Short Communication

Induction of Apoptosis in Low to Moderate-Grade Human Prostate Carcinoma by Red Clover-derived Dietary Isoflavones[1]

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Abstract

Epidemiological evidence suggests a geographical basis for the incidence of prostate cancer and dietary factors, including isoflavone consumption, may be linked to this phenomenon. This paper reports a nonrandomized, nonblinded trial with historically matched controls from archival tissue designed to determine the effects of acute exposure to a dietary supplement of isoflavones in men with clinically significant prostate cancer before radical prostatectomy. Thirty-eight patients were recruited to the study upon diagnosis of prostate cancer. Before surgery, 20 men consumed 160 mg/day of red clover-derived dietary isoflavones, containing a mixture of genistein, daidzein, formononetin, and biochanin A. Serum PSA, testosterone, and biochemical factors were measured, and clinical and pathological parameters were recorded. The incidence of apoptosis in prostate tumor cells from radical prostatectomy specimens was compared between 18 treated and 18 untreated control tissues. There were no significant differences between pre- and posttreatment serum PSA, Gleason score, serum testosterone, or biochemical factors in the treated patients ($P > 0.05$). Apoptosis in radical prostatectomy specimens from treated patients was significantly higher than in control subjects ($P = 0.0018$), specifically in regions of low to moderate-grade cancer (Gleason grade 1–3). No adverse events related to the treatment were reported. This report suggests that dietary isoflavones may halt the progression of prostate cancer by inducing apoptosis in low to moderate-grade tumors, potentially contributing to the lower incidence of clinically significant disease in Asian men. The assessment of new prostatic therapies aimed at increasing apoptosis should control for intake of dietary isoflavones.

Introduction

Prostate cancer is the most commonly diagnosed cancer in men (excluding nonmelanocytic skin cancer) and is the second most common cause of cancer-related death in men after lung cancer (1, 2). Dietary factors may be important as epidemiological evidence demonstrates an inverse relationship between the incidence of both BPH[3] and prostate cancer and dietary intake of foods rich in phytoestrogens (3, 4).

Although the incidence of microfocal cancer is identical in Western and Asian men, progression to clinical disease occurs more frequently in men from Western countries (5–9). The hypothesis that dietary factors may contribute to the different rates of progression is supported by a report that within two generations, Asian male migrants who relocated to the United States of America had the same rate of mortality from prostate cancer as United States-born native residents (10). A prospective study by Severson *et al.* (11) showed increased consumption of rice and tofu in men of Japanese ancestry living in Hawaii was associated with decreased risk of prostate cancer.

Urinary isoflavones excretion was higher in Japanese men compared with American men, who exhibited a greater incidence of clinically significant prostate cancer (12). The concentrations of isoflavones in plasma and prostatic fluids were higher in men from Hong Kong compared with those from European countries such as Britain or Portugal (13), indicative of their ability to concentrate in the prostate gland itself.

On the basis of the epidemiological evidence, there is renewed interest in the role of isoflavones in the chemoprevention of clinically significant prostate cancer. Isoflavones are nonsteroidal diphenolic plant compounds found almost exclusively in leguminous plants. The effects of isoflavones (summarized in several reviews; Refs. 4, 14, 15) include regulation of the activity of steroidogenic enzymes [such as 5α-reductase (16) and aromatase (17)], binding to both ER subtypes ER-α and ER-β, acting as estrogen agonists or antagonists (18), regulating steroid receptor (AR and ER) expression (19), inhibition of cell proliferation and DNA topoisomerase II (20), promotion of cell cycle arrest (21), and induction of cell differentiation (22) and apoptosis (23).

The effects of isoflavones such as genistein, daidzein, and biochanin A on the androgen-dependent and -independent prostate cancer cell lines (PC3, LNCaP, and DU145) include inhibition of cell proliferation and induction of apoptosis (24–26). Isoflavones also inhibited the growth of prostate cancer xenografts in several animal models (27–30), as well as reducing

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[3] The abbreviations used are: BPH, benign prostatic hyperplasia; ER, estrogen receptor; AR, androgen receptor; PSA, prostate specific antigen; TNM, Tumor-Node-Metastasis; AE, adverse event.

the incidence of poorly differentiated prostatic adenocarcinoma in the transgenic TRAMP mice (31). In young mice, soy consumption prevented the development of estrogen-induced premalignant changes to the prostate gland (32). Collectively, the data suggest that dietary isoflavones play a role in regulating cell proliferation and apoptosis in prostate cancer cells.

In humans, there are fewer studies on the effect of isoflavones in men with prostate cancer. A single case report described the effect of an isoflavone supplement (160 mg) derived from red clover taken for 7 days before radical prostatectomy after diagnosis of moderately high-grade adenocarcinoma. The resected specimen showed degenerative changes and increased apoptosis in the malignant tissue, whereas the surrounding nonmalignant tissue was unaffected (33). This preliminary finding supported *in vitro* data that showed isoflavones induced apoptosis in human prostate carcinoma cell lines.

