大豆黃酮類化合物抗癌機轉的研究(3/3)

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The Molecular Basis for Anti-angiogenic Effects of Soy Isoflavones

ABSTRACT

Soy isoflavones have been reported to exhibit tumor suppressor effects at the concentrations found in the range of human urine excretion. The cooperative action of isoflavones is more effective in dose-dependent growth inhibition and apoptosis induction than any single compound. This study was designed to identify the molecular basis underlying anti-angiogenic activities of soy isoflavones on human cancer. An immortalized E6 and five bladder cancer cell lines were tested—by immunoassay, flow cytometry, functional activity, reverse transcription-polymerase chain reaction, and immunoblotting—for the efficacy of soy isoflavones on angiogenesis inhibition in vivo. Factors analyzed included urokinase plasminogen activator (uPA), tissue plasminogen activator, plasminogen activator inhibitor-1 (PAI-1), vascular endothelial growth factor (VEGF_{165}), platelet-derived growth factor (PDGF), matrix metalloprotease-2 and 9 (MMP-2 and MMP-9), transmembrane MMP, cyclooxygenase-2 (COX-2), tissue factor (TF), and thrombospondin-1 (TSP-1). Genistein was among the most potent angiogenesis inhibitors of soy isoflavones in vivo. Both genistein and a cocktail regimen of isoflavones exhibited a dose-dependent inhibition of expression/excretion of angiogenic factors (VEGF_{165}, PDGF, and TF) and matrix-degrading enzymes (uPA, PAI-1, MMP-2, and MMP-9). However, there was
no altered expression of COX-2, transmembrane MMP, or TIMP-2. On the contrary, isoﬂavones up-regulate the expression of angiogenesis inhibitor TSP-1. Interestingly, we observed a differential inhibitory effect between immortalized uroepithelial cells and most cancer cell lines tested. The current investigation disclosed diverse pathways of angiogenesis inhibition induced by soy isoﬂavones, which support the hypothesis that they act as natural dietary inhibitors of angiogenesis for human cancer.

**Keywords:** Bladder cancer, soy isoﬂavones, anti-angiogenesis, angiogenic factors, proteases

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

Angiogenesis, the generation of new blood vessels, is required for the growth as well as expansion of solid tumors, especially those at 1 to 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 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 IC50 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). These results lead us to hypothesize that soy isoflavones 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). A number of biochemical targets and pharmacological actions have been proposed (37,38), and anti-angiogenesis is one of the important mechanisms responsible for anti-cancer effects (5,7,39,40). Using bladder cancer as a paradigm, this study was designed to identify the molecular mechanisms of the anti-angiogenic effect of soy isoflavones in the range of urine excretion.
MATERIALS AND METHODS

Chick chorioallantoic membrane (CAM) bioassay

The technique of CAM treatment was performed as described with slight modification (41). 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, NY), daidzein (Calbiochem-Novabiochem, CA), or biochanin-A (Sigma, MO) dissolved in Dimethyl sulfoxide (DMSO, Sigma) either as a single compound or as a cocktail mixture (genistein, daidzein, and biochanin-A in equal proportions). DMSO was kept below 0.05% (v/v) was used as control. The CAMs were photographed on day 11 using an Olympus stereomicroscope SZX9 (Fig. 1). Two independent observers (SJS and NHC) semi-quantitatively scored the degree of angiogenesis as follows: 0 = none, 1 = low, 2 = medium, and 3 = high (42). Average values for 6 embryos were recorded. The means of the scores were analyzed by the Wilcoxon Signed Rank test and pair-wise comparisons of the different treatment groups (CRUNCH Software Corporation).

Cell Culture

The E6 cell line was an immortalized human uroepithelium (43). Human bladder cancer cell lines RT4, J82, 5637, and T24 were obtained from the American Type
Culture Collection (Rockville, MD). TSGH8301 was established locally and has been reported in detail previously (44). 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 $1 \times 10^5$ cells/well and grown for experiment.

