospondin-1; TF, tissue factor; TIMP-2, tissue inhibitor of metalloproteinase-2; tPA, tissue-type plasminogen activator; MT-MMP, transmembrane MMP; uPA, urokinase-type plasminogen; and VEGF, vascular endothelial growth factor

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and progression of human cancer [2]. Hence, therapeutic agents are being devised either to interrupt the pathogenic steps of tumor angiogenesis or to directly destroy the tumor vasculature. Currently, chemicals that can cut off the tumor's blood supply, so-called angiogenesis inhibitors, are considered one of the most promising anticancer therapies.

A number of epidemiological studies show that the consumption of soy products may have a protective effect against human cancers of the breast, colon, or prostate in the Far East Asia [3,4]. The beneficial effects of a traditional Japanese diet are thought to come from isoflavones, the plant pigments found in soybeans. Experiments both in vitro and in vivo for bladder cancer have supported the notion that isoflavonoids, flavonoids, or lignans are able to suppress tumor growth [5–8]. We also found that isoflavone compounds tend to have a dose-dependent growth inhibition on human bladder cancer cells in vitro and a tumor suppressor effect in vivo [9]. It is interesting to note that the cooperative action of a mixture of isoflavone compounds generates greater anti-tumorigenic effects than any single compound does, and that the IC_{50} values of most cancer cell lines (3–5 μg/ml or 7.9–13.2 μM) are within the reach of the urine levels of daidzein (7,4′-dihydroxyisoflavone) (14.7 μM), and genistein (5,7,4′-trihydroxyisoflavone) (8.4 μM) following a soy challenge [10]. Nevertheless, genistein is one of the most potent soy isoflavones. These results have produced the hypothesis that soy isoflavones, especially the genistein, may be practical chemopreventives for human urinary tract cancer.

It is well-known that angiogenic activity can be detected in the urine of patients with bladder cancer [11,12]. In cases of flat dysplasia or non-invasive transitional cell carcinoma, there are also increased numbers of sub-urothelial capillaries, suggesting the existence of an angiogenic stimulus in the early stage of bladder tumorigenesis [13]. For this reason, bladder cancer is a particularly good paradigm to use for investigating anti-angiogenic agents. Many molecules released by tumor and host cells are known to play a role in tumor angiogenesis, and the final outcome in vivo depends on the net balance between positive (angiogenic factors) and negative (angiogenesis inhibitors) regulatory elements. Factors known to be involved in human bladder carcinogenesis include (1) angiogenic growth factors, such as vascular endothelial growth factor (VEGF) [14–16] and platelet-derived growth factor (PDGF) [17,18]; (2) other pro-angiogenic factors, such as cyclooxygenase-2 (COX-2) [19–21] and tissue factor (TF) [22]; (3) matrix-degrading enzymes, such as urokinase- and tissue-type plasminogen activator (uPA and tPA) [23–26], the matrix metalloproteinase (MMP) family [27–34], and their activator of transmembrane MMP (MT-MMP) [34]; and (4) angiogenesis inhibitors, such as tissue inhibitor of metalloproteinase-2 (TIMP-2) [28–30,33], plasminogen activator inhibitor-1 (PAI-1) [35], and thrombospondin-1 (TSP-1) [15,36], angiostatin [37], and endostatin. A number of biochemical targets and pharmacological actions have been proposed [38,39], and anti-angiogenesis is one of the important mechanisms responsible for anti-cancer effects of soy isoflavones [5,7,40,41]. Despite of the fact that in vitro anti-angiogenic mechanisms of genistein have been described in several cancer models, the information is far from complete. Using human bladder cancer as a paradigm, we performed this study to identify the molecular basis, especially the novel targets, for anti-angiogenesis of genistein in the range of urine excretion.

2. Materials and methods

2.1. Cell culture

The E6 cell line was an immortalized human uroepithelium [42]. Human bladder cancer cell lines RT4 (grade 1 bladder cancer), J82 (grade 3 bladder cancer), 5637 (grade 3 bladder cancer), and T24 (grade 3 bladder cancer) were obtained from the American Type Culture Collection (Rockville, MD). TSGH8301 (grade 2 bladder cancer) was established locally and has been reported in detail previously [43]. The E6 cells were grown in F12 medium (GIBCO BRL), the RT4 cells in McCoy's 5A medium (GIBCO BRL), and the rest of cancer lines were in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (GIBCO BRL) at 37 °C. Each cell line was seeded at 1×10^5 cells/well and grown for experiment. An immortalized human microvascular endothelial cell line, HMEC-1, was from Centers for Disease Control and Prevention and were grown in MCDB131 medium.

2.2. Nude mice xenograft model

The mice were maintained and all animal experiments were conducted according to institutional ethical standards established for, and have been approved by, the Animal Core Facility at the Medical College, National Cheng Kung University. TSGH8301 cells (1×10^7) were first injected subcutaneously into the flank of 6-week-old male mice as described previously [9]. Since we found that only intratumoral injection gives satisfactory suppressor effect compared with intra-peritoneal administration [9], engrafted tumors (approximately 5 mm in diameter) were treated with local injection of 0.025% DMSO (as a control), 10 μg of genistein, or a mixture regimen (10 μg of genistein, daidzein, and biochanin-A) every 3 days for 3 weeks. Five mice were used in each treatment group. The tumors were then excised and fixed for assessment of the degree of angiogenesis.

2.3. Immunohistochemistry and quantification of microvessel density (MVD)

For immunohistochemistry, the frozen tumors were fixed with acetone/methanol mixture at 4 °C, and blocked

for endogenous peroxidase by 3% hydrogen peroxide. The sections were incubated for 18 h at 4 °C with the appropriate dilution (1:100) of rat monoclonal anti-CD31 antibody (PharMingen). Then samples were incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody: peroxidase-conjugated goat antimouse IgG1 (Jackson ImmunoResearch Laboratory, Inc.). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Zymed Laboratory, Inc.). The sections were then washed with PBS and counterstained with Gill's hematoxylin (Biogenex Laboratories). Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. MVD was counted by two independent observers (CLH and NHC) and expressed as the average of the five highest areas identified within a single 200× field. Furthermore, for each tumor, the same hot spots were evaluated using an image-analysis system. Images were captured using a microscope coupled to a color video camera (Sony Dxc-M3a) and then processed with an image analysis device (Ibas 2000, Kontron Bild Analyse). The parameters automatically detected by the system were the number of microvessels, the area of the microvessels, and the overall area. For each tumor examined, the following measures were recorded: the mean area of individual vessels, the total microvascular area/field, corresponding to the sum of the areas of all $CD31^+$ structures located in each field, and the mean microvascular perimeter, corresponding to the mean of the perimeters of all $CD31^+$ structures identified.

2.4. Chick chorioallantoic membrane (CAM) bioassay

The technique of CAM treatment was performed as described with slight modification [44]. Briefly, fertilized White Leghorn chick eggs were incubated under constant humidity at a temperature of 37 °C. On the seventh day of incubation, a square window was opened in the eggshell. The CAM of the embryo was treated with 5 or 10 μg/ml genistein (GIBCO BRL) dissolved in dimethyl sulfoxide (DMSO) (Sigma) either as a cocktail mixture of genistein, daidzein (Calbiochem–Novabiochem), and biochanin-A (Sigma) in equal proportions). DMSO was kept below 0.05% (v/v) was used as control. The CAMs were photographed on day 11 using a laser-scanning confocal microscope (Leica model TCS2) after injection with FITC-dextrin 1 mg/ml (Cat#FD-2000S; Sigma). The mean fluorescent vascular density was measured by two independent observers (S.J.S. and C.L.H.) and scored the degree of angiogenesis with support of imaging software [45]. Average values for six embryos were recorded.

2.5. Cell migration assay

Cell migration assay was performed with 8 μm-pore size Transwell migration chamber (Corning Inc.) as previously described [46]. To examine the effect on cell migration, monolayer of T24 or TSGH8301 cells was formed in the lower chamber and treated with isoflavones for 12 h. After that, HMEC-1 (5×10^4) suspended in MCDB131 medium containing 0.5% FCS were added to the upper chambers. Endothelial cell migration was allowed to proceed for additional 18 h at 37 °C in CO_2 incubator. Then cells were removed from upper surface of the membranes with a cotton swab. All of the cells migrated to the lower surface were fixed in methanol and stained with hematoxylin. Dried membranes were cut out and mounted on glass slides in mounting medium. At least 15 random fields from each membrane were counted. All migration assays were repeated at least twice with similar results.

