

Soy Phytochemicals Prevent Orthotopic Growth and Metastasis of Bladder Cancer in Mice by Alterations of Cancer Cell Proliferation and Apoptosis and Tumor Angiogenesis

Ajita V. Singh,¹ Adrian A. Franke,² George L. Blackburn,¹ and Jin-Rong Zhou¹

¹Nutrition/Metabolism Laboratory, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts and ²Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii

Abstract

A role of dietary bioactive components in bladder cancer prevention is biologically plausible because most substances or metabolites are excreted through the urinary tract and are consequently in direct contact with the mucosa of the bladder. We first determined antigrowth activity of genistein against poorly differentiated 253J B-V human bladder cancer cells *in vitro*. Genistein inhibited the cell growth in a time- and dose-dependent manner via G₂-M arrest, down-regulation of nuclear factor κ B (NF- κ B), and induction of apoptosis. We also evaluated both genistin, which is a natural form of genistein, and the isoflavone-rich soy phytochemical concentrate (SPC) on the growth and metastasis of 253J B-V tumors in an orthotopic tumor model. Mice treated with genistin and SPC had reduced final tumor weights by 56% ($P < 0.05$) and 52% ($P < 0.05$), respectively, associated with induction of tumor cell apoptosis and inhibition of tumor angiogenesis *in vivo*. In addition, SPC treatment, but not genistin treatment, significantly inhibited lung metastases by 95% ($P < 0.01$) associated with significant down-regulation of NF- κ B expression in tumor tissues and reduction of circulating insulin-like growth factor-I levels, suggesting that SPC may contain other bioactive ingredients that have antimetastatic activity. The results from our studies suggest that further clinical investigation should be warranted to apply soy phytochemicals, such as SPC, as a potent prevention regimen for bladder cancer progression. This orthotopic human bladder tumor model also provides a clinically relevant experimental tool for assessing potential preventive activity of other dietary components against bladder tumor growth and metastasis. (Cancer Res 2006; 66(3): 1851-8)

Introduction

Bladder cancer is the fifth most commonly diagnosed malignancy in the United States in 2005 (1). Bladder cancer comprises a broad spectrum of tumors with various histologic types. Transitional cell carcinomas are by far the more prevalent tumors and represent nearly 95% of all bladder cancers in the Western Hemisphere. About 70% of the urinary bladder transitional cell carcinomas are diagnosed at presentation as well-differentiated superficial lesions and the rest correspond to highly invasive, poorly differentiated tumors. Although patients with superficial

disease initially responded successfully to transurethral resection via the cystoscope and additional treatment with intravesical chemotherapy or immune therapy, these patients are at high risk (~60%) of developing recurrent superficial bladder cancer. Of those, some (10-40%) progress to invasive/metastatic disease and are therefore potentially lethal (2).

The bladder cancer mortality varies in different countries. The highest rates are noted in European countries, such as Denmark, United Kingdom, Belgium, and Italy, the lowest rates in Asia countries, such as Japan, China, and Singapore (3). Populations in Southeast Asia have 4- to 10-fold lower incidence of and death from bladder cancer compared with those in the United States (4). Asian people migrating to the United States have increased risk in one generation to equal that of the Americans (5). Cigarette smoking is the major risk factor in affluent nations whereas exposure to chemical carcinogens in environment, particularly in workplace, is also a contributing factor (3, 6). However, definite etiology for bladder cancer is still unknown. With unknown etiologic factors for bladder cancer carcinogenesis and the fact that bladder cancer patients are facing both threat of metastases and uncontrolled local recurrence after treatment, additional efforts to define alternative intervention to prevent bladder cancer progression and metastasis are urgently needed.

Increased intake of soy products has been suggested to be associated in part with reduced risks of certain cancers, such as breast and prostate cancers, in Asian countries (7). Epidemiologic, *in vitro*, and laboratory animal studies have provided evidence for the hypothesis that certain soy phytochemicals, especially soy isoflavones, have anticarcinogenic and antitumorigenic properties (7, 8). Urine is a significant route for excretion of soy isoflavones and their metabolites (9-11) and provides an enriched resource of bioactive soy isoflavones. For example, the urinary level of soy isoflavones could reach 50 μ mol/L or higher after soy supplementation whereas the circulating level of soy isoflavones is only at a few micromolars (12). Therefore, soybean bioactive components may be particularly effective in the prevention of bladder cancer progression by providing higher levels of bioactive components to bladder tumor via both blood circulation and urinary exposure.

The epidemiologic association between soy consumption and bladder cancer development or progression has not been established. In a case-control study, soy juice intake was associated with a nonsignificant reduction of bladder cancer risk (13). In population-based cohort studies, soy consumption was associated with a significant increase of bladder cancer risk (14, 15). In contrast, experimental studies have shown that soy bioactive components, such as genistein, may have antibladder cancer activity. Genistein inhibited the growth of a bladder tumor xenograft (16). We evaluated the effects of several soy components such as genistein, soy phytochemicals, and soy protein on bladder

Requests for reprints: Jin-Rong Zhou, Nutrition/Metabolism Laboratory, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Burlington-5, 330 Brookline Avenue, Boston, MA 02215. Phone: 617-632-9222; Fax: 617-632-0275; E-mail: jrzhou@bidmc.harvard.edu.

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-05-1332

tumor growth and found that genistein and genistein-rich soy phytochemicals had more potent effects in inhibiting the growth of a murine bladder cancer xenograft (17). These results suggest that soy bioactive components may play an important role in the prevention of bladder cancer growth and progression. However, both studies have used s.c. implantation of bladder cancer cells. Therefore, it is imperative to evaluate the antibladder cancer activity of soy bioactive components by using a clinically relevant orthotopic bladder tumor model.

Materials and Methods

Materials. Soy isoflavone aglycones, genistein, glycitein, and daidzein, were purchased from LC Laboratories, Inc. (Woburn, MA) for the *in vitro* assays. Tissue culture media, fetal bovine serum (FBS), and trypsin were purchased from Life Technologies, Inc. (Grand Island, NY). Propidium iodide was from Sigma (St. Louis, MO) and RNase A and 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were from Promega (Madison, WI). Cell death detection ELISA kit was from Roche Diagnostics GmbH (Mannheim, Germany). Nuclear and cytoplasmic extraction and Trans AM ELISA kits were purchased from Active Motif North America (Carlsbad, CA). Antibodies against cyclin B1, nuclear factor κ B (NF- κ B), and β -actin were from Oncogene Research Products (Boston, MA); antibodies against cleaved caspase-3, cleaved poly(ADP ribose) polymerase (PARP), and I κ B- α were from Cell Signaling (Beverly, CA); antibodies against Bax, cyclin-dependent kinase 1 (Cdk-1), and Cdc-25C were from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies against Bcl-2 were from BD Pharmingen (San Diego, CA).

