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Inhibition of Murine Bladder Tumorigenesis by Soy Isoflavones via Alterations in the Cell Cycle, Apoptosis, and Angiogenesis[1]

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ABSTRACT

Soy isoflavones exhibit a number of biological effects, suggesting that they may have a role in cancer prevention. Our objectives are to determine whether components of soy products or purified soy isoflavones can inhibit the progression of bladder cancer. We compared the *in vitro* effects of pure soy isoflavones and soy phytochemical concentrate on growth curves, cell cycle progression, and apoptosis in murine and human bladder cancer cell lines. Pure soy isoflavones (genistein, genistin, daidzein, and biochanin A) and soy phytochemical concentrate exhibit dose-dependent growth inhibition of murine (MB49 and MBT-2) and human (HT-1376, UM-UC-3, RT-4, J82, and TCCSUP) bladder cancer cell lines, although the degree of inhibition varies among lines. Soy isoflavones induce a G_2-M cell cycle arrest in all human and murine lines evaluated by flow cytometry. In addition, some bladder cancer lines show DNA fragmentation consistent with apoptosis. We next evaluated the ability of genistein, soy phytochemical concentrate, and soy protein isolate, respectively, to inhibit the growth of transplantable murine bladder cancer *in vivo*. C57BL/6 mice were randomly assigned to treatment groups ($n = 12$/group): (*a*) AIN-76A diet; (*b*) AIN-76A diet plus genistein, i.p., 50 mg/kg body weight/day; (*c*) AIN-76 diet with soy phytochemical concentrate at 0.2% of the diet; (*d*) AIN-76 diet with soy phytochemical concentrate at 1.0% of the diet; and (*e*) AIN-76A diet with soy protein isolate, 20% by weight. Mice were inoculated s.c. with 5×10^4 syngeneic MB49 bladder carcinoma cells, and tumor growth was quantitated. Neither genistein nor soy products reduced body weight gain. Tumor volumes from mice treated with genistein, dietary soy phytochemical concentrate at 1%, or dietary soy protein isolate were reduced by 40% ($P < 0.007$), 48% ($P < 0.001$), or 37% ($P < 0.01$), respectively, compared with controls. We characterized the effects of treatment on several biomarkers in tumor tissue: proliferation index by proliferating cell nuclear antigen staining, apoptotic index by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling staining, and angiogenesis by microvessel quantitation. Soy products reduced angiogenesis, increased apoptosis, and slightly reduced proliferation while showing no histopathological effects on the normal bladder mucosa. Our data suggest that soy isoflavones can inhibit bladder tumor growth through a combination of direct effects on tumor cells and indirect effects on the tumor neovasculature. Soy products warrant further investigation in bladder cancer prevention and treatment programs or as antiangiogenic agents.

INTRODUCTION

Epidemiological, cell culture, and laboratory animal studies provide evidence supporting the hypothesis that products derived from soybeans contain substances exhibiting anticarcinogenic properties (1, 2). Much of the attention has focused on genistein and daidzein, which are the predominant isoflavones found in soy at concentrations of approximately 1–3 mg/g (3). In addition, protease inhibitors, inositol hexaphosphate (phytic acid), lignans, phytosterols, and saponins found in soy products may also have *in vivo* bioactivities relevant to the inhibition of carcinogenesis (1, 2, 4, 5). Several investigators have shown that soy isoflavones, particularly genistein, inhibit the proliferation of transformed cells in culture (6). Some *in vivo* studies, although not all (7–9), report that pure isoflavones or dietary soy protein inhibits tumorigenesis in rodent models (10–16). Several mechanisms have been proposed for the antitumor activity of soy isoflavones, including: agonist/antagonist effects on estrogen receptors (17), stimulation of sex hormone-binding globulin synthesis (17), inhibition of growth factor-associated tyrosine-kinase signal transduction (18), antioxidant properties (19), inhibition of DNA topoisomerase (20, 21), and antiangiogenic effects (22). Although the data described above are highly suggestive of beneficial effects for soy consumption with regard to cancer prevention, definitive intervention trials have not been completed, and caution should be practiced in our recommendations to the public or high-risk subgroups.

The presence of soy isoflavones in the urine provides an additional route of exposure for the uroepithelium lining the bladder and collecting system. Urine is a significant route for excretion of genistein, daidzein, and their metabolites (23–26). Several reports describe dose-dependent increases in urinary isoflavonoid excretion by Japanese men and women consuming soy-based food products (27) and Americans following consumption of isoflavone containing soy supplements in controlled studies (28–30). Indeed, 100- to 500-fold increases in urinary isoflavones are observed over basal concentrations with the addition of soy to the diet of Americans, who average less than 3 g/day of soy protein (26, 31, 32). In comparison, rural Japanese men and women consuming traditional diets average 39–54 g of soy foods per day (27), which may contain 10–20 mg of genistein and 5–6 mg of daidzein (33). A recent study shows that consumption of 20 g of soy protein/day (13 mg daidzein and 23 mg genistein), which is similar to a 4-oz serving of tofu or a 2-oz serving of tempeh, results in 10–36 μM urinary isoflavones excreted per day (30). Individuals whose diets are supplemented with a single dose of isoflavones in soy milk at 2 mg/kg showed mean urinary excretion of 76 μmol/day (28). Assuming an average of 1.5 liters of urine produced per day, these individuals have mean urine concentrations of soy isoflavones of approximately 50 μM, compared with the peak plasma concentrations of less than 5 μM. Therefore, the biologically relevant concentrations of isoflavones in studies exploring their effects on bladder cancer cells *in vitro* are 50 μM or less.

The presence of significant concentrations of soy isoflavones in the urine provides the rationale for investigating the effects of soy isoflavones on bladder cancer cells. Furthermore, the observations that soy products may have activity against tumors of some tissues (10–16) and perhaps enhance carcinogenesis in others (9) emphasizes the importance of examining each type of cancer individually. We report that soy isoflavones, particularly genistein, cause a dose-dependent growth inhibition of murine and human transitional cell carcinoma cell lines *in vitro* at concentrations observed in human urine. Furthermore, soy isoflavones produce G_2-M cell cycle arrest and enhance apoptosis of bladder cancer cells *in vitro*. Treatment of mice with genistein, dietary soy phytochemical extracts, or dietary soy protein isolate inhibits the progression of transplanted murine transitional cell

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carcinoma *in vivo*. The inhibition of bladder tumor growth is correlated with *in vivo* changes in tumor biomarkers, including reduced PCNA[3] labeling, reduced MVD as a marker of angiogenesis, and increased apoptosis. Our studies suggest that soy products should be explored in future chemoprevention studies in rodent models of *de novo* bladder carcinogenesis with the hope of proceeding to clinical trials for high-risk groups or as an adjunct to the treatment of superficial bladder cancer.