2.6. Immunoassay for angiogenic factors, MMPs, proteases and angiogenesis inhibitors

Supernatants of each cell line were collected after treatment with appropriate concentrations of genistein or controls (0.5% bovine serum albumin) for 12 h. The analysis included $VEGF_{165}$, PDGF-A, MMP-2, MMP-9 by using the Quantikine ELISA kit (Research and Diagnostics Systems, Inc.), angiostatin (Research and Diagnostics Systems, Inc.), endostatin (CHEMICON International, Inc.), and TSP-1 (Lab Vision Corporation). The immunoassay for proteases included uPA, tPA, or PAI-1 (American Diagnostica Inc.). Briefly, the microtiter plate was pre-coated by murine monoclonal antibody specific for each angiogenesis factor or protease. Both the recombinant standards and supernatants of culture medium (100 or 200 μl, according to the manufacturer's suggestion) were pipetted into the wells and reacted for 2 h at room temperature. After thorough washing, horseradish peroxidase-linked polyclonal antibody specific for angiogenic factors or proteases was added to the wells to "sandwich" the immobilized immune complexes. Substrate solution was then added to develop the color after washing off the unbound antibody-enzyme reagent. The reaction was terminated by the addition of stop solution (2N H_2SO_4) and the optical density was read using a spectrophotometer (Molecular Devices Corp.) set at 450 nm. At least two batches of conditioned media were tested, and the mean values were used as the final concentration. The sensitivities were 5 pg/ml for $VEGF_{165}$, 5 pg/ml for MMP-2, 1.7 pg/ml for PDGF-A, and 0.05 ng/ml for uPA and PAI-1. The values obtained after ELISA were corrected with a dilution factor and were finally expressed in pg/ml.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) amplification

The expression of COX-2, MT-MMP, and TIMP-2 in RT4, TSGH8301, and J82 cells was examined by RT-PCR. The expression of MMP-2 was analyzed in J82, 5637 and TSGH8301 cells, and TSP-1 or uPA in RT4 and

TSGH8301 cells. Briefly, total RNA was extracted from cells with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. We synthesized cDNA from 1 μg total RNA using a first strand cDNA synthesis kit (Amersham Pharmacia Biotech). The cDNA was amplified with AmpliTaq DNA polymerase (Perkin-Elmer/Applied Biosystems). Primers used for priming each gene were as the followings: the COX-2 gene, sense, 5′-GTG CCT GGT CTG ATG ATG TAT GC-3′, and anti-sense, 5′-CCA TAA GTC CTT TCA AGG AGA ATG-3′ (product size 402 bp) [47]; the MT-MMP gene, sense, 5′-CCCT-ATGCCTACATCCGTGA-3′, and anti-sense, 5′-TCCATC-CATCACTTGGTTAT-3′ (product size 550 bp) [48]; the TIMP-2 gene, sense, 5′-CCGAATTCTGCAGCTGCTCC-CCGGTGCACCCG-3′, and anti-sense, 5′-GGAAGCTTT-TATGGTCCCTCGATGTCGAG-3′ (product size 590 bp) [34]; the MMP-2 gene, sense, 5′-ACAAAGAGTGGCAG-TGCAA-3′, and anti-sense, 5′-CACGAGCAAAGGCAT-CATCC-3′ (product size 480 bp) [49]; the uPA gene, sense, 5′-AGAATTCACCACCATCGAGA-3′, and anti-sense 5′-ATCAGCTTCACAACAGTCAT-3′ (product size 474 bp); the PAI-1 gene, sense, 5′-ATGGGATTCAAGATTGAT-GA-3′, and anti-sense 5′-TCAGTATAGTTGAACTTGT-T-3′(product size 452 bp) [50]. the TSP-1 gene, sense, 5′-CGTCCTGTTCCTGATGCATG, and anti-sense, 5′-GGCA-GGACACCTTTTTGCAGA (product size 1000 bp) [51], and the PDGF-A gene, sense, 5′-CCCCTGCCCATTCG-GAGGAAGAG-3′, and the anti-sense, 5′-TTGGCCACCTT-GACGCTGCGGTG-3′ (product size 237 bp) [52]. The β-actin gene was amplified as an internal control. The RT-PCR conditions were as follows: for COX-2 were 94 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 2 min (25–30 cycles); for MT-MMP or TIMP-2 were 93 °C for 90 s, 58 °C for 90 s, and 72 °C for 3 min (40 cycles); for MMP-2 were 93 °C for 90 s, 58 °C for 90 s, and 72 °C for 3 min (40 cycles); for uPA and PAI-1 genes were 94 °C for 40 s, 48 °C for 1 min 30 s, and 72 °C for 1 min 20 s (25 cycles); for TSP-1 were 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min at (35 cycles); for PDGF-A were 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 60 s (25 cycles). Amplified products were separated on 1 or 2% agarose gel and stained with ethidium bromide. Stained bands on the gel were visualized under UV light and then photographed. Each RT-PCR test was confirmed by independent duplicate reactions. The amount of the RNA of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that of untreated cells as a control (taken as 100%).

2.8. Immunoblotting analysis

The procedure of Western blotting was performed as previously described [53]. Briefly, total cell lysate was prepared by direct adding 150 μl of 2× SDS loading buffer to confluent cells on a 10-cm dish, and detaching the cells with a rubber policeman. Protein concentration was determined by the Bio-Rad protein assay. Twenty-five micrograms of total cell lysates were separated by electrophoresis on a 8% SDS–polyacrylamide minigel. The proteins were electro-transferred onto a nitrocellulose filter at 100 mA for 2 h using an electroblot apparatus (Hoefer TE70 semidry transfer unit; Amersham Pharmacia Biotech). The nitrocellulose filter was first blocked with 5% skim milk (Difco Laboratories, Inc.) for 1 h and then probed with monoclonal antibody to MT-MMP and TIMP-2 (Fuji Chemical Industries), COX-2 (Cayman Chemical Inc.), angiostatin (Research and Diagnostics Systems, Inc.), endostatin (CHEMICON International, Inc.), $VEGF_{165}$ (CHEMICON International, Inc.), and TSP-1 (Lab Vision Corporation) for 2 h. The protein bands were visualized with an enhanced chemiluminescence detection kit (Amersham Corp.) using horseradish peroxidase-labeled secondary antibody, as suggested by the manufacturer. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that of untreated cells as a control (taken as 100%).

2.9. Measurement of TF activity and its expression on cell surface

A functional TF assay was performed using a one-stage clotting test as previously described [54]. Briefly, J82 cells were first treated with various concentrations of genistein for different periods of time. TF activity was then measured on 0.1 ml of cell lysate (10^6 cells/ml). It was incubated with 0.1 ml of pre-warmed human plasma and 0.1 ml 25 mM $CaCl_2$. The rate of fibrin formation was recorded on a coagulometer (Diagnostica Stago ST4). A linear standard curve (log TF versus log clotting time) was used to quantify TF activity. One unit of TF was defined as the concentration of extracted rabbit brain clotting time within 25 s. Generally, 100 U/ml of TF clotted normal human plasma in 12–14 s. Each experiment was performed in triplicate.

The assessment of TF expression on the cell surface of tumor cells was performed as previously described [24]. Briefly, J82 cells were labeled with 10 μl of FITC-conjugated anti-human tissue factor monoclonal antibody (#4508CJ; American Diagnostica) after treatment with appropriate concentrations of genistein for 12 h. The expression of TF on the cell surface was determined directly by flow cytometry. Ten milligrams of mouse IgG (FITC-conjugated; Dako) was used as a negative control. The percentage of positive fluorescence was determined in comparison to an isotype control for TF antigen.