The aim of this study was to determine the short-term effects of red clover-derived isoflavones in men with prostate cancer in the intervening period between diagnosis and radical prostatectomy.

Table 1 Comparison of treated and control patient pretreatment parameters

Parameter	Control ($n = 18$)[a]	Treated ($n = 18$)[b]
	Mean ± SD[c]	
Age at Surgery (yr)	60 ± 5.3	60.5 ± 6.6[c]
PSA (μg/liter)	8.7 ± 5.8	9.3 ± 7.0[c]
Gleason score	6.1 ± 1.3	6.6 ± 0.9[c]
	Clinical Stage[d]	
	n (%) patients	
TNM staging		
N_X, N_1, M_0	0	0
T_{1c}	12 (66.7)	10 (55.6)
T_{2a}	3 (16.7)	4 (22.2)
T_{2b}	3 (16.7)	4 (22.2)
T_{2c}	0	0
T_{3a}	0	0
T_{3b}	0	0
T_4	0	0

[a] $n = 18$ matched controls.
[b] $n = 18$ excluding patients ID 4 and ID 10 from treated group.
[c] Two sample *t* tests, control *versus* treated, not significant.
[d] All patients were N_0, M_0 unless otherwise stated.

Materials and Methods

Patient Selection. The nonrandomized, nonblind intervention study was conducted on a cohort of 20 men from Melbourne, Australia, who were diagnosed with clinically significant prostate cancer. The patients selected for study had nonmetastatic prostate cancer and a Gleason score of ≥5 based on pathological assessment from biopsy specimens. Patients who received neoadjuvant hormonal therapy or were vegetarians and/or soy users (consumption of soy based products, including soy milk, soy and linseed bread, soy sauce, and soy-based cereals as ascertained by dietary questionnaires) were excluded from the study. Of the 20 men, 2 did not have surgery [1 patient had lymph node metastatic disease (ID 10)]: one was excluded from the cohort and another withdrew because of preoperative medical complications (atrial fibrillation at anesthetic induction; ID 4) that were not related to the treatment. As such, pathology for the 18 patients who completed the study while pre- and posttreatment serum and urine analyses were available for 19 patients (ID 4 did not provide blood or urine samples at visit 3 but ID 10 did).

Postoperative specimens were used to compare the effects of isoflavone treatment: radical prostectomy specimens from men who took isoflavone supplementation were compared with those from a cohort of historically matched men ($n = 18$) who did not receive either isoflavone supplementation or neoadjuvant hormonal therapy. Because age, preoperative PSA, Gleason score, and TNM stage are parameters that are known prognostic markers of prostate cancer, it was considered appropriate to individually match patients for these preoperative factors and generate a control group for comparison to the treated group. Therefore, the cohort of untreated men was selected by individually matching each control patient to each treated patient for age (so that there was no more than 4 years of age difference between each matched patient), Gleason score, preoperative PSA, and TNM stage. The two groups therefore showed similar distributions in Gleason score, preoperative PSA, and TNM staging as shown in Table 1. Soy intake was also ascertained by the urologist in the untreated control patients and deemed to be within normal limits.

The studies were completed after approval from Cabrini Hospital and Monash Medical Centre Human Research Ethics Committees and a written informed consent obtained from each subject enrolled in the study. Control radical prostatectomy specimens were obtained and used under the guidelines of Cabrini Hospital and Monash Medical Centre Human Research Ethics committee.

Treatment. Each patient in the treated group consumed 160 mg of isoflavones daily consisting of 4 tablets/day (with the morning meal) of Trinovin (Novogen, Sydney, NSW, Australia) containing 40 mg of standardized red clover (*Trifolium pratense*)-derived isoflavones in the aglycone state, including predominantly formononetin and biochanin A with smaller amounts of genistein and daidzein in a ratio of biochanin A + genistein:formonentin + daidzein of 2:1. The length of treatment for each patient varied according to the time available before the scheduled radical prostatectomy (median, 20 days; range, 7–54 days; see Fig. 1 for treatment protocol). Patients were subjected to blood pressure, heart rate, and weight measurements at the beginning and the conclusion of the study, as well as regular medical assessments (including full physical examinations and digital rectal examinations) during the treatment interval (Fig. 1). Patients provided duplicate blood and urine samples pre- (Visit 2) and posttreatment (Visit 3) for analysis of several serum parameters (coagulation factors, biochemical and hematological factors, PSA, and testosterone; all parameters measured by standard pathological protocols), as well as excreted isoflavone concentrations. On the day before surgery (Visit 3), patients completed AE assessment based on the MedDRA coding system (Medical Dictionary for Regulatory Authorities); only 19 of 20 patients completed the assessment (patient ID 2 did not complete an AE assessment). At the end of the treatment interval, patients underwent radical prostatectomy (Visit 4). Pre- and posttreatment parameters were not available for the control patient group because they were not subjected to the same treatment protocol.