To evaluate the antibladder cancer activity of soy phytochemicals, an isoflavone-rich soy phytochemical extract, soy phytochemical concentrate (SPC), was used as the source of the soy phytochemical supplement to mimic soy phytochemical compositions commonly consumed by humans. It contained 51.9% soy isoflavones by weight (50.8% genistein aglycone equivalents, 40.5% daidzein aglycone equivalents, and 8.7% glycitein aglycone equivalents). Other phytochemicals in the SPC were not quantified. Genistin was used as the source of the natural glucoside form of the soy isoflavone genistein because >95% of genistein is present in the glucoside form. Genistin was purified from SPC and contained 100% soy isoflavones by weight (90.1% genistein aglycone equivalents, 9.1% daidzein aglycone equivalents, and 0.8% glycitein aglycone equivalents). Both SPC and genistin were provided by Archer Daniels Midland Company (Decatur, IL). The company used high-performance liquid chromatography to analyze isoflavone levels.

Cell culture. Highly metastatic human bladder cancer cell line 253J B-V was generously provided by Dr. Colin P.N. Dinney (University of Texas M.D. Anderson Cancer Center, Houston, TX). 253J B-V cell line was selected from the study because it develops a poorly differentiated and highly metastatic human bladder tumor that represents a highly lethal phenotype of transitional cell carcinoma *in vivo*. This cell line was established from 253J human bladder cancer cell line by orthotopic implantation and *in vivo* recycling (18). Monolayer culture of 253J B-V cells was maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS and antibiotics in a humidified atmosphere of 95% air and 5% CO₂.

Cell growth assay. The effects of genistein, daidzein, and glycitein on cell growth were determined by using Cell Titer 96 Aqueous One Solution Reagent, MTS (Promega). Briefly, cells were cultured in 96-well plates at a concentration of 3,000 per well and allowed to attach overnight. Cells were then treated with soy isoflavones at desired concentrations or the vehicle (DMSO) and incubated for 48 hours. MTS (20 μ L per well) was added and incubated for 2 to 4 hours at 37°C in 5% CO₂ and light absorbance of formazan was measured at 490 nm in a microplate reader (VersaMax, Molecular Device, Sunnyvale, CA). The experiments were done at least thrice, each in triplicate. Cell numbers were confirmed by using Z1 Coulter particle Counter (Beckman Coulter Company, Miami, FL).

Colonogenic survival assay. Cell survival after treatment with soy isoflavones was determined by a standard colony-forming assay. In brief, cells (250 cells) were plated in 35-mm tissue culture disc and allowed to

attach overnight, treated with desired concentration of isoflavone genistein or DMSO (vehicle), and incubated for 10 days. The colonies were stained with 0.5% crystal violet in methanol/acetic acid (3:1) and those composed of >50 cells were counted. The experiments were done at least twice, each in duplicate.

Cell cycle analysis. The effect of soy isoflavone genistein on cell cycle distribution was determined by flow cytometry following the described procedures (17).

Determination of apoptosis. Induction of 253J B-V cell apoptosis by genistein was determined by (a) quantitation of cells in sub-G₀ DNA content by flow cytometry following staining with propidium iodide; (b) quantitation of cytoplasmic histone-associated DNA fragments by using Cell Death Detection ELISA kit; and (c) Western blot showing PARP cleavage, active caspase-3, and Bax/Bcl-2 ratios. For analysis of cytoplasmic histone-associated DNA fragments, 1×10^5 cells were plated in T25 flasks, allowed to attach overnight, and treated with desired concentrations of genistein or DMSO for 48 hours. Both floating and adherent cells were collected, washed with PBS, and processed for cell death detection according to the instructions of the manufacturer (Roche Diagnostics). PARP cleavage and active caspase-3 expression were determined by Western blot analysis as described below.

Determination of NF- κ B activity. The NF- κ B activities in nuclear and cytoplasmic fractions were determined by using trans AM kit (Active Motif North America) according to the instructions of the manufacturer. Briefly, nuclear extracts and cytosolic fractions from the control- and genistein-treated 253J B-V cells were prepared based on the procedures provided by the manufacturer, added to the 96-well plate precoated with the oligonucleotide containing NF- κ B consensus sequence (5'-GGGACTTCC-3'), and incubated for 1 hour at 37°C. The primary antibody specific for NF- κ B/p65 was then added to each well and after 1-hour incubation, appropriate secondary antibody was added. Wells were washed three to four times with washing buffer and 100 μ L of developing solution were added; after 10-minute incubation, the absorbance was read at 450 nm using a micro plate reader (VersaMax).

Western blot analysis. Cells were treated with different concentrations of genistein for different time points, cell lysates were prepared, and protein expression was determined following the procedures we previously described (19). The protein levels were quantitated by using densitometric analysis by using NIH image analysis software and expressed as percentages of the control after being normalized with the housekeeping protein of β -actin. The primary antibodies used were cyclin B1 (1:200), Cdc-25C (1:500), PARP (1:200), Cdk-1 (1:500), Bcl-2 (1:100), Bax (1:500), cleaved caspase-3 (1:200), NF- κ B (1:500), I κ B- α (1:500), and β -actin (1:10,000). For NF- κ B protein expression in tumor tissues, the tumor tissue lysates were prepared and NF- κ B protein expression was determined following the procedures we previously described (19) and was quantitated by densitometric analysis.

Orthotopic implantation. Immediately before implantation, exponentially growing 253J B-V cells were trypsinized and resuspended in DMEM with 10% FBS, cell viability was determined by trypan blue exclusion, and a single-cell suspension with >90% viability was used for implantation. Female severe combined immune deficient mice were anesthetized, a lower midline abdominal incision was made, and the bladder was exteriorized. 253 J B-V cells (2×10^6 in 50 μ L medium) were carefully injected inside the bladder by means of a 30-gauge needle and the incision was closed by using a running suture of 5-0 silk.