MATERIALS AND METHODS

Soy Isoflavones, Soy Protein, and Phytochemical Extracts. Soy isoflavones (genistein, genistin, daidzein, and biochanin A) were purchased from Sigma Chemical Co. (St. Louis, MO). Soy protein isolate (Supro 670 HG, lot C5C-XPC-001; Protein Technology International, St. Louis, MO) contained 2.0 mg of isoflavones as aglycone equivalents (each isoflavone was calculated to reflect only the weight of the aglycone, because mixtures of free isoflavone and glycosylated forms are found in the product), including 0.64, 1.22, and 0.21 mg/g daidzein, genistein, and glycitein, respectively. A soy phytochemical concentrate containing approximately 300 mg of isoflavones/g was prepared at Archer Daniels Midland Co. (Decatur, IL). Whole soybeans were cracked, dehulled, and flaked by standard procedures, followed by a hexane extraction to remove the majority of lipid. The resulting defatted soy flour was extracted with aqueous ethanol (60%) to produce a mixture containing carbohydrates (60–70%), isoflavones (2%), fat (12%), ash (4%), and protein (5%). A proprietary extraction procedure was then used to remove the carbohydrates, and the remaining material was spray dried to form a powder called soy phytochemical concentrate and analyzed for isoflavones according to published methods (34). The final soy phytochemical concentrate used in our studies contained 30% isoflavones, 6% protein, 12% fat, and 4% ash, with the remaining 47% undefined but apparently rich in saponins. The isoflavone pattern in the soy phytochemical concentrate, expressed in mg/kg, was as follows: genistin, 126,000; daidzin, 121,000; glycitin, 25,600; malonyl genistin, 14,500; acetyl genistin, 7,630; malonyl daidzin, 4,070; daidzein, 1,210; and genistein, 791.

***In Vitro* Growth Curves.** Murine (MB49 and MBT-2) and human (HT-1376, UM-UC-3, TCCSUP, J82, and RT-4) bladder cancer cell lines were maintained as monolayer cultures in RPMI supplemented with 10% fetal bovine serum, 2 mmol L-glutamine, 100 units of penicillin/ml, and 100 mg streptomycin/ml in a 95% air/5% CO_2 water-saturated atmosphere. The *in vitro* studies were completed with 5×10^4 cells plated into 96-well microplates, treated with soy isoflavones or soy phytochemical concentrate dissolved in DMSO (final DMSO concentration ≤0.1%), and incubated for 72 h. DMSO vehicle controls were used in all studies. Growth curves were quantitated by the XTT assay (35). In brief, the XTT assay is an indirect measurement of viable cells based on the ability of dehydrogenase enzymes in metabolically active cells to cleave the tetrazolium salt XTT and yield a highly colored water-soluble formazan product that, with the addition of the electron-coupling agent phenazine methosulfate, potentiates the colorimetric reaction. All assays of growth curves were completed in triplicate, experiments were replicated, and results were confirmed by direct cell counting using a hemocytometer.

Analysis of Cell Cycle Progression and DNA Fragmentation. Cells were grown under conditions as described above, harvested by trypsinization and centrifugation, washed with PBS, and fixed with 80% ethanol. Cells were then washed with PBS, resuspended, stained by adding propidium iodide (at a final concentration of 50 μg/ml) and RNase (at a final concentration of 50 μg/ml), and incubated at 37°C for 30 min. Stained cells were analyzed by FACScan (Becton Dickinson, San Jose, CA) for fragmented DNA and cell cycle using programs provided by Becton Dickinson.

Diet Formulations and Treatment Groups. Soy phytochemical concentrate and soy protein isolate were used to prepare semipurified diets for five experimental groups (Table 1) by Research Diets, Inc. (New Brunswick, NJ):

Table 1 *Composition of experimental diets*

	Control[a]	Soy protein isolate[b]	0.2% soy phytochemical concentrate[c]	1.0% soy phytochemical concentrate[c]
Dietary component (g/kg)				
Casein	200	0	200	200
Soy protein isolate	0	200	0	0
DL-Methionine	3	3	3	3
Corn starch	150	150	150	150
Sucrose	500	500	498	490
Cellulose	50	50	50	50
Corn oil	50	50	50	50
Mineral mix[d]	35	35	35	35
Vitamin mix[e]	10	10	10	10
Choline bitartrate	2	2	2	2
Soy phytochemicals	0	0	2	10
Total	1000	1000	1000	1000
Isoflavones (mg/kg)[f]				
Total	0	400	341	1705
Genistein	0	234	159	794

[a] The control diet was also fed to mice receiving genistein by i.p. injection at a dose of 1.0 mg/mouse/day.
[b] Soy protein isolate contains 2.0 mg of total isoflavones/g.
[c] Soy phytochemical concentrate contains 300 mg of isoflavones/g.
[d] Mineral mix according to AIN-76 recommendations.
[e] Vitamin mix according to AIN-76 recommendations.
[f] Expressed as aglycone equivalents.

(*a*) AIN-76 diet (36, 37) as the control; (*b*) AIN-76A diet plus genistein, i.p., 50 mg/kg body weight/day (approximately 1 mg/day); (*c*) AIN-76A diet, with soy phytochemical concentrate at 0.2% of the diet providing 0.4 g of isoflavones/kg of diet; (*d*) AIN-76A diet, with soy phytochemical concentrate at 1.0% of the diet providing 2.0 g of isoflavones/kg of diet; and (*e*) AIN-76A diet, with casein replaced by soy protein isolate, 20% by weight, providing 0.4 g of isoflavones/kg diet.

Murine Tumor Studies. The MB49 cell line, grown as a s.c. implant, provides a rapid method for assessing the ability of dietary interventions to alter the growth of an invasive, poorly differentiated bladder tumor. Sixty female C57BL/6J mice (Taconic, Germantown, NY) at 8 weeks of age were randomly assigned to dietary treatment groups ($n = 12$) and fed their diets for 3 weeks. Mice were inoculated with 5×10^4 MB49 cells s.c. on the right flank and continued on the same treatments for an additional 3 weeks. Mice assigned to genistein treatment consumed the AIN-76A diet and were injected with genistein daily, i.p., at 50 mg/kg body weight (suspended in PBS at 2.5 mg/ml). The group treated with genistein by i.p. administration was included to provide an estimate of the maximal antitumor effect that could be observed for completely absorbed genistein and provide a comparison with the dietary soy products. Food intake, body weight, and tumor diameters were measured three times weekly. Tumor volumes were calculated by the following formula: tumor volume (cm^3) = 0.523 × (length × width × height). The experiment was terminated when mean tumor volume of controls exceeded 2 cm^3.