**Immunoassay for angiogenic factors, MMP, and proteases**

Supernatants of each cell line were collected after treatment with appropriate concentrations of isoflavones or controls (0.5% bovine serum albumin) for 12 hours. The analysis included VEGF, PDGF-AB, and MMP-9 by using the Quantikine ELISA kit (Research and Diagnostics Systems, Inc., Minneapolis, MN). The immunoassay for proteases included uPA, tPA, or PAI (American Diagnostica Inc., Greenwich, CT). 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 hours 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 H₂SO₄) and the optical density was read using a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) set at 450 nm. At least 2 batches of conditioned media were tested, and the mean values were used as the final concentration. The sensitivities were 5 pg/ml for VEGF₁₆₅, 5 pg/ml for MMP-9, 1.7 pg/ml for PDGF-AB, and 0.05 ng/ml for uPA and PAI. The values obtained after ELISA were corrected with a dilution factor and were finally expressed in pg/ml. The angiogenic factors and proteases in each group were compared and the significance of the results analyzed by the Student's *t*-test.

**Immunoblotting analysis**

The procedure of western blotting was performed as previously described (45). 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 µg 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 hours using an electroblot apparatus (Hoefer TE70 semidry transfer unit; Amersham Pharmacia Biotech, Piscataway, NJ). The nitrocellulose filter was first blocked with 5% skim milk (Difco Laboratories, Inc., Detroit, MI) for 1 hour
and then probed with monoclonal antibody to MT-MMP and TIMP-2 (Fuji Chemical Industries, Toyama, Japan) or COX-2 (Cayman Chemical Inc., Ann Arbor, MI) for 2 hours. The protein bands were visualized with an enhanced chemiluminescence detection kit (Amersham Corp., Arlington Heights, IL) using horseradish peroxidase-labeled secondary antibody, as suggested by the manufacturer. Equal loading of cell lysates was confirmed by staining the nitrocellulose membrane with amido black after western blotting analysis.

**Measurement of TF activity and surface expression on cancer cells**

A functional TF assay was performed using a one-stage clotting test as previously described (46). Briefly, J82 cells were first treated with various concentrations of isoflavones 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 CaCl₂. The rate of fibrin formation was recorded on a coagulometer (Diagnostica Stago ST4; Diagnostica Stago, Asnieres, France). 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 seconds. Generally, 100 U/ml of TF clotted normal human plasma in 12 to 14 seconds. 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 isoflavone compounds for 12 hours. The expression of TF on the cell surface was determined directly by flow cytometry. Ten mg of mouse IgG (FITC-conjugated; Dako, Carpinteria, CA) was used as a negative control. The percentage of positive fluorescence was determined in comparison to an isotype control for TF antigen.

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. Briefly, total RNA was extracted from cells with TRIzol reagent (Life Technologies, Glasgow, UK) 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, Foster City, CA). Primers used for priming the COX-2 gene were 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). Primers for MT-MMP were sense, 5′-CCCTATGCTACATCCGTGA-3′, and
anti-sense, 5'-TCCATCCATCACCTGGTTAT-3' (product size 550 bp) (48). Primers used for priming the TIMP-2 gene were sense, 5'-CCGAATTCTGCAGCTGGCCGGTGCACCCG-3', and anti-sense, 5'-GGAAGCTTTTATGGTCCCTCGGAG-3' (product size 590 bp) (34). The primers for TSP-1 were sense, 5'-CGTCCCTGCTTCATGATGCATG, and anti-sense, 5'-GGCAGGACACCTTTTTGAG (product size 1000 bp) (49). The β-actin gene was amplified as an internal control. RT-PCR conditions for COX-2 were 94 °C for 0.5 minutes, 60 °C for 0.5 minutes, and 72 °C for 2 minutes (25-30 cycles). The conditions for MT-MMP or TIMP-2 were 93 °C for 90 seconds, 58 °C for 90 seconds, and 72 °C for 3 minutes (40 cycles). The amplification conditions for TSP-1 were of 1' at 95 °C, 1' at 55 °C, and 1' at 72 °C (35 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.
RESULTS

We first tested the efficacy of soy isoflavones on angiogenesis in vivo. Using CAM bioassay, we found significantly reduced angiogenesis when the embryos were treated with 10 µg/ml genistein or a cocktail mixture of isoflavones (10 µg/ml) compared with controls (details in Table 1). On the contrary, no significant angiogenesis inhibition was noticed for daidzein or biochanin-A at 5 or 10 µg/ml levels (data not shown). The results are essentially consistent with growth inhibition and apoptosis induction effects of isoflavones in our prior report (9).

Given that tumor angiogenesis is the consequence of a net balance between pro- and anti-angiogenic factors, the effects of isoflavones on the production/excretion of matrix-degrading enzymes (uPA, tPA, and PAI-1), angiogenic factors (VEGF$_{165}$ and PDGF-AB), and MMP-9 were examined in the supernatants of an immortalized uroepithelial cell line (E6) and five human bladder cancer cell lines by immunoassay.