2.10. Statistical analysis

The means of the CAM scores and mean vessel areas were analyzed by the Wilcoxon Signed Rank test and pair-

wise comparisons of the different treatment groups. The differences in the MVD among xenografts treated with 10 μg/ml genistein, biochanin A, or daidzein or a mixture of 10 μg isoflavones and controls were analyzed by ANOVA test, and the differences between treatment groups and the controls were estimated by Mann–Whitney *U* test. The angiogenic factors and proteases in each group were compared and the significance of the results analyzed by the Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

First, we used a previously described xenograft model to evaluate the direct anti-angiogenic effect of isoflavones on bladder cancer cells [9]. A significantly reduced MVD of TSGH8301 cells in SCID mice was observed at 10 μg/ml genistein ($50.7 \pm 4.0/mm^2$) or 10 μg/ml isoflavone mixture ($28.4 \pm 5.3/mm^2$) ($P < 0.0001$ by ANOVA, respectively) (Fig. 1A). The difference between treatment groups and the controls ($70.2 \pm 7.6/mm^2$) was also statistically significant ($P < 0.001$). The mean area of individual vessels also showed a significant reduction after treatment with genistein ($0.19 \pm 0.09/mm^2$) or isoflavone mixture ($0.11 \pm 0.05/mm^2$) compared with controls ($0.32 \pm 0.15/mm^2$) ($P = 0.0001$ by ANOVA, respectively) (Fig. 1B). The results support the efficacy of isoflavones in the suppression of tumor angiogenesis in vivo. It is interesting to note that two of five control mice developed pulmonary metastasis compared to none in the treatment groups in vivo, implying that the cancer protective effect of isoflavones occurs at early stages of bladder carcinogenesis.

Next, we evaluated the direct effect of soy isoflavones on endothelial cells in vivo (details in Table 1). Using CAM bioassay, a significantly reduced angiogenesis was observed when the embryos were treated with 10 μg/ml genistein or a cocktail mixture of isoflavones (10 μg/ml) compared with controls ($P < 0.05$, respectively). A comparable reduced growth and DNA synthesis of HMEC-1 cells was detected (data not shown), supporting recent notion that isoflavones may also exert direct suppressor effect on endothelial cells. On the contrary, no significant angiogenesis inhibition was noticed for daidzein or biochanin-A at 10 μg/ml levels compared with controls (data not shown).

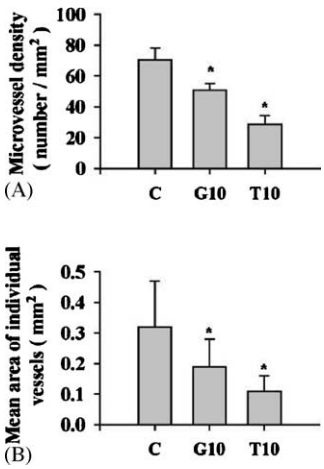

Fig. 1. Evaluation of the anti-angiogenesis of isoflavones in vivo. The engrafted tumors of TSGH8301 cells were taken for MVD measurement after treatment with isoflavones for 3 weeks. (A) The mean MVD (number/mm^2) was expressed as the average of the five highest areas within a 200× field. Tumor MVD was reduced significantly in mice treated with 10 μg/ml genistein (G10) or 10 μg/ml isoflavone mixture (T10) compared with controls ($P < 0.0001$, respectively). The difference between treatment groups and the controls was also statistically significant ($P < 0.001$). (B) For each tumor, the same hot spots were evaluated using an image-analysis system and analyzed for the mean area of individual vessels identified by CD31 immunohistochemistry. The mean area of individual vessels showed a significant reduction after treatment with genistein or isoflavone mixture compared with controls ($P = 0.0001$). The asterisk indicates the significance of the study group compared with controls.

Then, transwell co-culture systems were used to mimic the in vivo paracrine interaction between tumor cells and endothelial cells. A pronounced inhibition of endothelial migration was observed when T24 or TSGH8301 cells were treated with 10 μg/ml isoflavone mixture ($P = 0.0001$, respectively) (Fig. 2). The inhibitory effect at 5 μg/ml isoflavone mixture was observed in T24 cells ($P = 0.0001$), and genistein at 10 μg/ml observed in TSGH8301 cells ($P = 0.0001$). In TSGH8301 cells, biochanin-A at 10 μg/ml still had some migration inhibitory effect. But, daidzein produced the lowest level of angiogenic response. Altogether, genistein is among the most effective inhibitor of tumor-induced angiogenesis than daidzein or biochanin-A, and may well account for most of the reduced MVD of xenografts and migration inhibitory effect. The following model experiments in vitro thus were performed using genistein alone.

Given that tumor angiogenesis is the consequence of a net balance between pro- and anti-angiogenic factors, the effects of isoflavones on the expression/excretion of angiogenic factors ($VEGF_{165}$ and PDGF-A, COX-2 and TF), matrix-degrading enzymes (uPA, tPA, and PAI-1, MMP-2, MMP-9, MT-MMP, and TIMP-2), and angiogenesis inhibitors (endostatin, angiostatin, and TSP-1) were examined in an immortalized uroepithelial cell line (E6) and five human bladder cancer cell lines.

Table 1
The effect of isoflavones on angiogenesis assessed by CAM bioassay

Chemicals	Mean scores ± S.D.	*P*-values	
DMSO (control)	3.00 ± 0.10		
Daidzein (10 μg/ml)	2.70 ± 0.50	0.81[a]	0.01[b]
Biochanin-A (10 μg/ml)	2.37 ± 0.58	0.21[a]	0.03[b]
Genistein (10 μg/ml)	1.33 ± 0.62	0.02[a]	0.04[b]
Cocktail mixture (10 μg/ml)	0.40 ± 0.32	0.01[a]	

S.D.: standard deviation.
[a] Compared with the control group.
[b] Compared with those treated with isoflavone mixture.

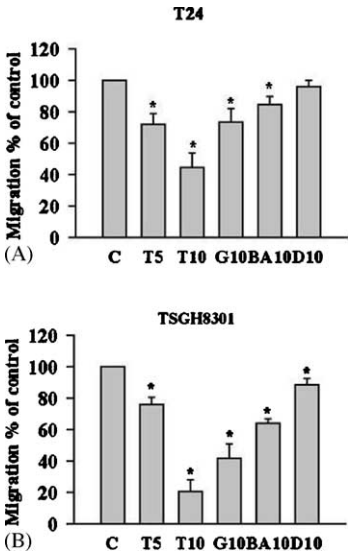

Fig. 2. The inhibitory effect of isoflavones on cell migration in vitro. The cancer cells in the lower transwell chambers were first treated with isoflavoens for 12 h. Then, HMEC-1 cells from upper chamber were allowed to migrate for additional 18 h at 37°. The numbers of endothelial cells present in the lower surface were counted. Error bars indicate standard deviations of duplicate experiments. The greatest inhibition of endothelial migration was observed at 10 μg/ml isoflavone mixture for both T24 (A) and TSGH8301 cells (B) ($P = 0.0001$, respectively). The inhibitory effect at 5 μg/ml isoflavone mixture was observed in T24 cells ($P = 0.0001$), and at 10 μg/ml genistein in TSGH8301 cells ($P = 0.0001$). Biochanin-A at 10 μg/ml still had some inhibitory effect on TSGH8301 cells. But, daidzein exhibited the lowest amount of tumor-induced angiogenic responses. (T5: 5 μg/ml isoflavone mixture; T10: 10 μg/ml isoflavone mixture; G10: 10 μg/ml genistein; BA10: 10 μg/ml biochanin-A; D10: 10 μg/ml daidzein). The asterisk indicates the significance of the study group compared with controls.

To establish the kinetics of angiogenic balance in response to isoflavones treatment, alterations of $VEGF_{165}$ and PDGF-A were assessed on a 24 h basis, respectively. The excretion of $VEGF_{165}$ was inhibited as early as 15 min after treatment with isoflavone mixture (10 μg/ml), but reverted to baseline levels 24 h later (data not shown). The most striking suppressive effect was observed between 15 min and 3 h after treatment with isoflavones. Thus, the subsequent in vitro experiments were basically analyzed 3 h after genistein treatment. It is interesting to note that each cell line has its own expression profile in the production/excretion of angiogenic factors and proteases. In general, those cell lines showing higher expression levels of angiogenic factors in the supernatants were selected in the following analyses. Basically, the response of each molecule to genistein was screened by immunoassay, and then examined by Western blot or RT-PCR (Table 2).

The Fig. 3 illustrated the alterations of $VEGF_{165}$ for RT4, TSGH8301 and T24 cells analyzed by immunoassay and Western blot, respectively. The dose-related suppression of $VEGF_{165}$ protein excretion was observed at 10 μg/ml genistein, with the most obvious inhibitory effect observed in RT4 cells. A comparable suppression of

Table 2
The inhibitory effects of genistein on angiogenic factors and matrix-degrading enzymes

Cell lines	$VEGF_{165}$	PDGF-A	uPA	PAI-1	MMP-2	MMP-9	TF
E6	ND	ND	+[a]	+++	ND	ND	+
RT4	++[b]	+[b]	++[b]	++[b]	+	+	−
TSGH8301	+[b]	+[b]	++[b]	−[c]	+[b]	+	+
T24	++[b]	+	+	++	+	+	−
J82	−	−	−	+	++[b]	++[b]	++[b]
5637	+[b]	+[b]	+	+	+++[b]	+++[b]	+++[b]

[a] Relative levels of angiogenic factors or matrix-degrading enzymes were arbitrarily categorized from "+++" to "+" scales, and "−" means non-detectable level by immunoassay (ND: not done).
[b] A significant inhibitory effect by soy isoflavones was observed.
[c] A significant stimulatory effect by soy isoflavones was observed.