To obtain tumor tissue for histology, we performed an identical second study, except that we did not include the 0.2% soy phytochemical group, and harvested tumors 10 days after inoculation. This approach allowed us to examine tumors that are smaller and more homogeneous in their histopathology, while avoiding the necrosis that occurs in the much larger tumors harvested after 21 days of growth. Tumor tissues were fixed in formalin, embedded in paraffin, and cut into 4-μm sections for histological and immunohistochemical evaluation. Bladders from mice fed control or soy-containing diets were also fixed, processed, and examined by light microscopy for histopathological changes. Animal care in all studies was provided in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication 86-23, 1985).

Immunohistochemical Determination of Angiogenesis (MVD). After deparaffinization, rehydration, and washing, sections were incubated with trypsin at 37°C for 30 min; quenched with 0.3% H_2O_2-methanol for 30 min; and blocked with 10% normal goat serum in a buffer containing 100 ml of PBS, 1.0 g of BSA, and 0.1 ml of Tween 20. The sections were treated with a rabbit polyclonal antibody directed against human factor VIII-related antigen (DAKO Corp., Carpinteria, CA), a 1:100 dilution with the PBS-BSA-Tween 20 buffer described above, followed by a biotinylated universal antibody at a 1:100 dilution (Vector Laboratories, Inc., Burlingame, CA). The sections were

[3] The abbreviations used are: PCNA, proliferating cell nuclear antigen; MVD, microvessel density; XTT, sodium 3′-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate; AI, apoptotic index; PI, proliferation index; TdT, terminal deoxynucleotidyltransferase; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

then treated with avidin-biotin complex (Vector Laboratories) followed by 3,3′-diaminobenzidine as a substrate for staining. Sections were counterstained with methyl green and mounted. MVD was calculated by counting microvessels on ×200 fields under light microscopy at three representative sites without necrosis of each section.

Immunohistochemical Determination of PI. PI was determined by calculating the proportion of cells with PCNA staining. After deparaffinization, rehydration, and washing, sections were soaked in 10 mM citrate buffer, heated for 5 min in a microwave oven, and cooled to room temperature. Sections were then stained following the procedures as described for factor VIII staining, using 10% horse serum for blocking and a PCNA mouse monoclonal antibody (DAKO) as primary antibody. Both PCNA-positive proliferating cells and total tumor cells were counted in three nonnecrotic areas of each section using light microscopy at ×400 magnification.

***In Situ* Apoptotic Cell Detection (TUNEL Assay).** Apoptotic cells were determined using the ApopTag *in situ* detection kit (Oncor, Gaithersburg, MD), following the manufacturer's procedures with modification. In brief, after deparaffinization, rehydration, and washing in PBS, sections were treated with 20 μg/ml of proteinase K for 20 min at room temperature and washed. Endogenous peroxidase activities in sections were quenched with 3% H_2O_2 in PBS for 5 min. Sections were applied with TdT labeled with digoxygenin peroxidase and incubated for 1 h at 37°C, and the reaction was stopped by stop/wash buffer. Sections were then incubated with antidigoxygenin peroxidase for 30 min at room temperature, washed, stained with 3,3′-deaminobenzidine substrate, counterstained with methyl green, and mounted. Positive and negative control slides were used for comparison. Substitution of TdT with distilled water was used as a negative control. Three representative areas without necrosis were selected for each tumor, and both apoptotic cells and tumor cells were counted under light microscopy at ×400 magnification. AI was expressed as a percentage (apoptotic nuclei/normal nuclei × 100).

Statistical Analysis. Cell culture studies were initially evaluated by ANOVA (38) followed by Fisher's protected least-significant difference (38). Tumor volume, AI, PI, and MVD were initially evaluated by ANOVA (38), followed by Fisher's protected least-significant difference (38) or Scheffés test (39), to evaluate pairwise comparisons among treatment groups using Statview 4.5 software (Abacus Concepts, Inc., Berkeley, CA) and Power Macintosh computers (Apple Computer, Cupertino, CA).

RESULTS

***In Vitro* Effects of Soy Isoflavones and Soy Phytochemical Concentrate on Bladder Cancer Cell Lines.** The dose-dependent effects of genistein on bladder cancer cell number after 72-h incuba-