Experimental diets and animal study. Five- to eight-week-old female severe combined immune deficient mice were purchased from Taconic (Germantown, NY) and housed at the animal facility of Beth Israel Deaconess Medical Center in a pathogen-free environment equipped with laminar flow hoods and standard vinyl cages with air filters. After 1 week of acclimatization and adaptation to the American Institute of Nutrition 93 (AIN-93) diet, mice were randomized into one of three experimental groups (in each group, $n = 8$) and received one of the experimental diets for 2 weeks. Mice were then implanted orthotopically with 253J B-V cells and continued on experimental diets throughout the experiments.

The experimental diets were (i) AIN-93M as the control, (ii) AIN-93M with the addition of SPC at 0.5% of the diet, and (iii) AIN-93M with addition of

genistin at 0.14% to provide the same amount of genistin as that in diet (ii). Food intake and body weight were measured weekly. The experiment was terminated 10 weeks after cell inoculation when the tumors in the control group were palpable and at the estimated weight of 2% to 5% of body weight. Final tumor weights were quantified, the lymph node metastases were counted, and the lungs were harvested for quantification of micrometastases. All procedures with animals were reviewed and approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. The same animal study was repeated once.

A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin embedded, and sectioned at 4- μ m thickness for histology and immunohistochemical determination of tumor biomarkers. Lymph nodes and lungs were fixed in buffer-neutralized formalin, paraffin embedded, sectioned, and H&E stained for determination of metastases.

To determine if the orthotopic bladder tumor model is more sensitive than the s.c. tumor model and to evaluate the antibladder cancer activity of soy bioactive components, we also conducted an addition animal study to compare in parallel the effects of SPC on the growth of 253J B-V tumors in these two models. In brief, female severe combined immune deficient mice were randomly assigned into four groups ($n = 8$ per group), two groups in each model, and pretreated with one of the two experimental diets [diet (i) or diet (ii)] for 2 weeks before cancer cell implantation orthotopically or s.c. and experimental treatments were continued throughout the study. At the end of the study, the animals were sacrificed and tumors were dissected and weighed. Lymph nodes and lungs were collected and evaluated for metastases.

In situ detection of apoptotic index, immunohistochemical determination of proliferation index, and immunohistochemical detection of microvessel density. Apoptotic cells in tumor samples were determined by a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay using the ApopTag *in situ* Apoptosis Detection System (Intergen, Purchase, NY) according to our previous procedures (17, 20, 21). Six representative areas of each section without necrosis were selected and both apoptotic cells and total nuclei cells were counted under a light microscope at $\times 400$ magnification. The apoptotic index was expressed as the percentage of positive apoptotic tumor cells to total tumor cells. Ki-67 was determined by automated immunohistochemical staining to quantify proliferation index as previously described (19). Both Ki-67-positive proliferating cells and total tumor cells were counted in six nonnecrotic areas of each section using light microscope at $\times 400$ magnification. The proliferation index was calculated as the percentage of Ki-67-positive tumor cells to total tumor cells. Microvessel density (MVD) was used as a marker for tumor angiogenesis and quantified by immunohistochemical staining of factor VIII following a previously described method (17, 20, 21). MVD was calculated by counting microvessels on $200\times$ fields under light microscopy at six representative sites without necrosis of each section.

Determinations of soy isoflavones and metabolites in urine and tumor tissue. A 24-hour urine sample was collected by housing the mouse in a metabolic cage. Urinary levels of soy isoflavones and metabolites were measured by high-performance liquid chromatography-mass spectrometry

method as previously described (21, 22). Because of small urine volume, the equal volume of urine from all animals in each group was pooled to form a pooled sample and analyzed.

For analysis of tumor tissue isoflavones and metabolites, 0.2-g frozen tissue was ground in liquid nitrogen and further homogenized in a tissue grinder after addition of water. After addition of triethylamine acetate buffer and incubation with protease, collagenase, β -glucuronidase, and arylsulfatase at 38°C for 16 hours in a total of 1.0 mL, the mixture was further homogenized in a tissue grinder. Fats were removed by partitioning with hexane and isoflavone aglycones were extracted by partitioning into ethyl ether twice. The ethyl ether extracts were combined, dried under nitrogen, and redissolved in 0.1-mL methanol followed by addition of 0.1-mL water. Liquid chromatography-mass spectrometric analysis was done as described for plasma analysis with the inclusion of equol and the use of triply labeled ^{13}C isotopes of isoflavones as internal standard according to our previous report (23).

Determinations of serum levels of insulin-like growth factor-I, insulin-like growth factor binding protein-3, and basic fibroblast growth factor. Circulating levels of insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3) were determined by ELISA following the instructions provided by the manufacturer (Diagnostic Systems Laboratories, Inc., Webster, TX). Blood basic fibroblast growth factor (bFGF) level was measured by ELISA (R&D Systems, Minneapolis, MN).

Statistical analysis. Tumor weight, metastases to lymph nodes and lungs, measured levels of serum and tumor biomarkers, and *in vitro* measurements were expressed as mean \pm SE. Statview 5.0 program (SAS Institute, Inc., Cary, NC) was used to calculate two-sided comparisons among experimental groups through initial ANOVA (24) followed by Fisher's protected least-significant difference (24). $P < 0.05$ was considered statistically significant.

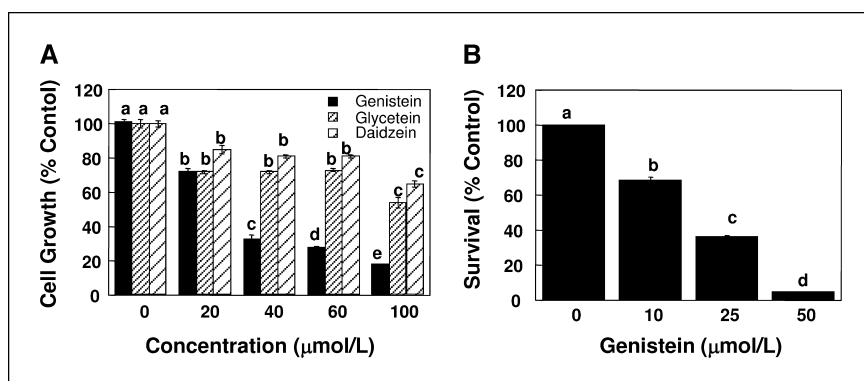
Results

Effects of soy isoflavones on growth of 253J B-V cells *in vitro*.