$VEGF_{165}$ protein excretion was confirmed in the RT4, T24 and TSGH8301 cells ($P < 0.05$). The dose-related suppression of PDGF-A by genistein was first demonstrated by immunoassay in TSGH8301 and 5637 cells, and then confirmed by RT-PCR (Fig. 4).

As for plasminogen activators and their inhibitor, no apparent inhibitory effect was observed for tPA in any cell line tested (data not shown). In contrast, genistein showed a significant dose-related suppression of uPA ($P < 0.05$). Representative results on uPA in RT4 and TSGH8301 cells

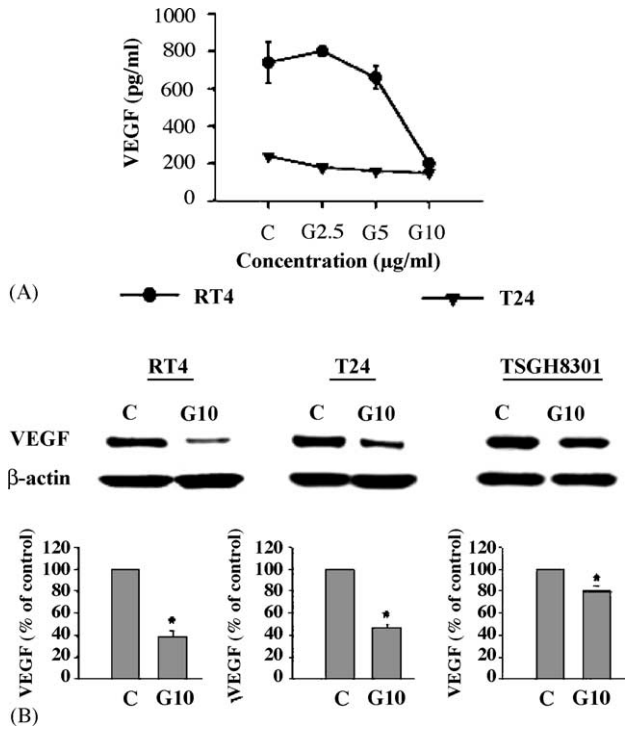

Fig. 3. The inhibitory effect of genistein on the production/excretion of $VEGF_{165}$. (A) Supernatants of RT4 and T24 cells were collected after treatment with genistein from 2.5 to 10 μg/ml (G2.5–G10), or 0.5% bovine serum albumin as a control (C) for 12 h. The dose-related suppression of $VEGF_{165}$ protein excretion was observed at 10 μg/ml genistein (G10), and RT4 cells showed the most pronounced inhibitory effect. (B) Alteration of $VEGF_{165}$ in response to genistein treatment was assessed by Western blot. A comparable suppression of $VEGF_{165}$ protein excretion was examined in RT4, T24 and TSGH8301 cells ($P < 0.05$). The asterisk indicates the significance of the study group compared with controls.

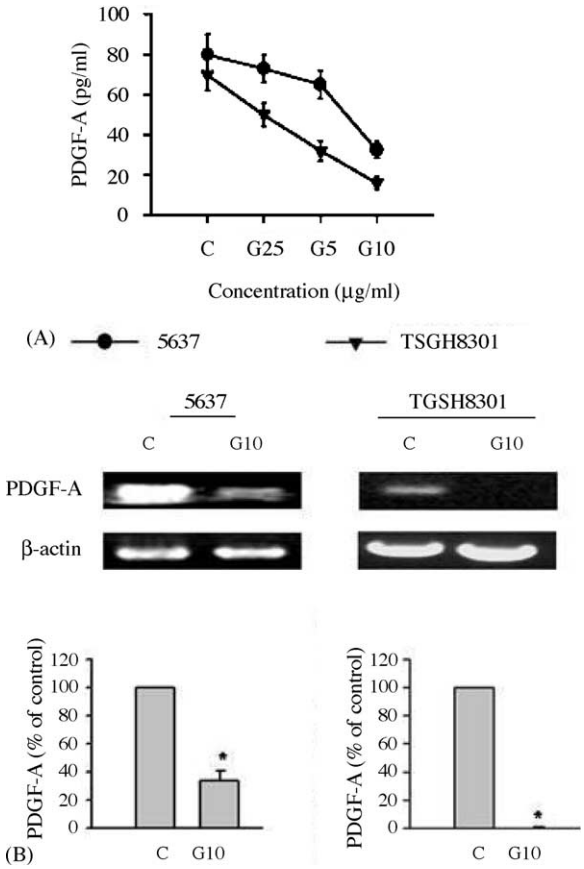

Fig. 4. Suppressive effect of genistein on the production/excretion of PDGF-A. (A) The dose-related suppression of PDGF-A was assessed on the supernatants of 5637 and TSGH8301 cells. Apparent inhibitory effect was observed at 10 μg/ml genistein (G10). (B) Alteration of PDGF-A in response to genistein was examined by RT-PCR. A comparable transcriptional suppression of PDGF-A was demonstrated in both 5637 and TSGH8301 cells ($P < 0.05$). The asterisk indicates the significance of the study group compared with controls.

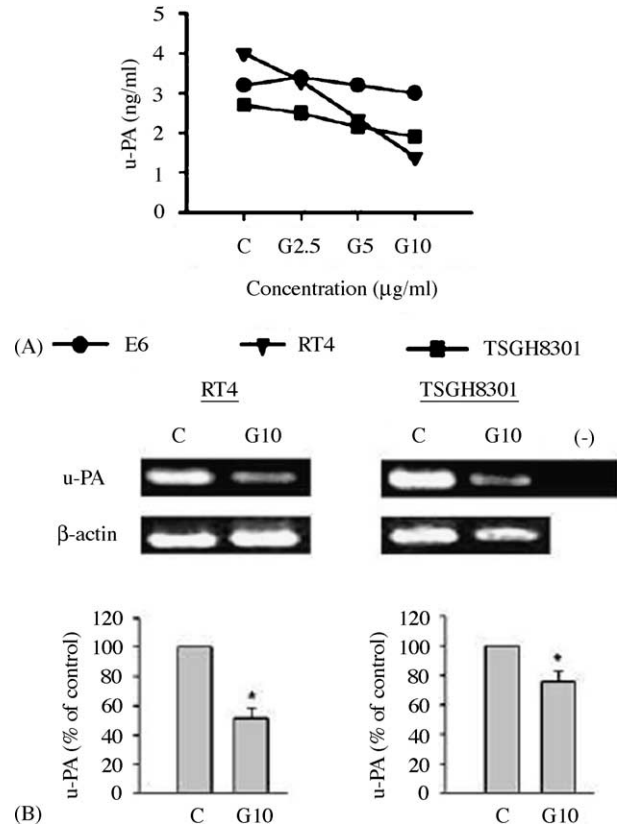

Fig. 5. The inhibitory effects of genistein on the matrix-degrading enzyme uPA. (A) Supernatants of E6, RT4 and TSGH8301 cells were collected after treatment with appropriate concentrations of isoflavones, from 2.5 to 10 μg/ml genistein (G2.5–G10), or 0.5% bovine serum albumin as a control (C). Genistein alone did not exhibit apparent inhibitory effect on proteases produced by E6 cells. In contrast, a significant dose-related suppression of uPA ($P < 0.05$) was demonstrated in RT4 cells, and TSGH 8301 cells. (B) Alteration of uPA RNA in response to genistein was assessed by RT-PCR. Comparable transcriptional suppression of uPA was present in both RT4 cells and TSGH8301 cells ($P < 0.05$, respectively). The asterisk indicates the significance of the study group compared with controls.

were shown in Fig. 5. The results for PAI-1 were shown in Fig. 6. Although RT4 showed a suppressive effect, TSGH8301 cells exhibited an induction of PAI-1 production by isoflavone treatment ($P < 0.05$). Of interest, E6 cells did not respond to genistein in terms of protease production, supporting our hypothesis regarding differential suppressive effect between cancer cells and non-neoplastic uroepithelium [9].