Sample Collection. Paraffin blocks of formalin-fixed radical prostatectomy specimens from all patients (treated and control) involved in the study were obtained from pathology at Monash Medical Centre, Cabrini and Royal Melbourne Hospitals (Melbourne, Victoria, Australia). The histological diagnosis, clinical staging based on the TNM system, and Gleason score were determined in each case during a routine pathological evaluation after surgery by a single pathologist (Dr. John Pedersen,

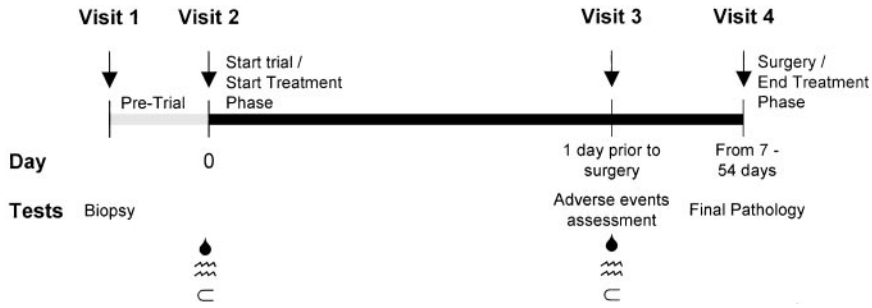

Fig. 1. Intervention study protocol for patients treated with dietary isoflavones; the treatment period varied for each patient and was between 7–54 days from the start of the treatment to surgery. ≈, urine collection; ♦, blood collection; ⊂, digital rectal examination.

Monash University). Tissue sections were obtained from the blocks involving carcinoma as selected by the pathologist, and 4-μm sections were cut and mounted on Superfrost Plus slides (Menzel-Glaser, Germany) for detection of apoptosis.

Detection of Apoptosis. Apoptosis was detected in radical prostatectomy specimens from treated and control patients by ApopTag *In Situ* Apoptosis Detection Kit (Integren, Purchase, NY). Sections were dewaxed, incubated in Equilibration Buffer (Integren), and then treated with terminal deoxynucleotidyl transferase enzyme in Reaction Buffer (Integren) for 1 h at 37°C. Sections were washed in Stop Wash Buffer (Integren) for 30 min at 37°C, treated with 3% (vol/vol) H_2O_2 in methanol for 15 min, and blocked with CAS block (Zymed Laboratories, San Francisco, CA). Apoptotic cells were detected by antidigoxigenin conjugate (Integren) for 30 min at room temperature and color reacted with 3,3′-diaminobenzidine tetrahydrochloride (Liquid substrate kit; Zymed). The reactions were stopped with water, and sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted.

Identification and Quantification of Apoptosis. To be sure the judges who scored the incidence of apoptosis were blinded to the treatment status, slides were independently code numbered by the personnel who prepared the slides. The blind scorer then identified apoptosis using two parameters. Firstly, positive immunolabeling as detected by apoptag staining. Secondly, cells that were positive by immunolabeling were then confirmed by morphological characteristics typical of cells undergoing programmed cell death, *i.e.*, including chromatin aggregation, nuclear, and cytoplasmic condensation as well as fragmentation of the dying cell into a cluster of membrane-bound segments or apoptotic bodies [as described by Kerr *et al.* (34)]. If a cell was judged to be apoptotic by both criteria, it was counted.

Apoptosis was then quantified based on a method that allowed an unbiased estimation of the percentage of cells in apoptosis in both treated and control patient samples. Random fields were systematically selected from the sections by computer-assisted software: Castgrid software (Olympus Danmark A/S, Albertslund, Denmark). Some regions throughout the tissue were subject to high background staining and these areas were excluded from the sampling during quantification. For each patient, an average of 2,500 cells were counted, and the number of sample fields varied according to the amount of tissue available. Each field typically contained 25–50 cells and was classified by pathology as nonmalignant BPH and prostatic intraepithelial neoplasia), low to moderate-grade (Gleason grade 1–3) or high-grade (Gleason grade 4–5) cancer. Initial analysis indicated that the incidence of apoptosis in BPH and prostatic intraepithelial neoplasia tissues were not different, so these pathologies were classed together as nonmalignant tissue. Each cell that was counted was recorded as epithelial or stromal and positively stained cells (confirmed by histological characteristics typical of cells undergoing programmed cell death) were represented as percentage of total cells.