Cells were treated with different concentrations (10-50 $\mu\text{mol/L}$) of genistein, glycitein, and daidzein for 48 hours. As shown in Fig. 1A, genistein was the soy isoflavone that most significantly inhibited the growth of 253J B-V cells in a dose-dependent manner whereas glycitein and daidzein had limited activities. The IC_{50} for genistein was ~ 30 $\mu\text{mol/L}$. Cell counting confirmed the growth inhibitory effect of genistein (data not shown). Genistein was further evaluated for its clonogenic survival effect. As shown in Fig. 1B, genistein significantly inhibited the clonogenic survival of 253J B-V cells with IC_{50} of <20 $\mu\text{mol/L}$. Genistein was then used for further mechanistic evaluation *in vitro*.

Effects of genistein on cell cycle progression of 253J B-V cells *in vitro*. Cells were treated with genistein at different concentrations for 48 hours and the cell cycle progression was measured. As shown in Fig. 2A, genistein inhibited cell growth by arresting cell

Figure 1. Effects of genistein on growth inhibition of 253J B-V cells determined by cell proliferation by using MTS assay (A) and colony formation (B) *in vitro*. Columns, mean of two independent experiments in triplicate; bars, SD. Within the isoflavone treatment, values not sharing a common letter are significantly different ($P < 0.05$).



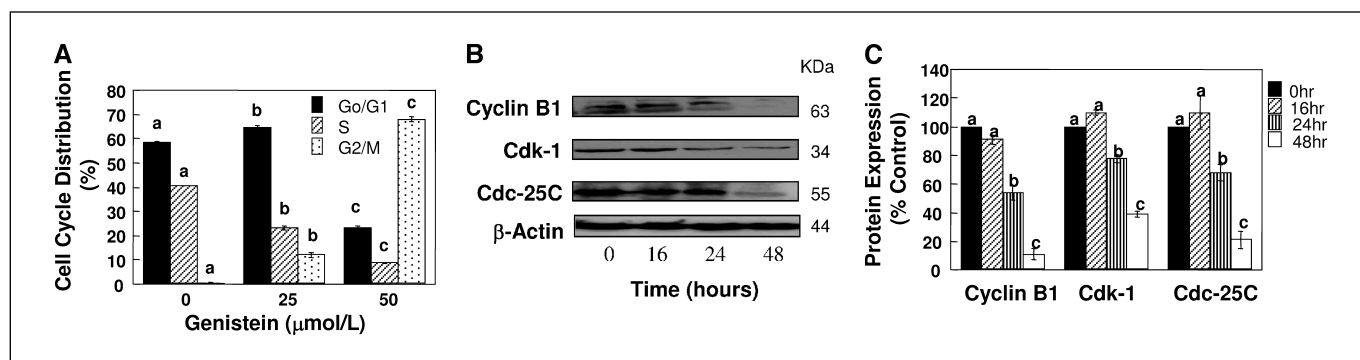


Figure 2. Effects of genistein on cell cycle distribution and cell cycle-related protein expression in 253J B-V cells *in vitro*. **A**, cell cycle distribution in DMSO-treated, control, and genistein-treated (25 or 50 μmol/L for 48 hours) 253J B-V cells. Columns, mean of three independent experiments in duplicate; bars, SD. Within the cell cycle phase (G₀-G₁, S, or G₂-M), values not sharing a common letter are significantly different ($P < 0.05$). **B**, representative Western blots showing the effect of genistein treatment on the expression of G₂-M regulatory protein, such as cyclin B1, Cdk-1, and Cdc-25C, and the housekeeping protein β-actin. 253J B-V cells were treated with genistein at 50 μmol/L for 0, 16, 24, and 48 hour time points before preparation of lysates. **C**, densitometric quantification of protein expression using NIH imaging analysis and by normalization to the β-actin ($n = 3$). The expression level in the control group was 100%. Within the same protein, values not sharing a common letter are significantly different ($P < 0.05$).

cycle at G₂-M phases in a dose-dependent manner. Consequently, cells accumulated at S and G₀-G₁ phases were reduced. Genistein also showed a time-dependent G₂-M arrest of cell cycle progression (data not shown).

To further elucidate the mechanisms by which genistein caused the G₂-M arrest, we determined the expression of G₂-M regulatory proteins that play key roles in G₂-M transition. As shown in Fig. 2B, genistein (50 μmol/L) treatment resulted in a decrease in the protein level of cyclin B1, Cdk-1, and Cdc-25C in a time-dependent manner. Cells treated with genistein for 48 hours significantly reduced expression of cyclin B1, Cdk-1, and Cdc-25C by 89%, 61%, and 79%, respectively, compared with the control (Fig. 2C).

Effects of genistein on apoptosis of 253J B-V cells *in vitro*.

The effect of genistein on apoptosis induction of 253J B-V cells was measured by accumulation of cells at sub-G₀, increased cytoplasmic histone-associated DNA fragmentation, and increased levels of

cleaved caspase-3, cleaved PARP, and Bax/Bcl-2 ratios. Genistein at 25 and 50 μmol/L significantly increased cell accumulation at sub-G₀ phase by 12- and 19-fold, respectively (Fig. 3A). Similarly, genistein treatment significantly increased histone-associated DNA fragmentation in a dose-dependent manner (Fig. 3B). Western blot analysis showed that cells treated with genistein at 50 μmol/L for 48 hours significantly increased levels of cleaved PARP and cleaved caspase-3, molecular markers for apoptosis induction (Fig. 3C). Genistein also reduced expression of Bcl-2 and increased expression of Bax (Fig. 3C and D), resulting in a time-dependent elevation of Bax/Bcl-2 ratio (Fig. 3D), another reliable marker for apoptosis.