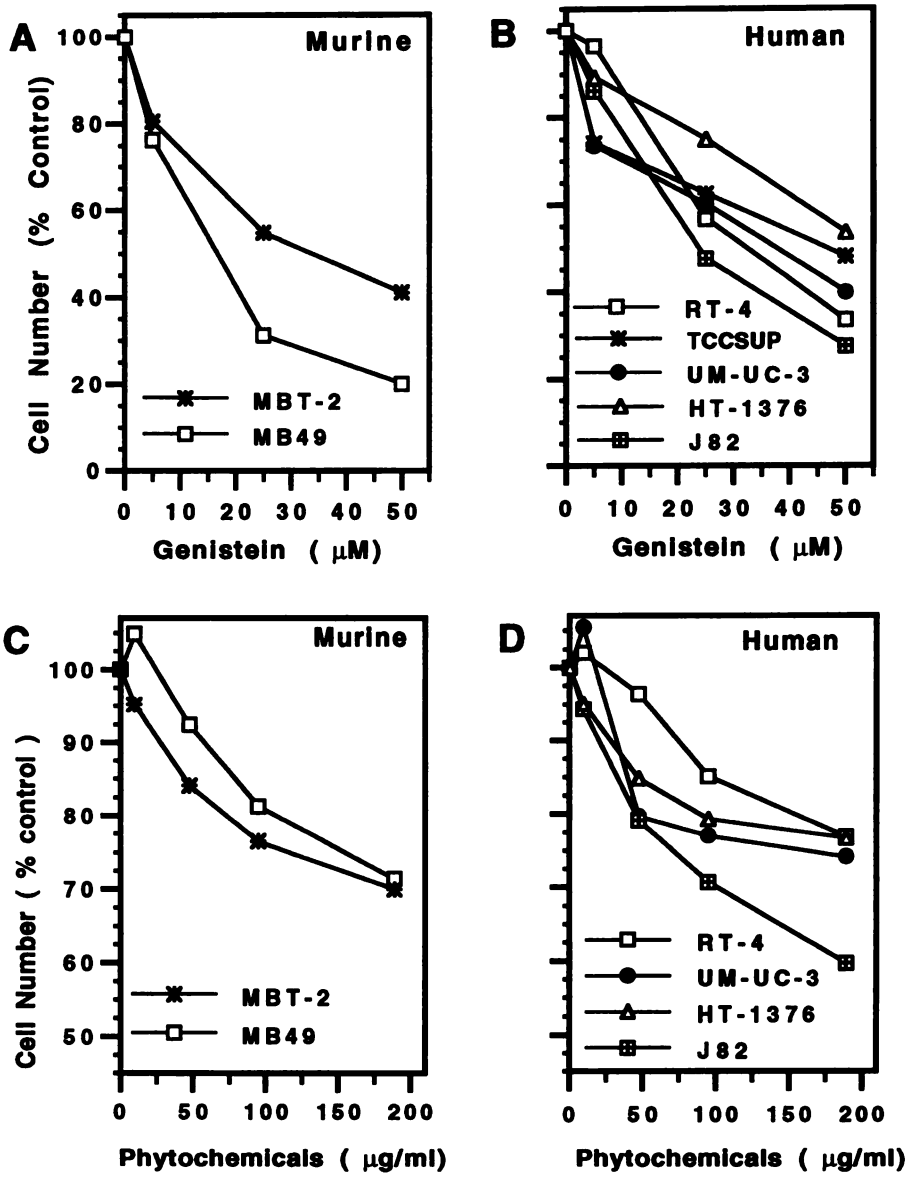

Fig. 1. Dose-dependent inhibition of cell number for murine and human bladder carcinoma cells *in vitro* by genistein or soy phytochemical concentrate. Cells were plated and treated for 72 h, and cell numbers were determined by XTT assay. Data represent means for quadruplicate samples; SEs are not shown for clarity of presentation but are less than 7% for each point. *A*, effects of genistein on murine cell lines (ANOVA; $P < 0.0001$ for main effect of dose, and $P < 0.0001$ for main effect of cell line; SE < 3.9 for each point). *B*, effects of genistein on human cell lines (ANOVA; $P < 0.0001$ for main effect of dose and no effect of cell line; SE < 3.2 for each point). Soy phytochemical concentrate was evaluated at 18–180 μg/ml, which contains 2.3–23 μM genistein or 5–50 μM total soy isoflavones (expressed as aglycone equivalents). *C*, effects of soy phytochemical extract on murine lines (ANOVA; $P < 0.0001$ for main effect of dose, and $P < 0.0001$ for pairwise comparisons of 0 *versus* each concentration greater than 48 μg/ml; SE < 2.9 for each point). MBT-2 cells were more sensitive to phytochemical extracts than were MB49 cells (ANOVA; $P = 0.0002$ for cell type). *D*, effects of soy phytochemical extract on human lines (ANOVA; $P < 0.0001$ for main effect of dose, and $P < 0.008$ for pairwise comparisons of 0 μg/ml *versus* each concentration greater than 48 μg/ml). We observed a significant difference in sensitivity to phytochemicals among human cell lines (ANOVA; $P < 0.001$ for main effect of cell type; SE < 6.0 for each point) that was primarily due to the greater sensitivity of the J82 line ($P < 0.005$ *versus* other lines).

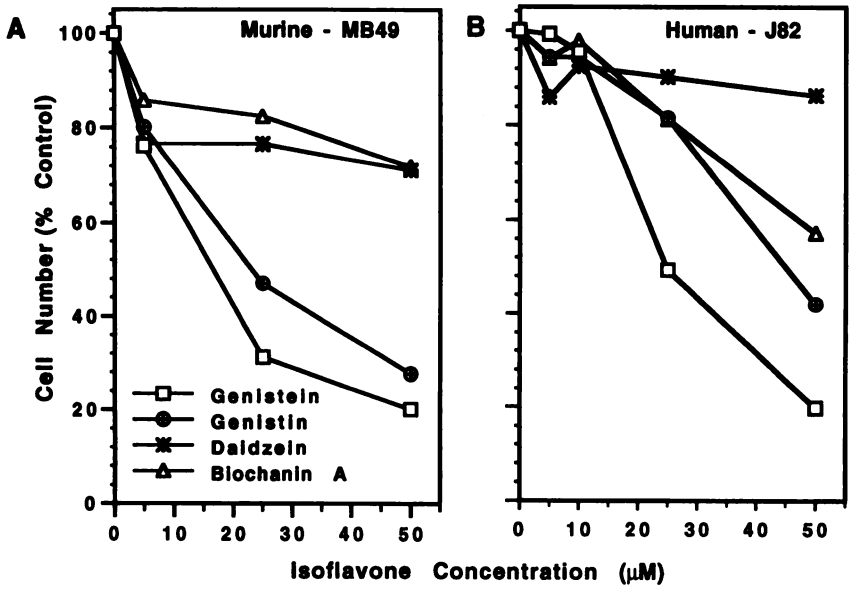

Fig. 2. Effects of different soy isoflavones (genistein, genistin, daidzein, and biochanin A) on murine and human bladder cancer cell lines *in vitro*. Cells were plated and treated for 72 h, and cell numbers were determined by XTT assay. Data represent means for quadruplicate samples; SE < 4.6 for each point. Experiments were repeated with identical conclusions, with cell number quantitated by direct cell counting. MB49 (*A*) and J82 (*B*) are representative murine and human bladder cancer cell lines, although similar results were observed with other bladder cancer cell lines. Daidzein and biochanin A are less potent than genistein or genistin for the inhibition of MB49 cells at 25 and 50 μM ($P < 0.0001$, daidzein or biochanin A *versus* genistein or genistin). The human J82 cell line was most sensitive to genistein ($P < 0.001$ *versus* others) and least sensitive to daidzein ($P < 0.001$ *versus* others), with biochanin A and genistin showing intermediate activity.

tions are presented in Fig. 1, *A* and *B*, respectively. Murine MB49 and MBT-2 cell lines showed reduced cell number with successive increases in genistein concentration (ANOVA and pairwise comparisons; $P < 0.0001$ for 0, 10, and 25 μM, and $P < 0.005$ for 25 μM *versus* 50 μM for each cell line). MB49 cells were more sensitive than were MBT-2 cells ($P < 0.001$ by ANOVA for main effect). Human bladder cancer cell lines were similarly influenced by successive increases in genistein concentration (ANOVA; $P < 0.0001$ for main effect of dose and for all pairwise comparisons of concentration). No significant differences between different human cell lines were observed (ANOVA; $P = 0.09$ for cell type).