Immunohistochemistry. Immunohistochemistry was performed in a cohort of randomly selected patients ($n = 10$ treated and $n = 10$ control patients) to assess the localization of the AR. Indirect avidin-biotin horseradish peroxidase immunohistochemistry was performed as described previously (35). Briefly, sections were dewaxed, rehydrated in graded ethanols, and subjected to microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 20 min at boiling temperature. Sections were then treated with hydrogen peroxide blocking solution (Dako Corporation, Carpinteria, CA) for 20 min and blocked with CAS block (Dako Corporation). Sections were then incubated with primary antibodies (AR-N20; Santa Cruz, CA; working concentration: 0.67 μg/ml) or concentration-matched rabbit IgG (Zymed; negative control sections) for 1 h at room temperature. Sections were then incubated with biotinylated goat antirabbit for 30 min at room temperature, followed by Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Sections were stained using 3,3′-diaminobenzidine and then counterstained and mounted as described above.

Measurement of Urinary Isoflavone Levels. Daidzein, genistein, biochanin, and formononetin were analyzed in the urine using a modification of previously published methods (36–38). Briefly, 10 ml aliquots of urine were mixed with 100 ml of glucuronidase. The mixture was incubated for 24 h at 37°C, after which it was extracted on a C-18 solid-phase extraction column (Waters Pty Ltd., Sydney, Australia). Isoflavones were eluted with 3 ml of methanol, and 10 ml of the extract were injected into the high-performance liquid chromatography system. The high-performance liquid chromatography system consisted of a 25 cm, 5 nM, C-18 stationary phase column (Symmetry; Waters Pty Ltd.) and a gradient acetonitrile/water mobile phase. The limit of detection of the assay for each of the isoflavones measured was 5 ng/ml.

Statistical Analysis. For Table 1, two sample *t* tests were used to test for pretreatment differences in age, PSA, and Gleason score between 18 of the treated patients and their matched controls. For Table 2, paired tests (either *t* tests or nonparametric sign tests, depending on the normality of the data) were used to test for changes from baseline (pretreatment) to posttreatment using all available data from all treated patients in the study. Some of the laboratory and isoflavone data were normally distributed and some were not, but for consistency, median and range have been presented for each of these parameters. All data for the percentage apoptosis were nonnormal, so Wilcoxon rank-sum tests were used to test for differences

Table 2 Comparison of pre- and posttreatment parameters in treated patients

Parameter	Baseline (pretreatment) $n = 20$	Posttreatment $n = 19$[a]
	Mean ± SD	
PSA (μg/liter)	10.9 ± 9.9	11.3 ± 10.5
Gleason score	6.6 ± 0.8	6.7 ± 1.0[b]
Testosterone (nmol/liter)	12.1 ± 3.5	12.1 ± 3.2[b]
	Median (range)	
	$n = 20$	$n = 19$[a]
Excreted Isoflavones		
Biochanin A (mg)	0.0 (0.0, 0.0)	2.1 (0.2, 5.5)[c]
Daidzein (mg)	0.3 (0.0, 5.7)	10.9 (1.4, 39.7)[c]
Formononetin (mg)	0 (0.0, 0.2)	2.1 (0.1, 7.9)[c]
Genistein (mg)	0.4 (0.0, 4.5)	3.6 (0.2, 13.7)[c]
Total isoflavones (mg)	0.7 (0.0, 10.2)	19.6 (1.9, 59.4)[c]
Coagulation factors		
INR[d]	1.0 (1.0, 1.2)	1.0 (1.0, 1.1)[b]
APTT (s)[e]	30.5 (27.0, 36.0)	30.0 (24.0, 36.0)[b]
Fibrinogen (g/liter)	2.7 (2.0, 4.3)	2.4 (1.9, 4.1)[b]
Hematology		
Hemoglobin (g/liter)	148.5 (119.0, 157.0)	132.0 (107.0, 149.0)[c]
White cell count ($\times 10^9$/liter)	6.3 (4.7, 9.2)	6.5 (4.8, 11.5)
Platelets ($\times 10^9$/liter)	217.5 (144.0, 430.0)	224.0 (169.0, 417.0)
Biochemistry		
Sodium (mmol/liter)	139.5 (137.0, 148.0)	141.0 (137.0, 145.0)
Potassium (mmol/liter)	4.1 (3.8, 4.9)	4.1 (3.6, 5.4)
Creatinine (μmol/liter)	90.0 (63.0, 122.0)	85.5 (65.0, 107.0)[b]
Urea (mmol/liter)	6.7 (3.7, 10.4)	6.2 (2.8, 7.6)[b]

[a] $n = 19$ excluding patient ID4.
[b] $n = 18$ excluding both patients ID4 and ID10.
[c] $P < 0.0001$: nonparametric sign test to test changes from pretreatment to posttreatment.
[d] INR: International Normalized Ratio.
[e] aPTT: Activated partial thromboplastin time.

between the control and treated patients. Pearson's or Spearman's (nonparametric) correlations were performed to investigate the relationship between selected parameters of interest in the study.

All tests were two-sided, and an overall P of <0.05 was considered statistically significant. To compensate for the fact the multiple tests were being performed, a Bonferroni adjustment was made to the critical significance level in the testing of apoptosis (three tests within each of graphs *A–D* in Fig. 3), isoflavones (five tests), and laboratory data. The critical significance level was set at $\alpha = 0.01$ for apoptosis and isoflavones, and for these tests only, Ps < 0.01 were considered statistically significant. For the laboratory parameters, only Ps < 0.001 were considered statistically significant.

