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, Adrian A. Franke, George L. Blackburn, and Jin-Rong Zhou

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 antitumor 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-κB (NF-κB), 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-κB expression in tumor tissues and reduction of circulating insulin-like growth factor-I levels, suggesting that SPC may contain other bioactive ingredients that have antitumor 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 most 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 4-10-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, 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)

Requests for reprints: Jin-Rong Zhou, Nutrition/Metabolism Laboratory, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, 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
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-kB (NF-kB), and -actin were from Oncogene Research Products (Boston, MA); antibodies against cleaved caspase-3, cleaved poly(ADP ribose) polymerase (PARP), and cleaved caspase-3 were from Cell Signaling (Beverly, CA); antibodies against Bax, cyclin-dependent kinase 1 (Cdk1), 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. Dimkey (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 Titre 96 Aqueous One Solution Reagent, MTS (Promega). Briefly, cells were cultured in 96-well plates at a density of 104 cells 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 µl 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-G1 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 x 106 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-kB activity. The NF-kB 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 preplated with the oligonucleotide containing NF-kB consensus sequence (5’-GGGACTTTCC-3’), and incubated for 1 hour at 37°C. The primary antibody specific for NF-kB/D56 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 µl 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 -actin. The primary antibodies used were cyclin B1 (1:200), Cdc-25C (1:500), PARP (1:2500), Cdk1 (1:500), Bcl-2 (1:1000), Bax (1:500), cleaved caspase-3 (1:200), NF-kB (1:500), IкB (1:500), and -actin (1:10,000). For NF-kB protein expression in tumor tissues, the tumor tissue lysates were prepared and NF-kB 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. 253J B-V cells (2 x 106 in 50 µl 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...
Effects of genistein at 0.14% to provide the same amount of genistein 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-μm 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 antitumor cancer activity of soy bioactive components, we also conducted an 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 ×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 ×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× fields under light microscopy at six representative sites without necrosis of each section.

**Determination 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 determined 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, α-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 equal and the use of triply labeled 13C isotopes of isoflavones as internal standard according to our previous report (23).

**Statistical analysis.** Tumor weight, metastases to lymph nodes and lungs, measured levels of serum and tumor biomarkers, and *in vitro* measurements were expressed as group means ± 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 μmol/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 IC50 for genistein was ~30 μmol/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 IC50 of ~20 μmol/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...
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 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 quantitation 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).

**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 z-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-κB activation in vitro. Genistein treatment (50 μmol/L) resulted in a time-dependent decrease in the NF-κB 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 IκBα, an inhibitor of NF-κB activation (Fig. 4B and C). Cells treated with genistein at 50 μmol/L for 48 hours significantly reduced cytosolic and nuclear levels of NF-κB by 44% and 98%, respectively, and significantly increased IκBα level by 160% (Fig. 4C). In parallel, genistein significantly inhibited DNA binding activity of NF-κB, a biomarker of NF-κB activity (Fig. 4D).

Effects of soy phytochemicals on orthotopic growth and metastasis of bladder tumors. The intrabladter 253J B-V human bladder tumor model was used as a clinically relevant in vivo model to evaluate the effects of SPC and genistin on tumor growth and metastasis. We used genistin because it is the natural form of genistin 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 genistin were reduced by 52% (P < 0.05) and 54% (P < 0.05), respectively, compared with the control group (Fig. 5A). Although SPC or genistin did not significantly reduce lymph node metastases (Fig. 5B), SPC and genistin 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 anti-growth 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 genistin 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 genistin 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 genistin 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-κB protein expression and blood levels of IGF-I, IGFBP-3, and bFGF. NF-κB 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 genistin treatment (Fig. 5D). Blood levels of IGF-I in mice treated with SPC and genistin 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 genistin diets were 1,483.2 μmol/L (3.92 μmol/mg creatinine) and 1,559.2 μmol/L (3.99 μmol/mg creatinine), respectively. Urinary levels of daidzein, glycitein, and O-desmethylangolensin in mice treated with the SPC diet were 1,849.0 μmol/L (4.89 μmol/mg creatinine), 339.4 μmol/L (0.90 μmol/mg creatinine), 900.0 μmol/L (2.38 μmol/mg creatinine), and 60.4 μmol/L (0.16 μmol/mg creatinine), respectively, whereas their levels in mice treated with the genistin diet were 200.0 μmol/L (0.51 μmol/mg creatinine), 10.5 μmol/L (0.03 μmol/mg creatinine), 36.0 μmol/L (0.09 μmol/mg creatinine), and 49.2 μmol/L (0.13 μmol/mg creatinine), respectively.

Figure 4. Effects of genistein on NF-κB pathway in vitro. A, NF-κB/P65 protein levels in the cytosolic extract as well as in the nuclear extract. B, expression of IκBα protein level. C, fold change in protein expression level relative to control. D, NF-κB/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.
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 ± 91.9, 6,494 ± 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 orthotropic 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-κB 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 μmol/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 μmol/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 μmol/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 orthotropic 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 genistein 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

![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-κB 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).](image)

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptosis index (%)</th>
<th>Proliferation index (%)</th>
<th>MVD (vessels/field)</th>
<th>IGF-I (ng/mL)</th>
<th>IGFBP-3 (ng/mL)</th>
<th>bFGF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (16)</td>
<td>5.1 ± 1.0a</td>
<td>57.4 ± 6.3a</td>
<td>2.6 ± 0.7</td>
<td>16.5 ± 0.7a</td>
<td>1,285.6 ± 423.6a</td>
<td>46.9 ± 6.6a</td>
</tr>
<tr>
<td>SPC (16)</td>
<td>18.5 ± 4.1b</td>
<td>48.7 ± 2.5a</td>
<td>1.7 ± 0.6</td>
<td>5.4 ± 2.1b</td>
<td>1,365.7 ± 321.1</td>
<td>60.9 ± 11.2a</td>
</tr>
<tr>
<td>Genistin (16)</td>
<td>18.6 ± 3.5b</td>
<td>44.8 ± 5.1a</td>
<td>1.3 ± 0.2</td>
<td>10.0 ± 3.8a</td>
<td>1,245.1 ± 216.2a</td>
<td>39.9 ± 4.7a</td>
</tr>
</tbody>
</table>

NOTE: Values are means ± 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 antitumor activity component(s) in SPC, and one of the future research projects should be further identification and characterization of the antitumor activity 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 genistein 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 genistein, 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-κB by inhibition of translocation of NF-κB to the nucleus and increased expression of its inhibitor IκB-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 and in vivo data.


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, Adrian A. Franke, George L. Blackburn, et al.