The soy phytochemical concentrate was examined for effects on bladder cancer cell lines at 18–180 μg/ml, which contains 2.3–23 μM genistein and 5–50 μM total isoflavones (Fig. 1, *C* and *D*). Murine MB49 and MBT-2 cell lines were inhibited by phytochemicals (ANOVA; $P < 0.0001$ for main effect of dose, and $P < 0.0001$ for pairwise comparisons of 0 *versus* each concentration greater than 48 μg/ml). MBT-2 cells were more sensitive to phytochemical concentrate than were MB49 cells (ANOVA; $P = 0.0002$ for cell type). Human bladder carcinoma cell lines were inhibited by soy phytochemicals (ANOVA; $P < 0.0001$ for main effect of dose, and $P < 0.008$ for pairwise comparisons of 0 μg/ml *versus* each concentration greater than 48 μg/ml). We observed a significant difference in sensitivity to phytochemicals among human cell lines (ANOVA; $P < 0.001$ for main effect of cell type) that was primarily due to the greater sensitivity of the J82 line ($P < 0.005$ *versus* other lines).

Other soy isoflavones (genistin, daidzein, and biochanin A) showed a similar ability to reduce cell number after 72 h of incubation (Fig. 2). Daidzein and biochanin A are less potent than genistein or genistin for the reduction of MB49 cell cell number ($P < 0.0001$). At concentrations of 25 or 50 μM, the human J82 cell line was most sensitive to genistein ($P < 0.001$ *versus* others) and least sensitive to daidzein ($P < 0.001$ *versus* others), with biochanin A and genistin showing intermediate activity.

Effects of Genistein and Soy Phytochemical Concentrate on DNA Fragmentation and Cell Cycle Progression *in Vitro*. Table 2 shows representative results of cell cycle analysis by flow cytometry in two murine and two human bladder cancer cell lines treated with genistein at 0, 10, and 50 μM (Table 2). We observed a 9-fold increase in hypodiploid DNA in the 50 μM genistein-treated MBT-2 cell line compared with controls ($P < 0.001$). The UM-UC-3 human line also showed a modest increase in fragmented DNA with genistein treatment at 50 μM ($P < 0.001$ *versus* controls). The MB49 and J82 cells were relatively resistant to genistein-induced nuclear fragmentation compared with the MBT-2 line. Representative histograms from bladder cancer cells treated with genistein are shown in Fig. 3, *A* and *B*, illustrating the increase in fragmented DNA consistent with apoptosis. Subsequent time course studies with MBT-2 cells treated with genistein at 50 μM showed hypodiploid DNA of 1.25% at baseline and 5.1% at 24 h, 33.8% at 48 h, and 48.6% at 72 h (Fig. 3*A*). G_2-M cell cycle arrest was observed in all murine and human cell lines tested (Table 2; Fig. 3, *A* and *B*). Additional time course studies indicated

Table 2 *The effects of genistein on cell cycle progression by flow cytometry in bladder cancer cell lines in vitro*[a]

Cell lines and treatment	Hypodiploid DNA (%)	Cell cycle phase		
		G_1(%)	S(%)	G2/M(%)
MB49				
Control	6.5 ± 2.3	56.1 ± 3.0	36.1 ± 4.2	7.9 ± 1.3
Genistein 10 μM	8.6 ± 5.0	66.7 ± 3.9[b]	27.5 ± 2.6[c]	5.9 ± 1.3
Genistein 50 μM	5.4 ± 1.3	46.2 ± 1.2[b,d]	8.2 ± 0.5[d,e]	45.6 ± 1.4[d,e]
MBT-2				
Control	3.6 ± 0.5	43.2 ± 1.2	48.2 ± 0.7	8.6 ± 0.9
Genistein 10 μM	5.0 ± 0.7	34.4 ± 1.3[f]	50.0 ± 1.3	15.6 ± 2.1
Genistein 50 μM	33.7 ± 8.1[d,e]	8.3 ± 2.1[d,e]	51.9 ± 12.3	39.8 ± 9.8[e,g]
J82				
Control	3.8 ± 2.2	68.0 ± 4.1	23.3 ± 2.6	8.8 ± 1.8
Genistein 10 μM	1.0 ± 0.1	69.0 ± 5.6	22.7 ± 2.2	8.3 ± 3.8
Genistein 50 μM	1.6 ± 0.6	43.8 ± 3.1[f,h]	13.8 ± 2.8[d]	42.4 ± 5.4[d,e]
UM-UC-3				
Control	2.2 ± 0.2	63.6 ± 6.8	26.9 ± 5.0	9.5 ± 2.1
Genistein 10 μM	3.0 ± 0.2	53.6 ± 4.0	32.5 ± 3.4	13.8 ± 0.6[b]
Genistein 50 μM	5.0 ± 0.4[f,i]	32.1 ± 1.1[e,i]	34.2 ± 1.6	33.8 ± 0.9[d,e]

[a] Data are means ± SE for the murine cell lines MB49 and MBT-2 and for human cell lines J82 and UM-UC-3. Hypodiploid DNA represents the Sub-G_0 fraction and is consistent with nuclear fragmentation or apoptosis. The proportions of remaining cells in specific phases of the cell cycle are calculated such that $G_1 + S + G_2M = 100\%$.

[b] Significantly different from control by ANOVA and pairwise testing ($P < 0.03$).

[c] Significantly different from control by ANOVA and pairwise testing ($P < 0.05$).

[d] Significantly different from genistein 10 μM by ANOVA and pairwise testing ($P < 0.0001$).

[e] Significantly different from control by ANOVA and pairwise testing ($P < 0.0001$).

[f] Significantly different from control by ANOVA and pairwise testing ($P < 0.001$).

[g] Significantly different from genistein 10 μM by ANOVA and pairwise testing ($P < 0.002$).

[h] Significantly different from genistein 10 μM by ANOVA and pairwise testing ($P < 0.001$).

[i] Significantly different from genistein 10 μM by ANOVA and pairwise testing ($P < 0.003$).