Results

Pre- and Posttreatment Analysis in Treated Patients. The mean age of the treated patients at surgery was 60.5 years ±6.69 (Table 1). Table 2 shows the effects of dietary isoflavones on individual patients before and after treatment, in terms of serum PSA, testosterone, and Gleason score. Overall, pre- and posttreatment serum PSA, testosterone, and Gleason score were not significantly different ($P > 0.05$; Table 2). Serum PSA levels were recorded in 19 patients, and 18 of 19 patients showed little change (within ±2 μg/liter/patient) between pre- and posttreatment time points, although 1 patient showed an increase in serum PSA from 25.90 to 31.30 μg/liter. For Gleason scores, 8 of 18 (44.4%) scores were unchanged, 6 of 18 (22.2%) were increased, and 4 of 18 (33.3%) were decreased, although each variation in posttreatment Gleason score was within ±2 grades of the pretreatment score. Despite variation in serum testosterone within the groups, there was no significant change from pre- to posttreatment levels (within 8 nmol/liter pre- and posttreatment; $n = 18$).

The excretions of the four main isoflavones (biochanin A, daidzein, formononetin, and genistein) were measured in urine samples of treated patients from pre- and posttreatment, and the data are summarized in Table 2. Very low concentrations of all four isoflavones were detected in pretreatment samples, indicating the cohort of patients had low isoflavone intake in their diets. Posttreatment levels of all four isoflavones were significantly increased from pretreatment levels ($P < 0.0001$), which demonstrated compliance with the treatment.

To assess changes indicative of cardiovascular complications and thromboembolic events, a number of coagulation factors, biochemical and hematological markers, were measured pre- and posttreatment (Table 2). There were no significant changes in any of the parameters recorded in Table 2, with the exception of hemoglobin. Hemoglobin levels showed a slight but significant decrease posttreatment.

There were some AEs experienced by the treated patients during the study, and these were recorded by the MedDRA coding system. One patient did not complete the AE assessment. Of the other 19 patients, 11 (57.9%) patients reported a total of 14 AEs during the treatment period, none of which were related to the study treatment. There were two serious AEs that resulted in the withdrawal of that patient from additional analysis; atrial fibrillation at anesthetic induction (ID 4), and a transient ischemic attack, possibly the result of an autologous blood donation before surgery. Neither event was thought to be related to the study treatment, and both patients recovered.

Incidence of Apoptosis in Radical Prostatectomy Specimens from Treated and Control Patients. The effects of dietary isoflavone intake on prostate morphology and incidence of apoptosis were analyzed using radical prostatectomy specimens from treated and control men. Apoptosis was identified in cells based on positive immunolabeling, as well as histological characteristics typical of cells undergoing programmed cell death, including chromatin aggregation, nuclear and cytoplasmic condensation, as well as fragmentation of the dying cell into a cluster of membrane-bound segments or apoptotic bodies (Fig. 2, *A–C*). Apoptotic cells were identified in nonmalignant (Fig. 2*A*), low to moderate-grade cancer (Fig. 2*B*), and high-grade cancer (Fig. 2*C*) tissue in radical prostatectomy specimens. Analysis of pathological grade/sample field in both a treated and a control group was used to identify the most commonly sampled pathology and to determine differences in the sampling populations between the treated and control groups. Overall, in both groups, histological examination showed predominantly nonmalignant tissue or low to moderate-grade carcinoma (Gleason grade 1–3) in the radical prostatectomy specimens, with relatively less high-grade cancer. The percentage of apoptosis was estimated for each pathological grade and each cell type in radical prostatectomy specimens from treated and control patients.

In the entire tissue (including all pathological grades and cell types), a significant difference was observed in the incidence of apoptosis in the treatment group, the median (represented by *bars* on Fig. 3) percentage apoptosis being 0.9% (range, 0.26–1.73%) compared with the control group, 0.25% (0–1.32%), $P = 0.0018$ (Fig. 3*A*). Patients who consumed the