It is interesting to note that genistein alone or isoflavone mixture did not exhibit any apparent inhibitory effect on proteases produced by E6 cells (Fig. 2). In contrast, soy isoflavones showed a significant dose-related suppression of uPA or PAI-1 production ($P < 0.05$) in RT4 cells (grade 1 bladder cancer). No such inhibitory effect was observed for TSGH8301 cells (grade 2 bladder cancer) or J82 cells (grade 3 bladder cancer). Soy isoflavones thus appear to repress the production of proteases in
low-grade bladder cancer cells without a concomitant detrimental effect on the non-neoplastic uroepithelium.

As shown in Figure 3, an anti-angiogenic effect was observed for VEGF, MMP-9, and PDGF-AB in some of the cell lines tested, with PDGF the most significantly suppressed by soy isoflavones. Except for a positive association with histological grading of cancer cell lines for MMP-9 expression, each cell line appeared to have its own expression profile in the production/excretion of angiogenic factors and proteases (Table 2). Only those cell lines showing higher expression levels of angiogenic factors in the supernatants were selected in the following analyses.

We also evaluated the involvement of pro-angiogenic factor TF in the tumor suppressor effect of soy isoflavones. As seen in Figure 4, each isoflavone compound 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 isoflavones were higher than 30 µg/ml. The flow cytometry further confirmed a dose-dependent inhibition of TF expression on the surface of J82 cells (Fig. 5).

The details of biomarkers showing inhibitory effect in by soy isoflavones at the protein level were recorded in Table 2. Taken together, soy isoflavones appears to exhibit suppressor effect on multiple angiogenesis-related pathways on human bladder cancer cells in vitro.
Next, the alterations of VEGF$_{165}$ and PDGF-AB in response to isoflavone treatment were examined on a 24-hour basis, respectively. Representative results on VEGF$_{165}$ in three model cell lines were shown in Figure 6. 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 hours later ($P < 0.05$). The most striking suppressive effect was observed at 3 hours after treatment with isoflavones ($P < 0.01$).

Then suppressive effect on MMP-2 was compared by RT-PCR at 30 minutes and 3 hours after treatment with isoflavone mixture, respectively. As shown in Figure 7A, expression of MMP-2 was apparently reduced in J82 and 5637 cells after treatment with isoflavones, but not in TSGH8301 cells (data not shown). We also examined the potential impact of isoflavones on the expression of angiogenesis inhibitor TSP-1 in human bladder cancer cell lines. Semi-quantitative RT-PCR revealed that up-regulation of TSP-1 was observed in RT4 cells as early as 30 minutes after treatment with isoflavones, but a weaker stimulatory effect was noticed for TSGH8301 cells only after 3 hours' treatment (Fig. 7B). No such stimulatory effect was observed for either J82 or TCCSUP cells (data not shown). However, there was no apparent inhibitory effect on the expression of COX-2, MT-MMP, or TIMP-2, respectively (data not shown). The lack of modulating effect on these three angiogenesis-related molecules was also verified by western blotting.
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 isoflavone mixtures, within the physiological range of urine excretion, inhibit the expression or secretion of angiogenic factors (such as VEGF₁₆₅, PDGF, and TF) and matrix-degrading enzymes (such as uPA, PAI-1, MMP-2, and MMP-9) of cancer cells. Hence, one of the effects of isoflavones on tumor angiogenesis is to reduce the proliferation and migration of tumor cells through ECM. The suppression of uPA, MMP-2, and MMP-9 possibly will also interrupt the breakdown of ECM surrounding the growing vessels and tumor. In addition, we demonstrated that soy isoflavones upregulate the expression of angiogenesis inhibitor TSP-1. As a result, soy isoflavones appears to restore the balance between pro- and
anti-angiogenesis on the side of angiogenesis inhibition, resulting in growth arrest of
the tumor.

However, such angiogenic inhibition of soy isoflavones might have cell-specific
relevance. Shao et al. (40) reported that genistein could inhibit the invasion of MCF-7
and MDA-MB-231 breast carcinoma cells in vitro by downregulating MMP-9 and
upregulating the tissue inhibitor of MMP. Genistein also inhibited angiogenesis by
decreasing vessel density and levels of VEGF_{iso} and TGF-β1. With limited available
information, both VEGF_{iso} and MMP-9 seem to be the common biological targets of
soy isoflavones for breast and bladder cancers in the literature. A more
comprehensive investigation is required to elucidate the universal biochemical targets,
as well as the tissue-specific mechanism(s), of soy isoflavones on human cancer.