To further determine the role of caspase-3 in genistein-induced apoptosis, the effects of z-DEVD-fmk, a specific inhibitor of caspase-3, on genistein-induced cleavage of procaspase-3 and PARP and increase in cytoplasmic histone-associated DNA fragmentation were determined. Genistein-induced cleavage of caspase-3 and

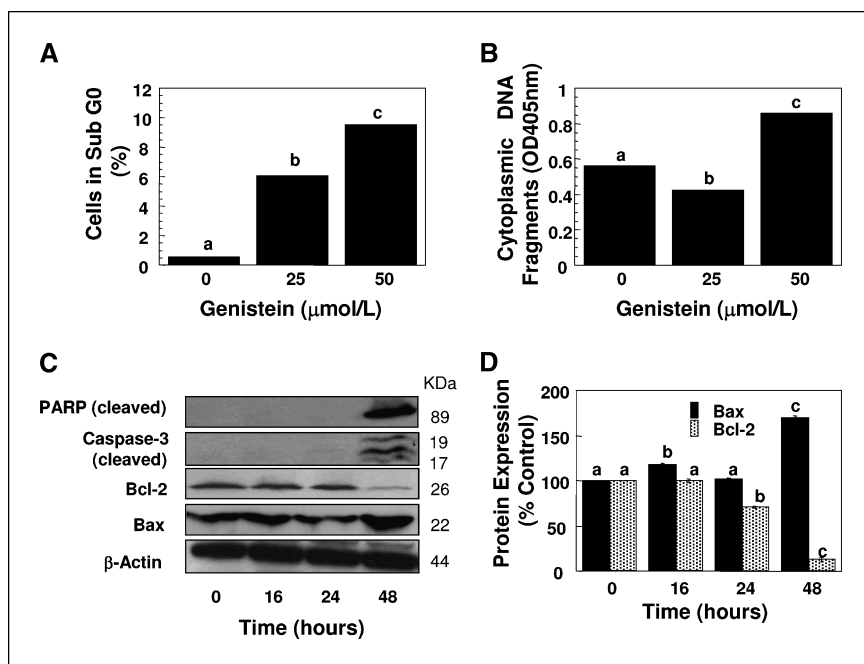


Figure 3. Effects of genistein on apoptosis induction *in vitro* measured by flow cytometric analysis of cells in sub-G₀ (A), analysis of histone-associated DNA fragments by using ELISA (B), and immunoreactive bands showing cleavage of PARP, active caspase-3, Bax, and Bcl-2 (C). D, quantitative results of Bax and Bcl-2 protein expression in (C). Columns, mean of two independent experiments in duplicate; bars, SD. In (A and B) or for the same protein (D), values not sharing a common letter are significantly different ($P < 0.05$).

PARP was fully blocked on 2-hour pretreatment of 253J B-V cells with α -DEVD-fmk (data not shown). Genistein-induced DNA fragmentation was also significantly inhibited by caspase-3 (data not shown). These data suggest the critical role of caspase-3 in genistein-induced apoptotic cell death.

Effect of genistein on NF- κ B activation *in vitro*. Genistein treatment (50 μ mol/L) resulted in a time-dependent decrease in the NF- κ B protein level in the cytosolic fraction and especially in the nuclear fraction (Fig. 4A and C). Genistein also stimulated in a time-dependent manner the expression of I κ B- α , an inhibitor of NF- κ B activation (Fig. 4B and C). Cells treated with genistein at 50 μ mol/L for 48 hours significantly reduced cytosolic and nuclear levels of NF- κ B by 44% and 98%, respectively, and significantly increased I κ B- α level by 160% (Fig. 4C). In parallel, genistein significantly inhibited DNA binding activity of NF- κ B, a biomarker of NF- κ B activity (Fig. 4D).

Effects of soy phytochemicals on orthotopic growth and metastasis of bladder tumors. The intrabladder 253J B-V human bladder tumor model was used as a clinically relevant *in vivo* model to evaluate the effects of SPC and genistein on tumor growth and metastasis. We used genistein because it is the natural form of genistein in soy. We used SPC because it represents soy phytochemicals that are consumed by humans. Dietary soy treatments did not significantly reduce body weight or food intake (data not shown). The effects of dietary treatments on tumor growth and metastasis are shown in Fig. 5. Tumors from those treated with SPC and genistein were reduced by 52% ($P < 0.05$) and 54% ($P < 0.05$), respectively, compared with the control group (Fig. 5A). Although SPC or genistein did not significantly reduce lymph node metastases (Fig. 5B), SPC and genistein reduced lung metastases by 95% ($P < 0.05$) and 70% ($P > 0.05$), respectively, compared with the control (Fig. 5C).

An additional animal study was also conducted to compare in parallel the effects of SPC on the growth and/or metastasis of 253J B-V tumors in both orthotopic and s.c. tumor models. SPC was evaluated because it showed both antigrowth and antimetastatic activities in an orthotopic tumor model. The SPC treatment inhibited the growth of s.c. tumor and orthotopic tumor by 25.1% ($P > 0.05$) and 55.5% ($P < 0.05$), respectively, compared with the

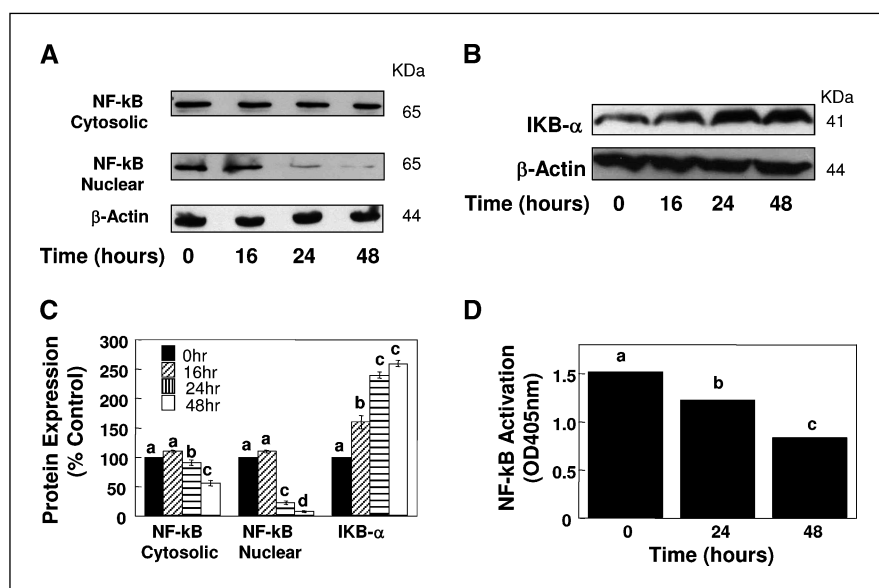
corresponding control. S.c. tumor had limited metastatic potential to lymph nodes or lungs; thus, comparison of the antimetastatic activity of SPC in these two models cannot be done.