The effect of isoflavones on MMP-2 was shown in Fig. 7. A comparable inhibitory effect was also observed for MMP-9 (data not shown). However, there was no apparent inhibitory effect on MT-MMP or TIMP-2. The lack of modulating effect on these two angiogenesis-related molecules was verified by Western blotting (data not shown).

We then evaluated the involvement of pro-angiogenic factor TF in the tumor suppressor effect of soy isoflavones. Then flow cytometry confirmed a dose-dependent inhibition of TF expression on the surface of J82 cells (Fig. 8). A comparable inhibitory effect on TF expression was also observed for 5637 cells (data not shown). Similarly, genistein exhibited a dose-dependent suppression of TF activity of J82 cells ($P < 0.05$). A trend toward time-related inhibition of TF activity by isoflavones was more apparent when genistein was higher than 30 μg/ml. Nevertheless, genistein did not exhibit any inhibitory effect on COX-2 expression (data not shown).

As for angiogenesis inhibitors, we examined the effect of genistein on the expression of endostatin, angiostatin and TSP-1. The potential stimulatory effect of genistein on the endostatin was supported by immunoassay and Western blot (Fig. 9). A parallel up-regulation of angiostatin expression induced by genistein was also noticed (Fig. 10). Furthermore, a significant dose-related stimulation of TSP-1 production/excretion was demonstrated in both RT4 and TSGH8301 cells ($P < 0.05$) (Fig. 11). Taken together, soy isoflavones, especially the genistein, appears to exhibit suppressor effect on multiple angiogenesis-related pathways on human bladder cancer cells in vitro.

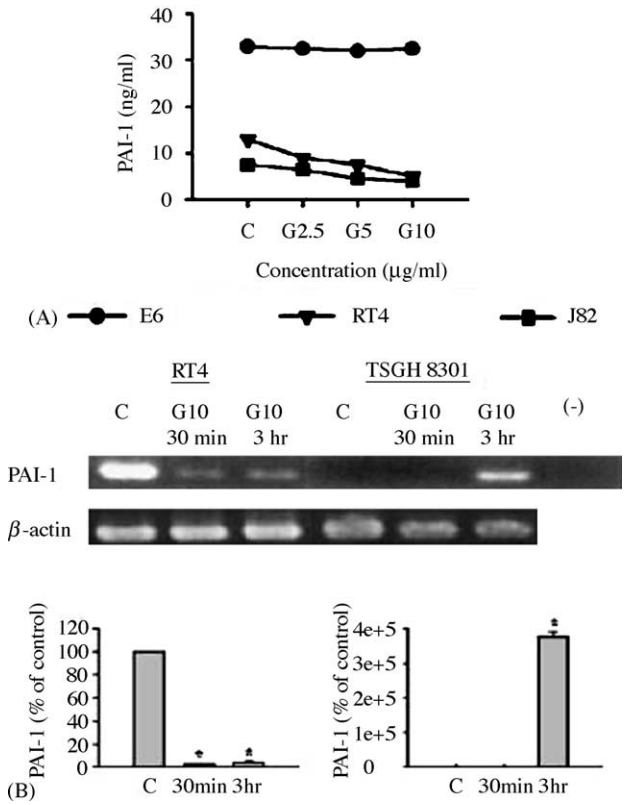

Fig. 6. The effects of genistein on the matrix-degrading enzyme PAI-1. (A) Supernatants of E6, RT4 and J82 cells were collected after treatment with appropriate concentrations of genistein from 2.5 to 10 μg/ml (G2.5–G10), or 0.5% bovine serum albumin as a control (C) for 12 h. Genistein alone did not exhibit apparent inhibitory effect on proteases produced by E6 cells, but exhibited a significant dose-related suppression of PAI-1 production in both RT4 cells and J82 cells ($P < 0.05$, respectively). (B) Alteration of PAI-1 RNA in response to genistein treatment was analyzed by RT-PCR. Although RT4 showed a suppressive effect, TSGH8301 cells exhibited an induction of PAI-1 production after treatment with isoflavones ($P < 0.01$, respectively). The asterisk indicates the significance of the study group compared with controls.

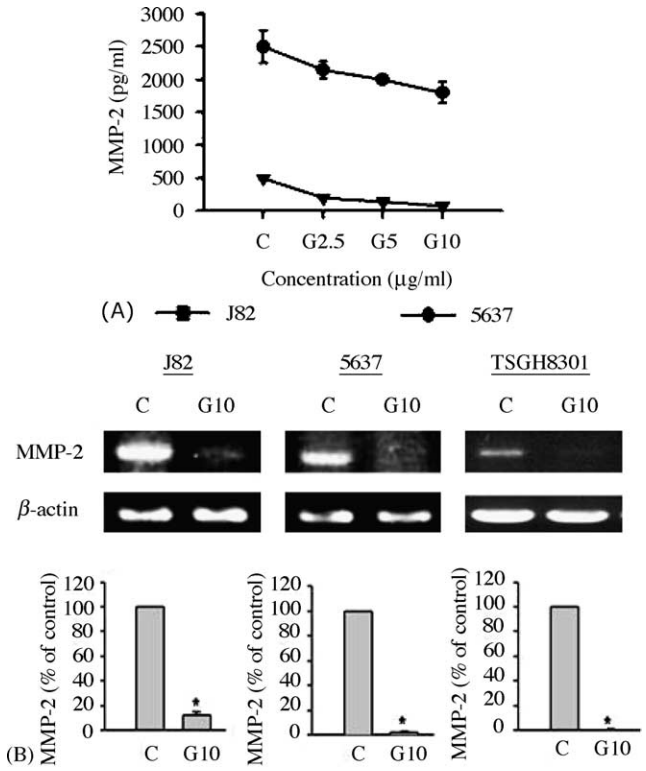

Fig. 7. The suppressive effects of genistein on the production/excretion of MMP-2. (A) Supernatants of J82 and 5637 cells were collected after treatment with appropriate concentrations of genistein or 0.5% bovine serum albumin as a control (C) for 12 h. Genistein alone, from 2.5 to 10 μg/ml (G2.5–G10), showed a significant dose-related suppression of MMP-2 production in both J82 and 5637 cells ($P < 0.05$, respectively). (B) Alteration of MMP-2 RNA in response to isoflavones was analyzed by RT-PCR. A comparable transcriptional suppression of MMP-2 was noticed in RT4, 5637 and TSGH8301 cells ($P < 0.01$, respectively). The asterisk indicates the significance of the study group compared with controls.

4. Discussion

Tumorigenesis of the human bladder is a multi-step and multifocal (field effect) process, possibly involving the spread of pre-malignant clones along the urothelial mucosa. As a result, human bladder cancer is uniquely suited to the development of chemoprevention, in which noncytotoxic drugs or nutrients are used to prevent, retard, or delay carcinogenesis. In this study, we demonstrated that both a cocktail mixture of isoflavones or genistein by itself drastically inhibit angiogenesis in vivo, as suggested previously [5,7,9]. Since angiogenic activity begins with a pre-neoplastic lesion of the human bladder, it is important to clarify the mechanism(s) of the anti-angiogenic effects of soy isoflavones.

We showed that genistein did not exhibit toxicity to normal bladder cells within the physiological range of urine excretion (10 μg/ml). The compound inhibits the expression or secretion of angiogenic factors (such as $VEGF_{165}$, PDGF-A, and TF) and matrix-degrading enzymes (such as uPA, MMP-2, and MMP-9) of human bladder cancer cells. On the contrary, genistein upregulates the expression of angiogenesis inhibitors (PAI-1, angiostatin, endostatin, and TSP-1). The results indicate that soy isoflavones can inhibit tumor angiogenesis through inhibition of cell proliferation and interruption of the breakdown of ECM surrounding growing vessels and tumor. Soy isoflavones, especially genistein, thus appear to restore the balance between pro- and anti-angiogenesis on the side of angiogenesis inhibition, resulting in growth arrest of the tumors.