A number of molecular mechanisms have been proposed to explain the tumor
suppressor effect of soy isoflavones. Genistein has been shown to reduce tumor cell
proliferation at micromolar doses (above 10–50 μM) (50) through cell-cycle
inhibition (51,52) and/or apoptosis induction (5,13,14). The primary intracellular
targets for apoptosis of cancer cells are tyrosine kinases, such as EGF receptor (53),
and topoisomerase (54), are although some recent reports have proposed direct action
on the mitochondrial permeability transition pore (55) or ion channel activity (56,57).
In our prior report, HER-2/neu is an additional biochemical target of soy isoflavones
It is interesting to note that both TF and TSP-1 are innovative targets for soy isoflavones. Expression of membrane TF on cancer cells was reported to be a malignant phenotype in human breast tissue (58), and is positively correlated 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 (22,58) as well as in bladder cancer (14-16). The results reinforce the significance of TF in the development of human bladder cancer.

TSP-1 is one of the important glycoproteins of the ECM and is known to be a potent inhibitor of angiogenesis in vitro and in vivo (15). Several reports suggest that TSP possesses tumor suppressor function, possibly through its ability to inhibit tumor neovascularization (15,36). Currently, loss of the inhibitory function of TSP-1 accounts for the development of an angiogenic phenotype in the early stage of bladder carcinogenesis (15). The upregulation of TSP-1 by isoflavones in RT4 (original grade 1 bladder cancer) and weakly in TSGH8301 (original grade 2 bladder cancer) cell lines further support the notion that soybean-based foods suppress angiogenesis in human bladder cancer at an early stage.

The results of the present study demonstrate that the anti-angiogenic effects of soy isoflavones may be diverse, although we failed to prove their effectiveness on
COX-2. In sharp contrast to the cancer cells considered in our model, endothelial cells, which are common to all solid tumors, may represent a preferential target for cancer therapy. Actually, some of the above-mentioned biochemical targets of soy isoflavones, such as TF (58), VEGF (59), PDGF (60), and MMP-2 (61), are also over-expressed in the endothelial cells of tumor tissue. For that reason, soy isoflavones are suspected of having a combination of direct effects on tumor cells and indirect effects on tumor neovascularure.

In conclusion, the current study identifies pleiotropic inhibitory activities of isoflavones on tumor angiogenesis. Taken together with their reported tumor suppressor effect *in vivo*, we support the role of soy isoflavones as natural dietary inhibitors of angiogenesis in human cancer.
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Table 1. The effect of isoflavones on angiogenesis assessed by CAM bioassay

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Mean scores ± SD</th>
<th>$P$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (control)</td>
<td>3.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Genistein (5 µg/ml)</td>
<td>2.50 ± 0.55</td>
<td>0.11 *</td>
</tr>
<tr>
<td>Genistein (10 µg/ml)</td>
<td>1.33 ± 0.62</td>
<td>0.03 *</td>
</tr>
<tr>
<td>Cocktail mixture (10 µg/ml)</td>
<td>0.33 ± 0.52</td>
<td>0.03 *</td>
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</table>

SD: standard deviation

*Compared with the control group.

†Compared with those treated with isoflavone mixture.
### Table 2. The inhibitory effects of isoflavones on angiogenic factors and matrix-degrading enzymes

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>tPA</th>
<th>uPA</th>
<th>PAI-1</th>
<th>TF</th>
<th>MMP-9</th>
<th>VEGF&lt;sub&gt;165&lt;/sub&gt;</th>
<th>PDGF</th>
</tr>
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<tbody>
<tr>
<td>E6</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>ND&lt;sup&gt;†&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RT4</td>
<td>+</td>
<td>+&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>+&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>ND</td>
<td>+</td>
<td>+++&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>TSGH8301</td>
<td>+&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>+&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T24</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>ND</td>
<td>+</td>
<td>++&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>J82</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>++&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5637</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+++&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>+</td>
<td>+&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*</sup>Relative levels of angiogenic factors were arbitrarily categorized as four scoring scales.