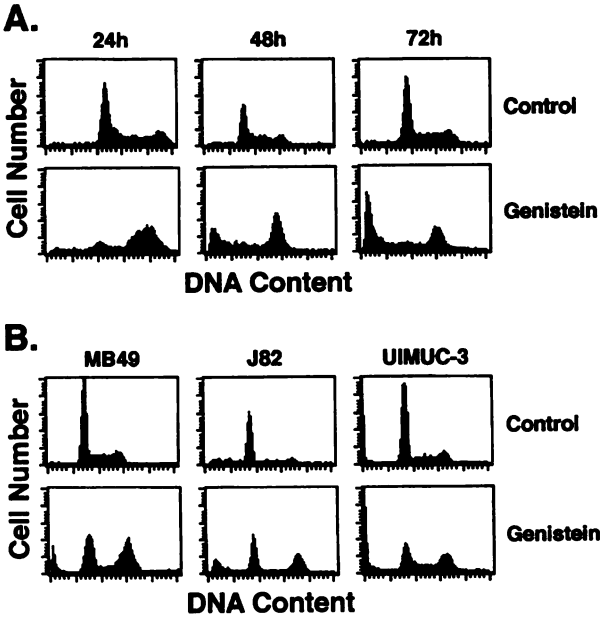

Fig. 3. *A, in vitro* effect of genistein (50 μM) or vehicle on DNA fragmentation and cell cycle progression of murine MBT-2 bladder cancer cells after 24, 48, and 72 h by flow cytometry. *A*, demonstration that the MBT-2 murine bladder cancer cell line rapidly shows G_2-M cell cycle arrest and that DNA fragmentation increases progressively over time. *B*, comparison of MB49, J82, and UM-UC-3 bladder cancer lines. Each *line* shows G_2-M cell cycle arrest following genistein (50 μM) treatment for 72 h. However, these lines are less susceptible to genistein-induced DNA fragmentation than the MBT-2 line is.

that these effects were present by 24 h of incubation and maintained for 72 h. Furthermore, genistein typically caused detectable accumulation of cells in the G_2-M phase at concentrations greater than 12.5 μM, and a plateau occurred between 25 and 50 μM (data not shown). Parallel studies (data not shown) with soy phytochemical concentrate also showed apoptosis in MBT-2 cells and cell cycle arrest in G_2-M for bladder cancer lines, although the magnitude of the response was attenuated compared with that of pure genistein. The results of the flow cytometry were consistent with the hypothesis that cell cycle arrest and apoptosis contribute significantly to the changes in the growth curves defined by the XTT assay and cell quantitation.

Dietary Soy Products and the Growth Inhibition of Transplantable Bladder Cancer. The growth of the murine MB49 transplantable bladder carcinoma in syngeneic C57BL/6J mice was used as an *in vivo* model to evaluate the effects of genistein- or soy-containing diets on tumor growth. Dietary soy treatments did not significantly reduce body weight or food intake (Table 3). The effect of dietary treatment on tumor growth rate (tumor volume) is shown in Fig. 4. Tumors from mice treated with soy protein isolate, genistein, soy phytochemicals at 0.2%, or high dietary soy phytochemicals at 1.0% were reduced in volume by 36% ($P < 0.01$), 39% ($P < 0.007$), 25% ($P > 0.05$), or 48% ($P < 0.001$) respectively, compared with the control group.

Effects of Dietary Soy Products on Tumor Apoptosis, Angiogenesis, and Proliferation. The MB49 murine bladder carcinoma is a rapidly growing, poorly differentiated, locally invasive, and metastatic tumor. All tumors from the above studies also show large areas of central necrosis at the time of sacrifice. These tumors are not ideal for evaluation of histological biomarkers, and to obtain more homogeneous tumor samples for microscopic analysis of biomarkers, we completed an identical experiment using the same procedures, except that mice were sacrificed at day 10 after tumor inoculation. Table 4 shows the effects of treatment on bladder tumor PI (PCNA staining), AI (TUNEL assay), and angiogenesis (MVD). Compared with controls, tumors from mice treated with genistein or fed soy protein or soy phytochemicals (1.0%) showed reduced proliferation rates of 14% ($P < 0.05$), 30% ($P < 0.001$), and 23% ($P < 0.005$), respectively. In contrast, tumor cell apoptosis rates were increased in mice treated with genistein or fed soy protein or soy phytochemicals by 51% ($P > 0.05$; not significant), 108% ($P < 0.05$), and 134% ($P < 0.005$), respectively, compared with controls. We observed that the rates of proliferation and apoptosis were inversely related, and we calculated a PI:AI ratio based on our hypothesis that this calculation may be a better biomarker of antitumor activity than either measure alone. We observed that the PI:AI ratio in mice treated with genistein or fed soy protein or soy phytochemicals was reduced by 41% ($P < 0.005$), 60% ($P < 0.001$), and 64% ($P < 0.001$) respectively, compared with that in controls. MVD as a marker of tumor angiogenesis was also affected by genistein, soy protein, and soy phytochemical concentrate, with reductions of 49% ($P < 0.05$), 44% ($P > 0.05$), and 34% ($P < 0.05$), respectively.

We have also examined bladders of mice fed control or soy-containing diets by light microscopy. We observed no change in the mucosa or muscle layers and no evidence of inflammatory responses. We conclude that soy products can modify bladder tumor growth while having no readily detectable toxicity for the normal bladder mucosa.

DISCUSSION

The objective of our *in vitro* studies with bladder cancer cells was to use a range of isoflavone concentrations relevant to *in vivo* exposures through the circulation or urine. The vast majority of publications examining effects of isoflavones *in vitro* use concentrations far beyond those achievable *in vivo* following dietary consumption of soy-based foods or products enriched in isoflavones. Interestingly, soy isoflavones can reach sustained urinary concentrations in the range of 50 μM in Asian populations consuming traditional diets or in volunteers fed defined doses of soy products (23, 26, 28–32). A single oral dose of pure genistein (20 mg/kg body weight) or a similar dose of genistein from an isoflavone-rich soy extract fed to rats results in a

Table 3 *Final body weight, total food intake, and total isoflavone intake for mice bearing the MB49 bladder cancer and randomized to various treatment groups*[a]