However, such angiogenic inhibition of genistein might have cell-specific relevance. The reported biological targets of genistein were MMP-9, $VEGF_{165}$, TGF-β1, and tissue inhibitor of MMP for breast cancer [41], MMP-2 and MMP-9 for head and neck cancer [55], VEGF for oral cancer [56], MMP-9, VEGF, uPA-R and TSP-1 for prostate cancer [57,58], and uPA, PAI-1, VEGF, or MMP-2 for endothelial cells [59]. Despite of the fact that MMP-2 appears to implicate in the anti-angiogenesis of genistein for bladder cancer cells, it was not relevant in oral and

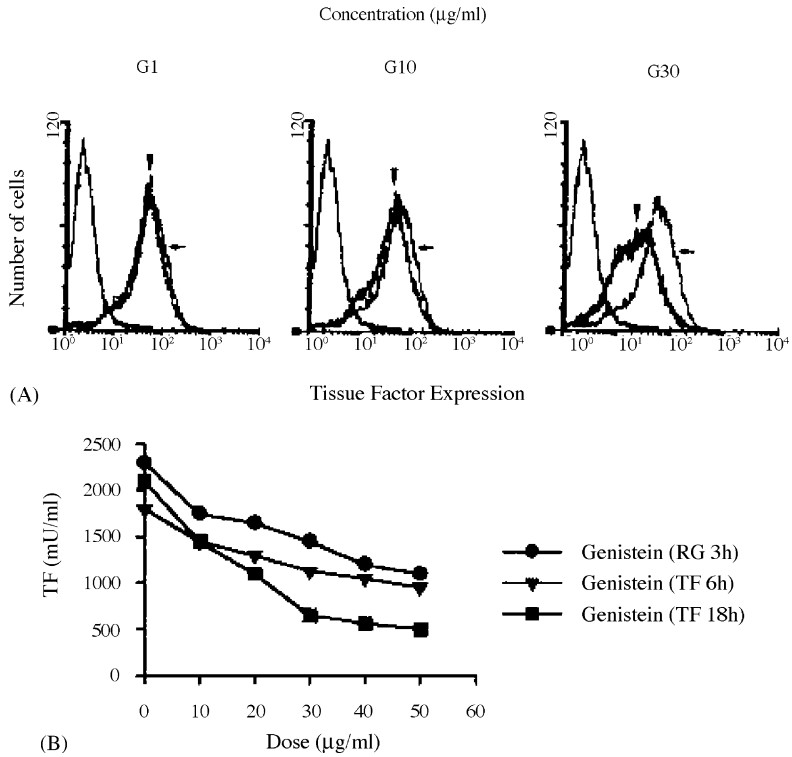

Fig. 8. The inhibitory effect of genistein on surface expression of TF and its biological activity. (A) J82 cells were labeled with FITC-conjugated anti-human TF monoclonal antibody after treatment with appropriate concentrations of genistein for 12 h. The expression levels of TF antigen on the cell surface was analyzed by flow cytometry. There was a dose-dependent inhibition of TF expression on the cell surface of J82 cells (arrowhead) compared with controls (arrow). (B) The functional assay of TF was performed using one-stage clotting test as previously described (54). J82 cells were first treated with genistein in various concentrations for different period of time (3, 6, and 18 h, respectively). Then TF activity was measured by incubation with 0.1 ml of pre-warmed human plasma and 0.1 ml 25 mM $CaCl_2$. The rate of fibrin formation was recorded on a coagulometer and a linear standard curve (log TF versus log clotting time) was used to quantify TF activity. There was a dose-dependent inhibition of TF activity by genistein. A trend toward time-related suppression of TF activity was also observed.

prostate cancer cells [56,57]. At present, both $VEGF_{165}$ and MMP-9 seem to be the common biological targets of soy isoflavones. Thus, a more comprehensive investigation is required to elucidate the common biochemical targets, as well as the tissue-specific mechanism(s), of soy isoflavones on human cancer.

Expression of membrane TF on cancer cells was reported to positively correlate with the metastatic potential of human cancer [22]. Evidence supporting TF as a marker of early angiogenesis comes from its co-localization with VEGF in breast cancer [22,60] as well as in bladder cancer [14–16]. The suppressive effects of genistein on the surface expression and activity of TF support the importance of TF in the pathogenesis of human bladder cancer and the tumor angiogenesis. Our data is the first proof that TF is an innovative target for soy isoflavones.

It is now known that a number of endogenous inhibitors of angiogenesis, such as angiostatin, endostatin, and TSP-1, could be generated by tumors. TSP-1 was thought to be a tumor suppressor, possibly through inhibition of tumor angiogenesis [15,36]. Actually, loss of TSP-1 accounts for the angiogenic phenotype in the early stage of bladder carcinogenesis (15). The up-regulation of TSP-1 by genistein in RT4 and TSGH8301 cell lines corresponds to Li and Sarkar [57] in their analysis of human prostate cancer cells.

Both endostatin and angiostatin are known to target endothelial cells and thus comprise the new blood vessel formation of tumors. In addition, endostatin exerts a direct anticancer action by blocking the activation of MMP-2, -9, and -13 in tumor cells [61]. Angiostatin was recently shown to significantly inhibit the growth and MVD of human bladder cancer in SCID mice [62]. The capability of genistein in up-regulation of both endostatin and angiostatin provides novel mechanisms for isoflavones to reverse the angiogenic switch of epithelial cancer.

The results of the present study indicate that the anti-angiogenic effects of soy isoflavones may be diverse. In sharp contrast to the cancer cells considered in our model, isoflavones also suppress the growth and DNA synthesis of endothelial cells in vitro in our preliminary survey. Actually, some of the above-mentioned biochemical targets of soy isoflavones, such as TF [60], VEGF [63], PDGF [64], and MMP-2 [65], are over-expressed in the endothelial cells of tumor tissue. In addition, the stromal cells and/or inflammatory cells of tumor tissue have also been report to express VEGF [14,16], PDGF [18], PAI-1 [23], MMP-2, and MMP-9 [27]. Accordingly, soy isoflavones are

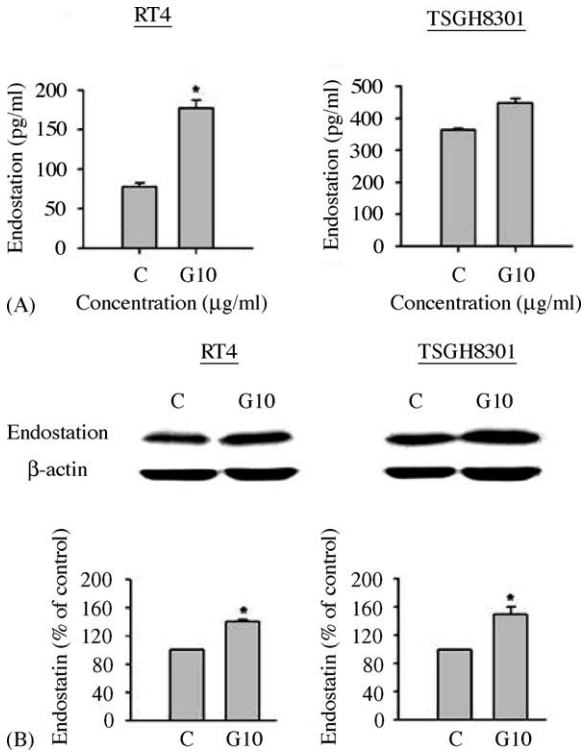

Fig. 9. The effect of genistein on the expression of endostatin. Cell lysates of RT4 and TSGH8301cells were collected after treatment with 10 μg/ml genistein or 0.5% bovine serum albumin as a control (C) for 12 h. (A) Concentrations of endostatin in RT4 and TSGH8301 cells were measured by immunoassay. The stimulatory effect on endostatin expression by genistein in RT4 cells ($P < 0.01$). (B) Alteration of endostatin expression was examined by Western blot. An elevation of endostatin expression was demonstrated in RT4 and TSGH8301 cells at 10 μg/ml genistein ($P < 0.05$). The asterisk indicates the significance of the study group compared with controls.