Effects of soy phytochemicals on tumor apoptosis, angiogenesis, and proliferation. Tumor cell apoptotic index and tumor angiogenesis were measured by TUNEL assay and factor VIII staining, respectively. Apoptotic indices of primary tumors in mice treated with SPC and genistein were significantly increased by 263% ($P < 0.01$) and 265% ($P < 0.01$), respectively, whereas microvessel densities of primary tumors in mice treated with SPC and genistein were significantly reduced by 35% ($P < 0.05$) and 50% ($P < 0.05$), respectively, compared with the control (Table 1). Treatment of tumors with SPC and genistein also reduced tumor cell proliferation, as measured by Ki-67 staining, by 15% ($P > 0.05$) and 22% ($P > 0.05$), respectively, compared with the control (Table 1).

Effects of soy phytochemicals on tumor NF- κ B protein expression and blood levels of IGF-I, IGFBP-3, and bFGF. NF- κ B protein expression in tumor tissues of SPC treated animals was significantly down-regulated by 43% ($P < 0.05$; Fig. 5D) compared with that of the control. On the other hand, its expression was not significantly down-regulated by genistein treatment (Fig. 5D). Blood levels of IGF-I in mice treated with SPC and genistein diets were reduced by 67% ($P < 0.05$) and 39% ($P > 0.05$), respectively, compared with the control (Table 1) whereas blood levels of IGFBP-3 and bFGF were not altered by the dietary treatments (Table 1).

Effects of soy phytochemicals on isoflavone levels in urine and tumor tissue. Urinary levels of total genistein in mice treated with SPC and genistein diets were 1,483.2 μ mol/L (3.92 μ mol/mg creatinine) and 1,559.2 μ mol/L (3.99 μ mol/mg creatinine), respectively. Urinary levels of daidzein, glycitein, equol, and *O*-desmethy-langolensin in mice treated with the SPC diet were 1,849.0 μ mol/L (4.89 μ mol/mg creatinine), 339.4 μ mol/L (0.90 μ mol/mg creatinine), 900.0 μ mol/L (2.38 μ mol/mg creatinine), and 60.4 μ mol/L (0.16 μ mol/mg creatinine), respectively, whereas their levels in mice treated with the genistein diet were 200.0 μ mol/L (0.51 μ mol/mg creatinine), 10.5 μ mol/L (0.03 μ mol/mg creatinine), 36.0 μ mol/L (0.09 μ mol/mg creatinine), and 49.2 μ mol/L (0.13 μ mol/mg creatinine), respectively.

Figure 4. Effects of genistein on NF- κ B pathway *in vitro*. A, NF- κ B/p65 protein levels in the cytosolic extract as well as in the nuclear extract. B, expression of I κ B- α protein level. C, fold change in protein expression level relative to control. D, NF- κ B/p65 activity determined by an ELISA-based assay. Within each protein, data not sharing a common letter are significantly different ($P < 0.05$). A and B, representative of at least two independent experiments with similar results.



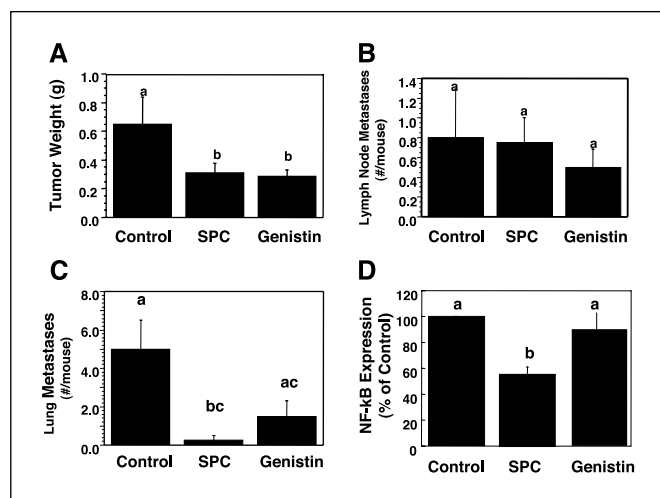


Figure 5. Effects of SPC and genistin on final tumor weight ($n = 16$; A), lymph node metastases ($n = 16$; B), lung metastases ($n = 16$; C), and NF- κ B protein expression ($n = 6$; D) of 253J B-V bladder tumors in an orthotopic bladder tumor mouse model. Columns, means; bars, SE. Values not sharing a common letter are significantly different ($P < 0.05$).

Tumor samples from both s.c. and orthotopic tumors treated with 0.5% SPC were analyzed for isoflavones and their metabolites. Levels of daidzein, genistein, equol, glycitein, desmethylangolensin, dihydrodaidzein, and dihydrogenistein in s.c. bladder tumors were 120 ± 9.4 , 668.1 ± 15.3 , <6.7 , 22.9 ± 4.1 , <7.0 , <12.8 , and <13.5 ng/g tissue, respectively, and the levels of these isoflavones in orthotopic tumors were $1,121.1 \pm 91.9$, $6,494 \pm 387$, 20.5 ± 19.3 , 88.3 ± 3.9 , <7.0 , 17.4 ± 4.8 , and <13.5 ng/g tissue, respectively. Daidzein and genistein levels in orthotopic bladder tumors are almost 10-fold higher than those in s.c. tumors.

Discussion

In this report, we conducted *in vitro* studies and found that genistein significantly inhibited the growth of poorly differentiated and highly metastatic bladder cancer cell line 253J B-V via cell cycle arrest in G₂-M phases and induction of apoptosis. We further applied a clinically relevant orthotopic bladder tumor model to evaluate the preventive effects of genistin and SPC on the growth and metastasis of bladder tumors and found that both genistin and SPC significantly inhibited orthotopic growth of bladder tumor by induction of tumor cell apoptosis and reduction of tumor angiogenesis. In addition, SPC significantly inhibited lung metastases, down-regulated the expression of NF- κ B in tumor tissues,

and reduced circulating IGF-I levels, suggesting that SPC may have other bioactive ingredients that have antimetastatic activity by targeting different signaling pathways. It is the first report, to the best of our knowledge, that the orthotopic bladder tumor model was applied to evaluate the preventive effects of soy bioactive components or other dietary components on bladder tumor growth and metastasis.