Groups	Body weight[b] (g)	Food intake (g/day)	Isoflavone intake (mg/day)	Genistein intake (mg/day)	Daidzein intake (mg/day)
Control	20.9 ± 0.6	2.6 ± 0.1	0.00 ± 0.00[d]	0.00 ± 0.00[d]	0.00 ± 0.00[e]
Genistein	22.7 ± 0.8[c]	2.7 ± 0.1	1.00 ± 0.01[d]	1.00 ± 0.01[d]	0.00 ± 0.00[e]
Soy protein	21.0 ± 0.8	2.7 ± 0.1	1.08 ± 0.01[d]	0.63 ± 0.01[d]	0.32 ± 0.01[d]
Soy phytochemicals (0.2%)	20.7 ± 0.6	2.6 ± 0.1	0.89 ± 0.01[d]	0.41 ± 0.01[d]	0.36 ± 0.01[d]
Soy phytochemicals (1.0%)	21.0 ± 0.7	2.6 ± 0.1	4.43 ± 0.17[d]	2.06 ± 0.08[d]	1.83 ± 0.07[d]

[a] Values are means ± SE.
[b] Body weight data represent final weight of mouse before necropsy minus final tumor weight. Starting weights were 17.7 ± 0.4 g.
[c] Significantly different from controls, or 0.2% soy phytochemicals ($P < 0.05$).
[d] Significantly different from all treatment groups ($P < 0.05$).
[e] Significantly different from soy protein, 0.2% soy phytochemicals, or 1.0% soy phytochemicals ($P < 0.05$).

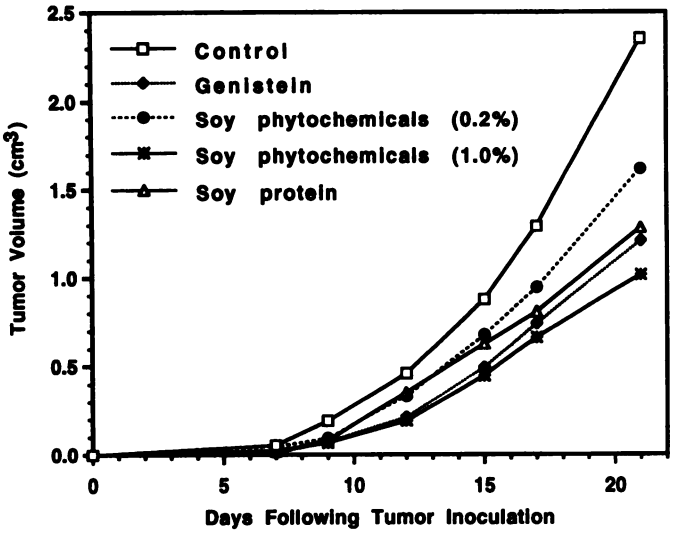

Fig. 4. Effects of genistein injection, dietary soy protein (20% of diet), and dietary soy phytochemical extract at 0.2 or 1.0% on the growth of a MB49 murine bladder cancer in syngeneic C57BL/6 female mice. Data represent mean tumor volume; SEs range from 0.20 to 0.34 at 21 days.

plasma genistein concentration at 2 h after dosing of 11.0 μM and 4.93 μM, respectively (40). Twenty-four h later, plasma genistein was almost completely eliminated (40). Urinary recoveries over than 48 h in genistein- and soy extract-treated rats were similar, *i.e.*, 17.9 and 17.5%, respectively (40). i.v. injection of genistein in mice as a single dose of up to 54 mg/kg body weight resulted in a rapid increase of plasma genistein concentration to 237 μM at 3 min after injection followed by a rapid decline of more than 250-fold over a period of only 50 min (41). In summary, soy isoflavones are rapidly cleared from the serum, and sustained concentrations of more than 5–10 μM after ingestion of soy-based foods or by oral consumption of a pure isoflavone are unlikely. In contrast, urinary isoflavones have consistently been reported to reach concentrations in the range of 25–50 μM (27–30). Our *in vitro* studies typically observed effects of soy isoflavones on bladder cancer cell cycle progression, apoptosis, and growth curves *in vitro* within the range of isoflavone concentrations observed in urine. However, much remains to be understood concerning the exposure of early bladder tumors to circulating and urinary isoflavones as well as the specific metabolites involved.

We report that pure genistein, soy protein isolate, and a soy phytochemical extract can inhibit the growth of an aggressive and rapidly growing murine bladder carcinoma *in vivo*. We chose pure genistein by injection to assure bioavailability, because absorption of isoflavones is incomplete. The soy protein isolate and phytochemical extracts were chosen for evaluation, because both products are suitable for incorporation into experimental diets and human foods for future chemoprevention studies. Furthermore, we chose these products for our initial studies precisely because they contain a diverse array of biologically active compounds that could potentially interact to provide more potent anti-bladder cancer activity. These hypothetical benefits would not be appreciated in studies of pure compounds. Readers should be cautious about extrapolating these effects to other bladder cancer models, other histological types of cancer, or potential effects in humans. The *in vivo* inhibition of cancer incidence or progression by soy products or pure isoflavones has been reported for gastric cancer (15), leukemia cells (16), breast cancer (10, 11), and others (1). In contrast, some studies have not found *in vivo* inhibitory effects of soy on tumorigenesis (1, 7, 8). Of concern, some studies report that soy-based dietary treatments had tumor-promoting effects. Consumption of a soybean-based diet (defatted soybean meal, 33.3% by weight providing 20% dietary protein) (42) or administration of genistein (9) resulted in increased colon tumor incidence and tumor burden in rats treated by chemical carcinogens. In addition, it has been hypothesized that the estrogenic properties of soy isoflavones may stimulate breast tumor growth under some conditions (43). Investigators, clinicians, and commercial enterprises should use caution in universally recommending soy supplements enriched in isoflavones for cancer prevention or therapy, except in the context of clinical studies.