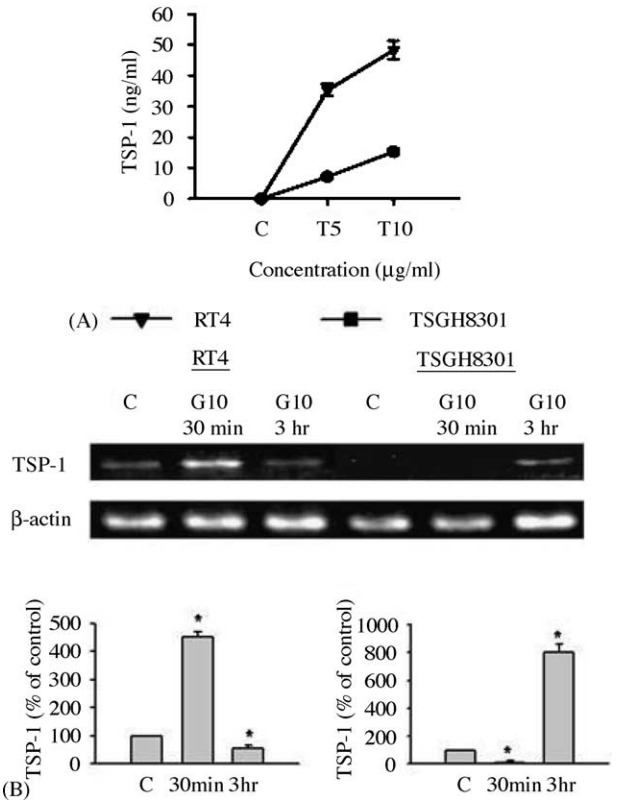

Fig. 11. The stimulatory effect of TSP-1 production/excretion induced by genistein. (A) Supernatants of RT4 and TSGH8301 cells were collected after treatment with appropriate concentrations of genistein or 0.5% bovine serum albumin as a control (C) for 12 h. Genistein from 5 to 10 μg/ml showed a significant dose-related stimulation of TSP-1 production in both cell lines tested ($P < 0.05$). (B) Alteration of TSP-1 RNA in cell lysates was measured by RT-PCR. A comparable transcriptional activation of TSP-1 by genistein was demonstrated in both RT4 (30 min) and TSGH8301 cells (3 h) ($P < 0.01$). The asterisk indicates the significance of the study group compared with controls.

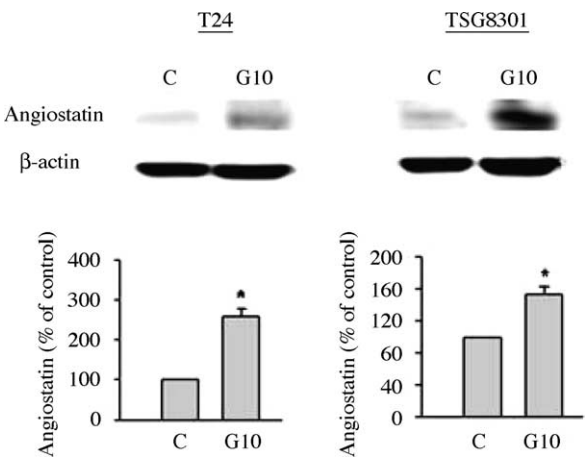

Fig. 10. The effect of genistein on the angiostatin expression. Cell lysates of T24 and TSGH8301cells were collected after treatment with 10 μg/ml genistein or 0.5% bovine serum albumin as a control (C) for 12 h. Alteration of angiostatin expression was examined by Western blot. Both T24 and TSGH8301 cells exhibited stimulatory effect after treatment with 10 μg/ml genistein ($P < 0.05$). The asterisk indicates the significance of the study group compared with controls.

suspected of having a combination of suppressor effects on tumor cells, the stromal compartment of tumor and the neovasculature.

In conclusion, we demonstrated the involvement of pleiotrophic regulators of angiogenesis in the anti-angiogenic activities of genistein. Among them, tissue factor, endostatin, and angiostatin are novel molecular targets of genistein. Taken together with their tumor suppressor effect in vivo, we support the relevance of soy-based foods as natural dietary inhibitors of angiogenesis for human cancer.

Acknowledgements

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References

[1] Folkman J. Tumor angiogenesis factor. Cancer Res 1974;34: 2109–13.

[2] Bochner BH, Cote RJ, Weidner N, Groshen S, Chen SC, Skinner DG, et al. Angiogenesis in bladder cancer: relationship between microvessel density and tumor prognosis. J Natl Cancer Inst 1995;87:1603–12.

[3] Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect 1995;103:103–12.

[4] Messina M, Barnes S. The role of soy products in reducing risk of cancer. J Natl Cancer Inst 1991;83:541–6.

[5] Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, et al. Genistein, a dietary-derived inhibitor of in vitro angiogenesis. Proc Natl Acad Sci USA 1993;90:2690–4.

[6] Fotsis T, Pepper M, Adlercreutz H, Hase T, Montesano R, Schweigerer L, et al. A dietary ingested isoflavonoid, inhibits cell proliferation and in vitro angiogenesis. J Nutr 1995;125:790S–7S.

[7] Zhou JR, Mukherjee P, Gugger ET, Tanaka T, Blackburn GL, Clinton SK. Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. Cancer Res 1998;58:5231–8.

[8] Mokhtar NM, el-Aaser AA, el-Bolkainy MN, Ibrahim HA, el-Din NB, Moharram MZ. Effect of soybean feeding on experimental carcinogenesis-III carcinogenicity of nitrite and dibutylamine in mice: a histopathological study. Eur J Cancer Clin Oncol 1988;24:403–11.

[9] Su SJ, Yeh TM, Lei HY, Chow NH. The potential of soybean foods as a chemoprevention approach for human urinary tract cancer. Clin Cancer Res 2000;6:230–6.

[10] Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, et al. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr 1980;54:1093–100.

[11] Chodak GW, Haudenschild C, Gittes RF, Folkman J. Angiogenic activity as a marker of neoplastic and preneoplastic lesions of the human bladder. Ann Surg 1980;192:762–71.

[12] Chodak GW, Scheiner CJ, Zetter BR. Urine from patients with transitional-cell carcinoma stimulates migration of capillary endothelial cells. N Engl J Med 1981;305:869–74.

[13] Deen S, Ball RY. Basement membrane and extracellular interstitial matrix components in bladder neoplasia—evidence of angiogenesis. Histopathology 1994;25:475–81.

[14] Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, et al. Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol 1993;143:1255–62.

[15] Campbell SC, Volpert OV, Ivanovich M, Bouck NP. Molecular mediators of angiogenesis in bladder cancer. Cancer Res 1998;58:1298–304.

[16] Chow NH, Liu HS, Chan SH, Cheng HL, Tzai TS. Expression of vascular endothelial growth factor in primary superficial bladder cancer. Anticancer Res 1999;19:4593–7.

[17] Mizutani Y, Okada Y, Yoshida O. Expression of platelet-derived endothelial cell growth factor in bladder carcinoma. Cancer 1997;79:1190–4.

[18] O'Brien TS, Fox SB, Dickinson AJ, Turley H, Westwood M, Moghaddam A, et al. Expression of the angiogenic factor thymidine phosphorylase/platelet-derived endothelial cell growth factor in primary bladder cancers. Cancer Res 1996;56:4799–804.

[19] Shirahama T. Cyclooxygenase-2 expression is up-regulated in transitional cell carcinoma and its preneoplastic lesions in the human urinary bladder. Clin Cancer Res 2000;6:2424–30.

[20] Shirahama T, Arima J, Akiba S, Sakakura C. Relation between cyclooxygenase-2 expression and tumor invasiveness and patient survival in transitional cell carcinoma of the urinary bladder. Cancer 2001;92:188–93.

[21] Ristimaki A, Nieminen O, Saukkonen K, Hotakainen K, Nordling S, Haglund C. Expression of cyclooxygenase-2 in human transitional cell carcinoma of the urinary bladder. Am J Pathol 2001;158: 849–53.

[22] Amirkhosravi A, Meyer T, Warnes G, Amaya M, Malik Z, Biggerstaff JP, et al. Pentoxifylline inhibits hypoxia-induced upregulation of tumor cell tissue factor and vascular endothelial growth factor. Thromb Haemost 1998;80:598–602.

[23] Hasui Y, Suzumiya J, Marutsuka K, Sumiyoshi A, Hashida S, Ishikawa E. Comparative study of plasminogen activators in cancers and normal mucosae of human urinary bladder. Cancer Res 1994;49:1067–70.

[24] Hasui Y, Marutsuka K, Nishi S, Kitada S, Osada Y, Sumiyoshi A. The content of urokinase-type plasminogen activator and tumor recurrence in superficial bladder cancer. J Urol 1994;151:16–9.

[25] Hudson MA, McReynolds LM. Urokinase and the urokinase receptor: association with in vitro invasiveness of human bladder cancer cell lines. J Natl Cancer Inst 1997;89:709–17.

[26] Fujiyama C, Jones A, Fuggle S, Bicknell R, Cranston D, Harris AL. Human bladder cancer invasion model using rat bladder in vitro and its use to test mechanisms and therapeutic inhibitors of invasion. Br J Cancer 2001;84:558–64.