We hypothesize that increased intake of soy bioactive components may provide an effective nutritional regimen for the prevention of bladder cancer progression because soy bioactive components can exert their effects via both blood circulation and especially direct contact with the mucosa of the bladder. Urinary levels of total genistein in mice treated with the SPC and genistin diets were at 1,483.2 and 1,559.2 μ mol/L, respectively. Although >95% of isoflavones and metabolites are present in urine and blood as conjugated forms (25), urinary levels of free form of genistein can reach 50 μ mol/L or higher. At which levels, genistein showed significant growth inhibition, G₂-M arrest, and apoptosis induction of 253J B-V cells *in vitro*. Thus, the doses of genistein used in the *in vitro* studies were achievable *in vivo*. In contrast, the blood levels of total genistein in animals treated with the same diets were at 1.84 and 1.57 μ mol/L, respectively (21). The orthotopic bladder tumor model mirrors tumor cell-host interactions in humans and is thus more clinically relevant than the s.c. tumor model. Therefore, the effect of soy bioactive components or other nutritional ingredients on bladder cancer growth and progression should be appropriately evaluated in orthotopic bladder tumor models. Because urinary levels of soy bioactive components are higher than that in blood, and isoflavones daidzein and genistein in orthotopic tumors are almost 10-fold higher than that in s.c. tumors, it is expected that the antibladder cancer activity would be more potent in an orthotopic bladder tumor model than in a s.c. tumor model. Indeed, SPC treatment inhibited the growth of orthotopic tumor by ~52% to 55.5% but inhibited the growth of s.c. tumor by 25%.

By using a clinically relevant orthotopic bladder tumor model, we found that SPC and genistin had similar effects on growth inhibition of final tumor weight (Fig. 5A). Because these two groups contained similar levels of genistein, the data suggest that genistin is the major bioactive component in SPC. On the other hand, SPC treatment significantly reduced the lung metastases by 95% whereas the genistin effect was not significant. It suggests that SPC may contain other components that have antimetastatic activity. This finding is consistent with our previous finding in the prostate cancer study (21) in which SPC (0.5%), but not genistin (0.14%), significantly inhibited metastases of LNCaP prostate tumor to the lymph nodes by 82% and to the lungs by 91% in an

Table 1. Effects of soy phytochemicals on tumor cell apoptosis and proliferation, tumor angiogenesis, and blood levels of IGF-I, IGFBP-3, and bFGF

Treatment	Apoptosis index (%)	Proliferation index (%)	MVD (vessels/field)	IGF-I (ng/mL)	IGFBP-3 (ng/mL)	bFGF (ng/mL)
Control (16)	5.1 ± 1.0^a	57.4 ± 6.3^a	2.6 ± 0.5^a	16.5 ± 0.7^a	$1,285.6 \pm 423.6^a$	46.9 ± 6.6^a
SPC (16)	18.5 ± 4.1^b	48.7 ± 2.5^a	1.7 ± 0.6^b	5.4 ± 2.1^{bc}	$1,365.7 \pm 321.1^a$	60.9 ± 11.2^a
Genistin (16)	18.6 ± 3.5^b	44.8 ± 5.1^a	1.3 ± 0.2^b	10.0 ± 3.8^{ac}	$1,245.1 \pm 216.2^a$	39.9 ± 4.7^a

NOTE: Values are means \pm SE. Within a column, means not sharing a common superscript letter are significantly different ($P < 0.05$). See text for P values for specific comparisons.

orthotopic prostate tumor model. Together, our results suggest the presence of potent antimetastasis component(s) in SPC, and one of the future research projects should be further identification and characterization of the antimetastatic soy phytochemical component(s).

Soy isoflavones have been suggested to be in part associated with the anticancer activity of soybeans and soy products. Among soy isoflavones, the chemopreventive property of genistein has been the subject of extensive *in vitro* and *in vivo* research. The experimental studies have shown that genistein inhibits the growth of bladder cancer cells by inhibiting proliferation *in vitro* (16, 17, 26–28) and *in vivo* (16, 17, 28), inducing apoptosis *in vitro* (17) and *in vivo* (17), and inhibiting angiogenesis *in vitro* (28, 29) and *in vivo* (20, 28). In this report, we found that both genistin and SPC significantly induced bladder tumor cell apoptosis and inhibited tumor angiogenesis *in vivo* (Table 1). Our *in vivo* biomarker determination showed that the antiproliferation activities of SPC and genistin, although 15% and 22%, respectively, were not significant (Table 1).

The *in vitro* studies have shown that genistein possesses multiple biological and biochemical functions that may be related to its anticancer activities. These functions include inhibition of tyrosine kinase activities (30) and epidermal growth factor receptor signal transduction pathways (27); induction of G₂-M cell cycle arrest and inhibition of cdc-2 kinase activity (16); down-regulation of the angiogenic factors vascular endothelial growth factor (28, 31), platelet-derived growth factor (28), tissue factor (28), urokinase plasminogen activator (28), and matrix metalloproteinases (28); and up-regulation of angiogenesis inhibitors plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1 (28). Our *in vitro* studies in this report showed that genistein inhibited bladder cancer cell proliferation by inducing G₂-M arrest via modulation of G₂-M phase-related regulators, such as cyclin B1, Cdk-1, and Cdc-25C (Fig. 2), and induced bladder cancer cell apoptosis by activation of caspase-3 via several pathways, such as inactivation of NF- κ B by inhibition of translocation of NF- κ B to the nucleus and increased expression of its inhibitor I κ B- α (Fig. 4) and up-regulation of Bax/Bcl-2 (Fig. 3).

Despite the above shown biological functions of genistein *in vitro*, limited studies have been conducted to determine which markers are modulated by genistein *in vivo*. One of the possibilities is that genistein concentrations used in the *in vitro* studies are higher than

those achievable *in vivo*; thus, some of these biomarkers are not significantly modulated in the *in vivo* samples. Another possibility may be due to limited amounts of tumor samples that can be used for *in vivo* biomarker determination. In this report, we have primarily focused on characterization of cellular markers. Clearly, further evaluation of *in vivo* modulation of molecular markers is expected to provide crucial information on understanding the molecular mechanisms of action of soy bioactive components.