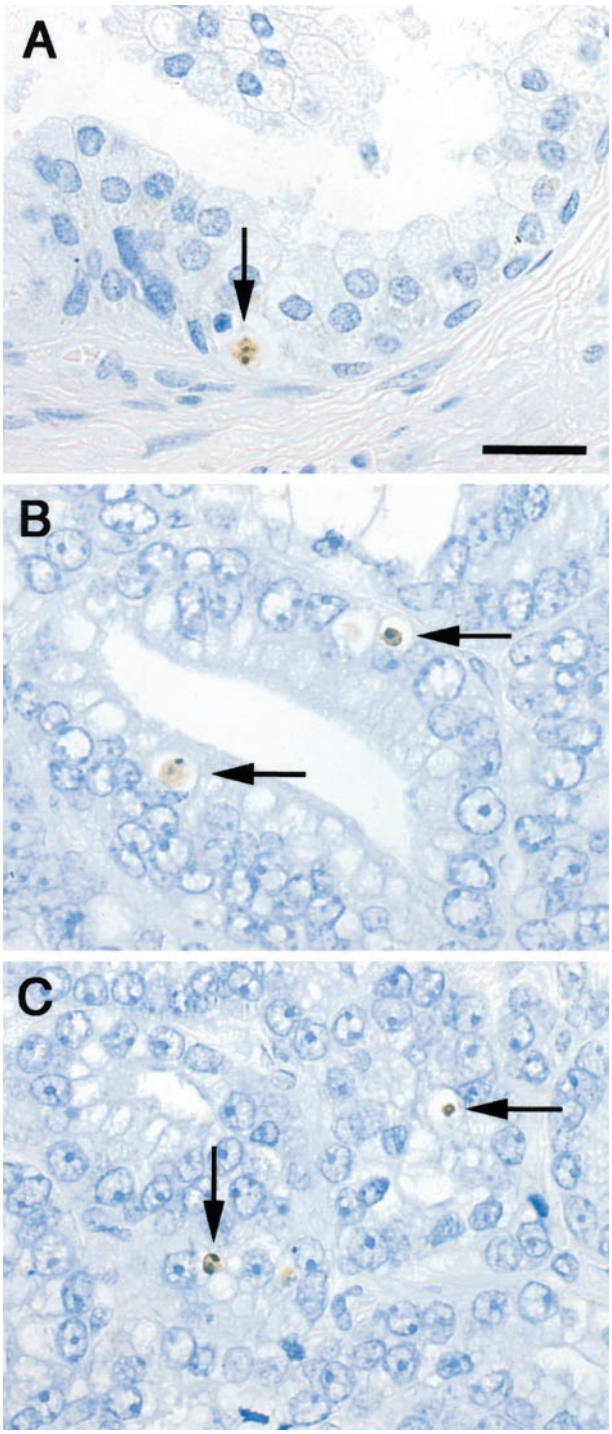

Fig. 2. Apoptag labeling in radical prostatectomy specimens from patients after dietary isoflavone consumption. Brown nuclear staining and morphological characteristics of apoptotic cells (indicated by *arrows*) were observed in **(*A*)** BPH, **(*B*)** low to moderate-grade cancer, and **(*C*)** high-grade cancer. (*Bar* = 25 μm).

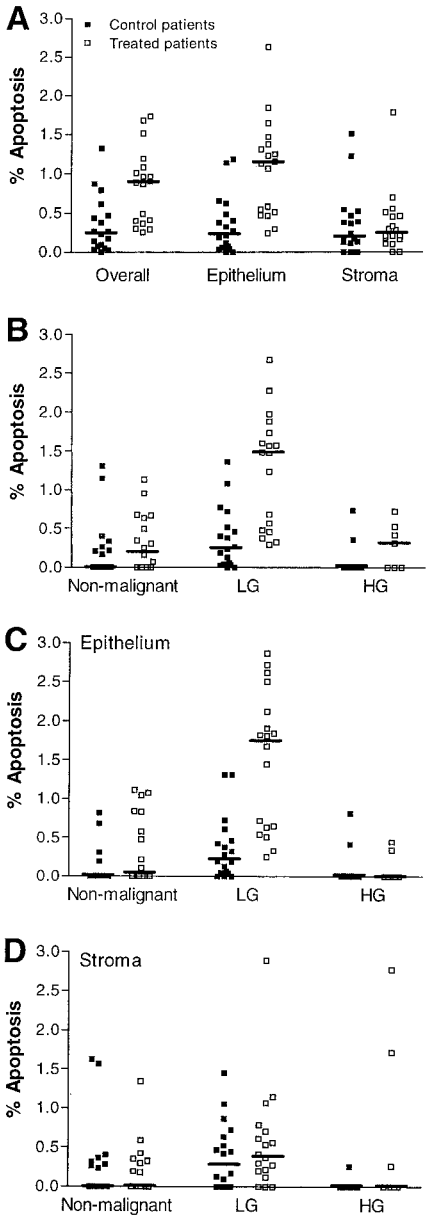

Fig. 3. Quantification of apoptosis in radical prostatectomy specimens from treated and control patients. (■), control patients; (□), treated patients; *bar*, median. *A*, the incidence of apoptosis (percentage) throughout the radical prostatectomy specimen, including all cell types (overall) and specifically in the epithelium and the stroma. *B*, the incidence of apoptosis in all cell types in regions of nonmalignant tissue and low to moderate-grade and high-grade cancer. *C*, the incidence of apoptosis in epithelial cells in regions of nonmalignant tissue and low to moderate-grade and high-grade cancer. *D*, the incidence of apoptosis in stromal cells in regions of nonmalignant tissue and low to moderate-grade and high-grade cancer.

dietary isoflavones showed a significantly higher percentage of apoptosis in epithelial cells throughout the prostate, where the median (range) percentage apoptosis was 1.14% (0.24–2.63%), $P = 0.0007$, compared with the control group, 0.24% (0–1.19%), however, the amount of apoptosis in the stromal tissue was the same in both control and treatment groups, $P = 0.7$ (Fig. 3*A*).