[27] Davies B, Waxman J, Wasan H, Abel P, Williams G, Krausz T, et al. Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion. Cancer Res 1993;53:5365–9.

[28] Grignon DJ, Sakr W, Toth M, Ravery V, Angulo J, Shamsa F, et al. High levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) expression are associated with poor outcome in invasive bladder cancer. Cancer Res 1996;56:1654–9.

[29] Kanayama H, Yokota K, Kurokawa Y, Murakami Y, Nishitani M, Kagawa S. Prognostic values of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression in bladder cancer. Cancer 1998;82:1359–66.

[30] Furukawa A, Tsuji M, Nishitani M, Kanda K, Inoue Y, Kanayama H, et al. Role of the matrix metalloproteinase and tissue inhibitors of metalloproteinase families in noninvasive and invasive tumors transplanted in mice with severe combined immunodeficiency. Urology 1998;51:849–53.

[31] Ozdemir E, Kakehi Y, Okuno H, Yoshida O. Role of matrix metalloproteinase-9 in the basement membrane destruction of superficial urothelial carcinomas. J Urol 1999;161:1359–63.

[32] Sier CF, Casetta G, Verheijen JH, Tizzani A, Agape V, Kos J, et al. Enhanced urinary gelatinase activities (matrix metalloproteinases 2 and 9) are associated with early-stage bladder carcinoma: a comparison with clinically used tumor markers. Clin Cancer Res 2000;6:2333–40.

[33] Hara I, Miyake H, Hara S, Arakawa S, Kamidono S. Significance of matrix metalloproteinases and tissue inhibitors of metalloproteinase expression in the recurrence of superficial transitional cell carcinoma of the bladder. J Urol 2001;165:1769–72.

[34] Hamasaki T, Hattori T, Kimura G, Nakazawa N. Tumor progression and expression of matrix metalloproteinase-2 (MMP-2) mRNA by human urinary bladder cancer cells. Urol Res 1998;26:371–6.

[35] Hudson MA, McReynolds LM. Urokinase and the urokinase receptor: association with in vitro invasiveness of human bladder cancer cell lines. J Natl Cancer Inst 1997;89:709–17.

[36] Grossfeld GD, Ginsberg DA, Stein JP, Bochner BH, Esrig D, Groshen S, et al. Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression. J Natl Cancer Inst 1997;89:219–27.

[37] Westphal JR, Van't Hullenaar R, Geurts-Moespot A, Sweep FC, Verheijen JH, Bussemakers MM, et al. Angiostatin generation by human tumor cell lines: involvement of plasminogen activators. Int J Cancer 2000;86:760–7.

[38] Peterson G. Evaluation of the biochemical targets of genistein in tumor cells. J Nutr 1995;125:784S–9S.

[39] Middleton Jr E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000;52:673–751.
[40] Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL, Clinton SK. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. J Nutr 1999;129:1628–35.
[41] Shao ZM, Wu J, Shen ZZ, Barsky SH. Genistein exerts multiple suppressive effects on human breast carcinoma cells. Cancer Res 1998;58:4851–7.
[42] Reznikoff CA, Belair C, Savelieva E, Zhai Y, Pfeifer K, Yeager T, et al. Long-term genome stability and minimal genotypic and phenotypic alterations in HPV16 E7-, but not E6-, immortalized human uroepithelial cells. Genes Dev 1994;8:2227–40.
[43] Yeh MY, Yu DS, Chen SC, Han SH, Wang YC. Establishment and characterization of a human urinary bladder carcinoma cell line (TSGH-8301). J Surg Oncol 1988;37:177–84.
[44] Sheu JR, Chang CF, Tsai ML, Chuang WJ. Effect of U-995, a potent shark cartilage-derived angiogenesis inhibitor, on anti-angiogenesis and anti-tumor activities. Anticancer Res 1998;18:4435–42.
[45] Miller WJ, Kayton ML, Patton A, O'Connor S, He M, Vu H, et al. A novel technique for quantifying changes in vascular density, endothelial cell proliferation and protein expression in response to modulators of angiogenesis using the chick chorioallantoic membrane (CAM) assay. J Transl Med 2004;2:4.
[46] Senger DR, Perruzzi CA, Streit M, Koteliansky VE, de Fougerolles AR, Detmar M. The alpha(1)beta(1) and alpha(2)beta(1) integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. Am J Pathol 2002;160:195–204.
[47] Mizutani Y, Kamoi K, Ukimura O, Kawauchi A, Miki T. Synergistic cytotoxicity and apoptosis of jte-522, a selective cyclooxygenase-2 inhibitor, and 5-fluorouracil against bladder cancer. J Urol 2002;168:2650–4.
[48] Hamasaki T, Hattori T, Kimura G, Nakazawa N. Tumor progression and expression of matrix metalloproteinase-2 (MMP-2) mRNA by human urinary bladder cancer cells. Urol Res 1998;26:371–6.
[49] Hamasaki H, Hattori T, Kimura G, Nakazawa N. Tumor progression and expression of matrix metalloproteinase-2 (MMP-2) mRNA by human urinary bladder cancer cells. Urol Res 1998;26:371–6.
[50] Champelovier P, Boucard N, Levacher G, Simon A, Seigneurin D, Praloran V. Plasminogen- and colony-stimulating factor-1-associated markers in bladder carcinoma: diagnostic value of urokinase plasminogen activator receptor and plasminogen activator inhibitor type-2 using immunocytochemical analysis. Urol Res 2002;30:301–9.
[51] Vikhanskaya F, Bani MR, Borsotti P, Ghilardi C, Ceruti R, Ghisleni G, et al. G. p73 overexpression increases VEGF and reduces thrombospondin-1 production: implications for tumor angiogenesis. Oncogene 2001;20:7293–300.
[52] Basciani S, Mariani S, Arizzi M, Ulisse S, Rucci N, Jannini EA, et al. Expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, and PDGF receptor-alpha and -beta during human testicular development and disease. J Clin Endocrinol Metab 2002;87:2310–9.
[53] Su SJ, Lai MD, Yeh TM, Chow NH. Overexpression of HER-2/neu enhances sensitivity of human bladder cancer cells to urinary isoflavones. Eur J Cancer 2001;37:1413–8.
[54] Su SJ, Chang KL, Lin TM, Huang YH, Yeh TM. Uromodulin and Tamm-Horsfall protein induce human monocytes to secrete TNF and express tissue factor. J Immunol 1997;158:3449–56.
[55] Alhasan SA, Aranha O, Sarkar FH. Genistein elicits pleiotropic molecular effects on head and neck cancer cells. Clin Cancer Res 2001;7:4174–81.
[56] Myoung H, Hong SP, Yun PY, Lee JH, Kim MJ. Anti-cancer effect of genistein in oral squamous cell carcinoma with respect to angiogenesis and in vitro invasion. Cancer Sci 2003;94:215–20.
[57] Li Y, Sarkar FH. Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. Cancer Lett 2002;186: 157–64.
[58] Li Y, Sarkar FH. Gene expression profiles of genistein-treated PC3 prostate cancer cells. J Nutr 2002;132:3623–31.
[59] Kim MH. Flavonoids inhibit VEGF/bFGF-induced angiogenesis in vitro by inhibiting the matrix-degrading proteases. J Cell Biochem 2003;89:529–38.
[60] Contrino J, Hair G, Kreutzer DL, Rickles FR. In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. Nat Med 1996;2:209–15.
[61] Nyberg P, Heikkila P, Sorsa T, Luostarinen J, Heljasvaara R, Stenman UH, et al. Endostatin inhibits human tongue carcinoma cell invasion and intravasation and blocks the activation of matrix metalloproteinase-2, -9, and -13. J Biol Chem 2003;278:22404–11.
[62] Beecken WD, Fernandez A, Joussen AM, Achilles EG, Flynn E, Lo KM, et al. Effect of antiangiogenic therapy on slowly growing, poorly vascularized tumors in mice. J Natl Cancer Inst 2001;93:382–7.
[63] Eisma RJ, Spiro JD, Kreutzer DL. Vascular endothelial growth factor expression in head and neck squamous cell carcinoma. Am J Surg 1997;174:513–7.
[64] de Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer I: an inventory in search of autocrine and paracrine loops. J Pathol 1998;184:44–52.
[65] St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, et al. Genes expressed in human tumor endothelium. Science 2000;289:1197–202.