Our studies with murine bladder tumors *in vivo* suggest that biomarkers of proliferation, apoptosis, and angiogenesis are sensitive to dietary soy products and may be useful in future clinical dietary/nutritional intervention trials in which histopathological samples of the bladder can be obtained via the cystoscope. We found that the inhibition of tumor growth by soy was associated with a modest decrease in proliferation rate. Interestingly, proliferation rates in human bladder carcinoma as assessed by PCNA immunostaining has been reported to be an independent predictor for survival (44). We observed that markers of apoptosis may be even more sensitive indicators of the bioactivity of soy products than PI. In our studies, mice fed soy products exhibited significant increases in the proportion of tumor cells exhibiting TUNEL staining. Our observations with bladder lines *in vitro* are supported by previous studies in malignant and normal cells of certain histological types. Genistein at 50 μM can induce apoptosis, possibly through suppression of focal adhesion kinase activity and regulating c-*myc* function in prostate carcinoma cells (45). Genistein at 30 μM has been shown to induce apoptosis of immature human thymocytes *in vitro* by inhibiting topoisomerase II (46), which was further supported by studies showing genistein to induce apoptosis of mouse thymocytes (47), human stomach cancer cell lines (15), or leukemia cells (48). Because we observed that specific bladder cancer cell lines have differing sensitivity to isoflavone-induced apoptosis *in vitro*, we hypothesize that the pattern of oncogene or tumor suppresser gene mutations within each specific cell line may underlie this response. We are in the process of determining whether p53, bcl-2, $p21^{WAF1}$, and cyclin expression are related to the ability of soy isoflavones to induce apoptosis in addition to cell cycle arrest. The observation that the MB49 cell line is relatively insensitive to soy-induced apoptosis *in vitro*, but that apoptosis was significantly increased *in vivo*, allows us to speculate concerning additional mechanisms or modulating factors. For example,

Table 4 *The effects of genistein or dietary soy products on immunohistochemical evaluation of bladder tumor biomarker*[a]

Treatment group	Proliferation Index (%)	Apoptotic Index (%)	PI:AI ratio	Vascular density (vessels/field)
Control	58.6 ± 3.7[b]	2.6 ± 0.1[f]	22 ± 1[b]	40.3 ± 9.2[b]
Genistein	50.3 ± 2.1[c]	4.0 ± 0.5[g]	13 ± 2[h]	20.4 ± 2.6[e]
Soy protein	40.7 ± 1.6[d]	5.5 ± 1.1[e]	9 ± 3[e]	22.7 ± 5.2[e]
Soy phytochemicals	45.0 ± 2.1[e]	6.2 ± 0.6[d]	8 ± 1[d]	26.5 ± 2.7[e]

[a] Mice were inoculated with 5×10^4 MB49 bladder tumor cells and harvested after 10 days for evaluation. Values are means ± SE.
[b] Significantly different from genistein, soy protein, or soy phytochemicals with at least $P < 0.05$. See text for P values for specific comparisons.
[c] Significantly different from control and soy protein groups with at least $P < 0.05$. See text for P values for specific comparisons.
[d] Significantly different from control and genistein groups with at least $P < 0.05$. See text for P values for specific comparisons.
[e] Significantly different from control groups with at least $P < 0.05$. See text for P values for specific comparisons.
[f] Significantly different from soy protein and soy phytochemical groups with at least $P < 0.05$. See text for P values for specific comparisons.
[g] Significantly different from soy phytochemical group ($P < 0.05$).
[h] Significantly different from control ($P < 0.005$) and soy phytochemical groups ($P < 0.05$).

cells may be more sensitive to apoptosis *in vivo*, where growth factors, oxygen, and nutrient availability may be more tenuous than in cell culture media optimized for maximal growth. However, these observations are also consistent with the hypothesis that apoptosis *in vivo* is a secondary or indirect effect of soy isoflavones on other processes related to tumor progression, such as tumor angiogenesis.

Our *in vitro* studies with a series of murine and human bladder lines, showing dose-dependent effects of soy isoflavones, provide insight into potential mechanisms relevant to our *in vivo* observations in the transplantable bladder cancer model in rodents. It is probable that soy isoflavones are inhibiting growth factor-induced signal transduction in bladder cancer cells (18, 49, 50). We observe that the inhibition of proliferation in murine and human bladder cancer cell lines by soy isoflavones is consistently associated with a dose-dependent G_2-M cell cycle arrest. It is unclear what molecular mechanisms account for these findings. We propose that soy isoflavones influence phosphorylation and dephosphorylation of proteins involved in cell cycle progression, such as cdc2. It has been shown that apigenin, a flavonoid with a structure similar to that of genistein, arrests keratinocytes at G_2 and M phases by inhibiting the cdc2 kinase activity (51). Additional efforts to identify molecular targets of soy isoflavone activity may lead to the characterization of new biomarkers that can be used in future clinical studies as surrogate end points.

Folkman (52) has characterized the growing tumor as having two compartments that can be targeted for therapy, the malignant clone and the neovasculature. Our studies suggest that soy isoflavones can target both proliferating tumor cells and the associated tumor neovasculature. Angiogenesis, the generation of new blood vessels, is a very important event in cancer growth and metastasis. Angiogenesis in primary bladder is significantly correlated with tumor stage and the presence of vascular invasion and suggested to be an independent prognostic indicator for patients with invasive transitional cell carcinoma of the bladder (53) and a predictor of death from the disease (54). Angiogenic growth factors are increased in the urine of patients with bladder cancer, particularly those with a higher stage of disease (55). We observe that soy significantly inhibits bladder tumor angiogenesis based on MVD as a surrogate marker. Furthermore, our *in vitro* studies (data not shown) confirm the work of others (22, 56) showing that soy isoflavones or the soy phytochemical extract inhibits growth factor-induced endothelial proliferation *in vitro*. The reduced MVD, the greatly enhanced apoptosis, and the only slight reductions in PI in response to soy isoflavones are very similar to the effects observed in rodent tumor models following treatment with antiangiogenic agents such as angiostatin (57). The mechanisms whereby soy products alter the angiogenic switch and the balance between angiogenic growth factors and inhibitors *in vivo* are under investigation. Regardless of the mechanism, we believe that markers of angiogenesis may be useful in rodent and human studies of soy products and bladder cancer.

In summary, we observe that soy extracts and isoflavones have direct effects on bladder cancer cell lines *in vitro* by inhibiting growth rates, blocking cell cycle progression, and enhancing apoptosis. These observations are supported by rodent studies documenting the inhibition of tumor growth by soy products in association with enhanced tumor cell apoptosis, reduced proliferation, and reduced tumor angiogenesis. Furthermore, these effects on bladder tumors *in vivo* occur with no histopathological evidence for toxicity to the normal bladder mucosa. Our laboratory studies and the investigations in humans showing high concentrations of urinary isoflavones following consumption of soy-containing foods suggests that soy products or pure isoflavones may have unique benefits for those at risk of primary or recurrent superficial bladder cancer. To further define the anti-bladder cancer properties of soy products and pure isoflavones, we are currently undertaking studies of *de novo* chemical carcinogenesis and transplantable bladder cancer placed orthotopically within the bladder. These experiments will provide the preclinical data necessary to extend our work to human intervention trials.

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