When the incidence of apoptosis was analyzed according to the pathological grade, neither the nonmalignant tissue ($P = 0.3$) nor the high-grade cancer tissue ($P = 0.1$) showed any statistical differences between control and treated groups (Fig. 3*B*). However, there was a significant difference in the inci-

dence of apoptosis in low to moderate-grade cancer tissue between the treatment group, where the median (range) percentage apoptosis was 1.48% (0.30–2.68%), and the control group was 0.25% (0–1.36%), $P = 0.0007$ (Fig. 3*B*). The incidence of apoptosis in low to moderate-grade cancer was found to be significantly higher in the treated group for the epithelial cell compartment, where the median (range) percentage apoptosis was 1.74% (0.26–2.87%), $P = 0.0003$ than in the control group 0.24% (0–1.32%), but this was not the case in nonmalignant, $P = 0.1$ or high-grade cancer, $P = 0.7$ (Fig. 3*C*). There was no significant difference between the control and treated groups in the incidence of apoptosis in stromal cells from nonmalignant, $P = 0.9$ low to moderate-grade, $P = 0.3$ or high-grade cancer tissue, $P = 0.2$, although the incidence appeared to be slightly higher in the low to moderate-grade tissue of the treated group compared with the controls (Fig. 3*D*).

Correlations between Duration of Treatment and Apoptosis in Treated Patients. Several correlations were performed to determine whether the duration of treatment or change in excreted isoflavone levels had any influence on posttreatment PSA, serum testosterone, Gleason score, or the incidence of apoptosis in radical prostatectomy specimens, and no significant correlations were identified.

Immunolocalization of AR on Radical Prostatectomy Specimens from Treated and Control Patients. Immunolocalization of AR was performed to assess whether the isoflavone treatment had any influence on AR expression and therefore androgen signaling. Immunolocalization of AR protein in radical prostatectomy specimens was predominantly in epithelial cells of all pathologies examined (nonmalignant, low to moderate-, and high-grade cancer) from control and treated patients. The level and pattern of AR expression was similar between the cohorts of patients examined from each group, specifically, there was no evidence of down-regulation of AR after isoflavone treatment at any pathological stage (data not shown).

Discussion

This manuscript reports the results of an intervention study designed to identify the short-term effects of red clover-derived isoflavones on men with clinically significant prostate cancer. A comparison of pre- and posttreatment parameters showed no significant changes to serum PSA, serum testosterone, or Gleason score, no clinically significant changes to biochemical factors and minimal adverse effects related to treatment. However, analysis of the radical prostatectomy specimens from treated patients showed a significant increase in apoptosis, specifically in regions of low to moderate-grade cancer (Gleason grade 1–3) compared with controls. Experimental and epidemiological evidence indicates that increased consumption of specific dietary compounds may have the potential to inhibit the progression and, therefore, incidence of clinically significant prostate cancer, and this study implicates dietary isoflavones as one of those factors.

A single case report has previously described similar findings after consumption of a red clover-derived isoflavone supplement (160 mg) taken for 7 days before radical prostatectomy where the resected specimen showed degenerative changes and increased apoptosis in the malignant tissue, whereas the surrounding nonmalignant tissue was unaffected (33). This study used a larger patient cohort and more rigorous detection and quantification techniques to identify apoptosis and has consolidated the previous findings by Stephens *et al.* (33). In this study, we compared postoperative radical prostatectomy specimens from treated specimens to historically matched untreated controls. We felt this approach was a more accurate comparison than a comparison between preoperative biopsy specimens and postoperative radical prostatectomy specimens in the isoflavone-treated group because the prostate gland exhibits significant heterogeneity that is not reflected in the biopsy sample. It is possible that the use of historically matched control patients and a lack of blind randomization may have had some confounding influences on the study.

Traditionally, estrogen therapy in prostate cancer patients was associated with disseminated intravascular coagulation and increased platelet aggregation, which frequently led to cardiovascular complications and thromboembolic events, after therapy (39–41). In this study, none of the patients who consumed the isoflavone supplement showed clinically significant changes in serum coagulation factors similar to that found after estrogen therapy.