Although we found that genistein significantly down-regulated the expression of NF- κ B *in vitro*, we did not confirm its down-regulation by genistin treatment *in vivo*. On the other hand, we found that SPC treatment significantly down-regulated the expression of NF- κ B protein *in vivo*. In addition, we found that SPC treatment, but not genistin treatment, significantly reduced blood level of IGF-I (Table 1). It suggests that SPC may contain other bioactive components and this mixture of natural soy components may be a potent agent against bladder cancer growth and particularly metastasis via, in part, its effects on NF- κ B and IGF-I.

In conclusion, we found that genistin and SPC significantly inhibited the growth of a poorly differentiated human bladder tumor associated with induction of tumor cell apoptosis and inhibition of tumor angiogenesis. In particular, SPC, but not genistin, significantly inhibited lung metastases and reduced NF- κ B expression and circulating level of IGF-I. The results from this study, together with our previous studies, suggest that SPC may contain potent antimetastasis component(s). The results from our studies suggest further clinical studies should be warranted to apply soy phytochemicals, such as SPC, as a potent prevention regimen for bladder cancer progression. This orthotopic human bladder tumor model also provides a clinically relevant experimental tool for assessing potential preventive activity of other dietary components against bladder tumor growth and metastasis.

Acknowledgments

Received 4/15/2005; revised 11/4/2005; accepted 12/2/2005.

Grant support: National Cancer Institute, NIH grants R01 CA92546 and R03 CA112640 (J-R. Zhou).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Colin P. Dinney (University of Texas M.D. Anderson Cancer Center) for providing the 253J B-V cell line.

References

- American Cancer Society. Cancer facts and figures 2005. Atlanta: American Cancer Society; 2005.
- Heney NM, Ahmed S, Flanagan MJ, et al. Superficial bladder cancer: progression and recurrence. *J Urol* 1983; 130:1083–6.
- Paneau C, Schaffer P, Bollack C. Epidemiology of bladder cancer. *Ann Urol* 1992;26:281–93.
- Silverman DT, Hartge P, Morrison AS, Devesa SS. Epidemiology of bladder cancer. *Hematol Oncol Clin North Am* 1992;6:1–30.
- Dunn JE. Cancer epidemiology in populations of the United States—with emphasis on Hawaii and California—and Japan. *Cancer Res* 1975;35:3240–5.
- Nakata S, Sato J, Ohtake N, Imai K, Yamanaka H. Epidemiological study of risk factors for bladder cancer. *Hinyokika Kyo* 1995;41:969–77.
- Messina MJ, Persky V, Setchell KD, Barnes S. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. *Nutr Cancer* 1994;21:113–31.
- Kennedy AR. The evidence for soybean products as cancer preventive agents. *J Nutr* 1995;125:733–43S.
- Adlercreutz H, Honjo H, Higashi A, et al. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am J Clin Nutr* 1991;54:1093–100.
- Kelly GE, Nelson C, Waring MA, Joannou GE, Reeder AY. Metabolites of dietary (soya) isoflavones in human urine. *Clin Chim Acta* 1993;223:9–22.
- Kirkman LM, Lampe JW, Campbell DR, Martini MC, Slavin JL. Urinary lignan and isoflavonoid excretion in men and women consuming vegetable and soy diets. *Nutr Cancer* 1995;24:1–12.
- Xu X, Wang HJ, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr* 1994;124: 825–32.
- Lu CM, Lan SJ, Lee YH, et al. Tea consumption: fluid intake and bladder cancer risk in Southern Taiwan. *Urology* 1999;54:823–8.
- Sun CL, Yuan JM, Arakawa K, et al. Dietary soy and increased risk of bladder cancer: the Singapore Chinese Health Study. *Cancer Epidemiol Biomarkers Prev* 2002; 11:1674–7.
- Sun CL, Yuan JM, Wang XL, et al. Dietary soy and increased risk of bladder cancer: a prospective cohort study of men in Shanghai, China. *Int J Cancer* 2004;112: 319–23.
- 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.
- Zhou J-R, Mukherjee P, Gugger ET, et al. The inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. *Cancer Res* 1998;58:5231–8.
- Dinney CP, Fishbeck R, Singh RK, et al. Isolation and characterization of metastatic variants from human transitional cell carcinoma passaged by orthotopic implantation in athymic nude mice. *J Urol* 1995;154: 1532–8.
- Zhou J-R, Yu L, Zerbini LF, Libermann TA, Blackburn

- GL. Progression to androgen-independent LNCaP human prostate tumors: cellular and molecular alterations. *Int J Cancer* 2004;81:800-6.
20. Zhou J-R, Gugger ET, Tanaka T, et al. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. *J Nutr* 1999;129:1628-35.
21. Zhou J-R, Yu L, Zhong Y, et al. Inhibition of orthotopic growth and metastasis of androgen-sensitive human prostate tumors in mice by bioactive soybean components. *Prostate* 2002;53:143-53.
22. Franke AA, Custer LJ, Wilkens LR, et al. Liquid chromatographic-photodiode array mass spectrometric analysis of dietary phytoestrogens from human urine and blood. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002;777:45-59.
23. Blair RM, Appt SE, Franke AA, Clarkson TB. Treatment with antibiotics reduces plasma equol concentration in cynomolgus monkeys (*Macaca fascicularis*). *J Nutr* 2003;133:2262-7.
24. Steel RGD, Torrie JH. Principles and procedures of statistics: a biometrical approach. 2nd ed. New York: McGraw-Hill Book Company, Inc.; 1980.
25. Adlercreutz H, van der Wildt J, Kinzel J, et al. Lignan and isoflavonoid conjugates in human urine. *J Steroid Biochem Mol Biol* 1995;52:97-103.
26. Lu LJ, Lin SN, Grady JJ, Nagamani M, Anderson KE. Altered kinetics and extent of urinary daidzein and genistein excretion in women during chronic soya exposure. *Nutr Cancer* 1996;26:289-302.
27. Theodorescu D, Laderoute KR, Calaoagan JM, Guilding KM. Inhibition of human bladder cancer cell motility by genistein is dependent on epidermal growth factor receptor but not p21ras gene expression. *Int J Cancer* 1998;78:775-82.
28. Su SJ, Yeh TM, Chuang WJ, et al. The novel targets for anti-angiogenesis of genistein on human cancer cells. *Biochem Pharmacol* 2005;69:307-18.
29. Fotsis T, Pepper MS, Aktas E, et al. Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res* 1997;57:2916-21.
30. Akiyama T, Ishida J, Nakagawa S, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987;262:5592-5.
31. Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem* 1996;271:2746-53.