<sup>†</sup>ND: not done

<sup>‡</sup>A significant inhibitory effect by soy isoflavones was observed.
**Figure Legends**

**Figure 1.** The inhibitory effect of isoflavones on chick CAM angiogenesis in vivo. A: Representative appearance of chick CAMs in the control group was seen in the left half of figure (scored as high level of angiogenesis). B: The CAM angiogenesis in one of experimental embryos showing markedly decreased angiogenesis (scored as low level of angiogenesis). The embryo had been treated with 10 µg/ml of genistein for four days. The results were photographed on day 11 of the experiment.

**Figure 2.** The inhibitory effects of isoflavones on the production/excretion of matrix-degrading enzymes uPA, tPA, and PAI-1. Supernatants of each cell line were collected after treatment with appropriate concentrations of isoflavones or 0.5% bovine serum albumin as a control (C) for 12 hours. Representative results on uPA, tPA, and PAI-1 were shown here. Either genistein alone or isoflavone mixture did not exhibit apparent inhibitory effect on proteases produced by E6 cells from 2.5-10 µg/ml genistein (G2.5-G10) to 1.5-5 µg/ml isoflavone cocktail mixture (T1.5-T5). There was no apparent inhibitory effect was observed for tPA in any cell line tested. Soy isoflavones had a significant dose-related suppression of uPA or PAI-1 production (p < 0.05) in RT4 cells, but not in TSGH 8301 cells.

**Figure 3.** Suppressive effects of isoflavones on the production/excretion of
angiogenic factors and MMPs. Supernatants of each cell line were collected after treatment with appropriate concentrations of isoflavones or 0.5% bovine serum albumin as a control (C) for 12 hours. Only those cell lines with higher expression levels of angiogenic factors in the supernatants were selected in this experiment. Representative results on VEGF₁₆₅, MMP-9, and PDGF-AB are shown in the figure. A dose-dependent inhibition of VEGF₁₆₅, MMP-9, and PDGF-AB was observed in RT4 and 5637 cells, respectively, from 2.5-10 µg/ml genistein (G₂.₅-G₁₀) to 1.5-5 µg/ml isoflavone cocktail mixture (T₁.₅-T₅).

**Figure 4.** The inhibitory effect of isoflavones on TF activity. The functional assay of TF was performed using one-stage clotting test as previously described (44). J82 cells were first treated with biochanin A, genistein or daidzein in various concentrations for different period of time (3, 6, and 18 hours, respectively). Then TF activity was measured by incubation with 0.1 ml of pre-warmed human plasma and 0.1 ml 25 mM CaCl₂. 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 all three isoflavone compounds. A trend toward time-related suppression of TF activity was also observed when isoflavone concentrations were higher than 30 µg/ml.
Figure 5. The inhibitory effect of isoflavones on the surface expression of TF by flow cytometry. J82 cells were labeled with FITC-conjugated anti-human TF monoclonal antibody after treatment with appropriate concentrations of isoflavone compounds for 12 hours. The expression levels of TF antigen on the cell surface was analyzed by flow cytometry. All three types of isoflavone compounds exhibited a dose-dependent inhibition of TF expression on the cell surface of J82 cells (arrowhead) compared with controls (arrow).

Figure 6. The kinetics of angiogenesis inhibition induced by soy isoflavones on human bladder cancer cells. The suppressive effect of soy isoflavones on VEGF$_{165}$ production/excretion was measured by immunoassay on RT4, TSGH8301 and T24 cells. Supernatants of each cell line ($1 \times 10^5$) were determined for VEGF$_{165}$ levels after treatment with 10 µg/ml isoflavone mixture during 24 hours period. The excretion of VEGF$_{165}$ were inhibited as early as 15 minutes after treatment, but was reverted to baseline levels when tumor cells had been treated with isoflavones for more than 16 hours. The most striking suppressive effect was observed at 3 hours after treatment. The results are expressed as the mean ± S.D. for three independent experiments.
Figure 7. RT-PCR screening of suppressive effect of isoflavones on the expression of MMP-2 and TSP-1. Total RNA was extracted from cells with TRIzol reagent from bladder cancer cell lines with/without treatment with isoflavone mixture 10 µg/ml. A. Semi-quantitative RT-PCR revealed inhibition of MMP-2 expression in both J82 and 5637 cells (lines 1 & 4) after treatment with isoflavones for 30 minutes (lines 2 & 5) or 3 hours (lines 3 & 6). B. Isoflavone mixture up-regulated the TSP-1 expression in RT4 cells (line 1) as early as 30 minutes after treatment (line 2). However, such stimulatory effect in TSGH8301 cells (line 4) was observed only after 3 hours’ treatment (line 6).