The mechanism by which dietary isoflavones induce apoptosis in prostate cancer cells is not clear. Dietary isoflavones are phytoestrogens that directly bind and activate ERs, particularly ER-β (42), and it is possible that the effects are mediated via either ER subtype. In previous studies, this laboratory indicated that signaling through ER-α-induced squamous metaplasia and proliferative changes to the murine prostate gland, but squamous metaplasia was not observed in the patients consuming dietary isoflavones (43, 44), and antiproliferative effects were observed. Therefore, it is unlikely that these compounds activate ER-α. A biological action on the prostate, mediated through ER-β, was more difficult to assess because a definitive biological response mediated through ER-β (such as an antiproliferative effect) is equivocal (45). The normal human prostate epithelium expresses ER-β, but a recent report showed a progressive loss of expression in prostatic hyperplasia and invasive prostate cancer (46). Whether or not the induction of apoptosis in patients who received the dietary supplement occurs via ER-β remains speculative.

Other biological effects of isoflavones include inhibition of steroid metabolism and androgen synthesis, leading to lower levels of androgens and the induction of apoptosis. In this study, serum testosterone levels were unchanged after consumption of dietary isoflavones; local levels within the human prostate tissue are unknown. A reduction in 5α-reductase activity and decreased conversion to the more potent androgen di-hydrotestosterone, would initiate apoptosis by androgen withdrawal. Previous studies showed that isoflavones reduced 5α-reductase activity in human genital skin fibroblast monolayers and homogenates of BPH tissue (16). If the induction of apoptosis observed in this study was a response to androgen withdrawal, it is difficult to explain why the increased incidence of apoptosis was restricted to low to moderate-grade cancer cells and was not recorded in the adjacent nonmalignant epithelium.

Alternatively, the regional increase in apoptosis could occur by localized reduction in expression of AR. However, our results showed expression of AR was similar in the different regions of radical prostatectomy specimens from men treated with dietary isoflavones, compared with controls. Therefore, the mechanism by which dietary isoflavones specifically target low to moderate-grade prostate cancer cells remains unclear, but given the lack of change to testosterone and AR expression, it seems unlikely to be androgen or AR mediated. Alternate mechanisms include inhibition of tyrosine kinase (47) or DNA topoisomerase II activity (20), which were not examined in this study.

Regardless of dietary supplementation, it was noted that

the incidence of apoptosis in regions of high-grade tumor was not significantly elevated compared with nonmalignant tissue regions or regions of low to moderate-grade cancer. This observation contrasts with previous reports of an increased incidence of apoptosis with increased Gleason score (48–50) and is most likely because of a low frequency of sampling of high-grade tumor tissue. Treated patients examined in this study had a mean Gleason score of 6, and therefore low to moderate-grade tumor tissue is more prevalent than high-grade tumor tissue. Overall, only 11 of 18 control patient samples and 7 of 18 treated patient samples contained high-grade cancer regions. Aside from this sampling bias, it may be possible that the response to dietary isoflavones has been specifically targeted to low to moderate-grade cancer cells, reflecting an effect on prostate cancer initiation rather than promotion or progression. Future studies are required to examine a larger cohort of patients (both control and treated) that were selected based on more advanced malignancy.

As yet, the follow-up interval for each of the treated patients is too brief to permit an assessment of the long-term effects of preoperative treatment with dietary isoflavones. However, based on our study, a complete carefully controlled, randomized, double-blinded, clinical trial using multiple doses of isoflavones and with extensive follow-up is warranted to determine whether dietary isoflavones alter the rate of progression of prostate cancer. These data do lend support to the hypothesis that dietary isoflavones may contribute to the lower prevalence of clinical prostate cancer between Asian and Western populations, because as the incidence of subclinical prostate cancer is the same in Asia as in Western countries but some factor(s) halt or delay the progression to clinically significant, invasive malignancy in Asia (5).

Finally, and most importantly, this study demonstrates that dietary isoflavones have the potential to confound results of clinical trials of therapeutics that induce apoptosis in prostate carcinoma. It has become fashionable for older men with benign and malignant prostate disease to consume dietary supplements such as isoflavones as alternative therapies. Data from this study shows that consumption of these compounds are sufficiently effective if taken in high doses to alter prostate pathology by increasing the incidence of apoptosis in regions of low to moderate grade-cancer. Therefore, it will be necessary for urologists recruiting patients into clinical trials of new therapeutic agents to assess or control for the consumption of dietary supplements.

In summary, results from this intervention study show that red clover-derived isoflavones in dietary supplements increased apoptosis in low to moderate-grade prostate carcinoma with minimal adverse effects. The results lend additional experimental support for the geographical and epidemiological difference in the incidence of prostate cancer between Asian and Western populations of men. The potential role of dietary isoflavone supplements in the chemoprevention of prostate cancer requires additional investigation. This study is an observational study on a small cohort of Australian men. Formal health benefits from dietary isoflavone intake can only be determined by a reduction of mortality in men regularly taking soy products, in a large, multicenter-randomized, double-blind, preventative study, which should be conducted in the future. In addition, the results highlight the potentially confounding effects of dietary isoflavones in making valid assessments of new therapeutic strategies aimed at increasing apoptosis.

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