Characterization of Soy-based Changes in Wnt-Frizzled Signaling in Prostate Cancer

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Abstract. Background: A soy-based diet has been associated with a decreased risk of prostate cancer through its antiandrogenic effects. Because the Wnt/beta catenin pathway has been associated with aggressive prostate cancer, we have sought to further evaluate this pathway with respect to soy protein and prostate cancer. Materials and Methods: Previously we have treated rat and human prostate cancer cell lines with soy protein isolates or purified genistein and used gene expression profiling and cross species analysis to identify genes with similar expression changes. One pathway that was identified included the Wnt/beta-catenin pathway. Here the initial data are evaluated and extended with immunohistochemistry in human prostate cancer, and Western blotting, small interfering ribonucleic acid (siRNA) inhibition and bromodeoxyuridine (BrDU) labeling in prostate cancer cell lines. Results: The Wnt/beta-catenin pathway is modulated by both soy protein isolates and genistein in the genomic results. Immunohistochemistry demonstrated staining of Wnt pathway component molecules, in particular frizzled 3, glycogen synthase kinase 3 (GSK-3), and beta-catenin, in prostate tumors. Western blotting noted increased GSK3 and decreased expression of beta-catenin in soy treated prostate cancer PC3 cells. Supporting this finding, siRNA blocking of GSK3 accelerated growth whereas inhibition of frizzled 3 suppressed growth based on growth curves and BrDU labeling. Conclusion: Soy protein appears to regulate prostate cancer via the Wnt/beta-catenin pathway. These data demonstrate that the effect of soy protein effect on prostate cancer may occur through the frizzled 3 receptor with activation of GSK3 leading to increased degradation of beta-catenin and cell growth.

Prostate cancer is the second most common type of cancer and 1 in 6 men will be diagnosed during their lifetime (1). Previous epidemiological studies have shown and increased risk of prostate cancer relating to diet, most notably seen between the Asian and American populations. Asian men have a lower incidence of prostate cancer however when exposed to an American diet over time the risk increases to that of American men (2, 3). Other than lower saturated fat consumption, high soy protein consumption has been hypothesized to be involved in the lower incidence of prostate cancer (4). In animal studies, consumption of soy isolate has prevented spontaneous development of prostate cancer metastasis in Lobund-Wistar rat (5). Genistein, the most abundant and well studied isoflavone isolated from soy protein, has also shown favorable inhibition of prostate cancer in the Lobund-Wistar rats and the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (6, 7). Studies have shown that treating the TRAMP mouse with genistein can prevent poorly differentiated carcinoma relating to similar human pathology seen in Asian countries (8). The mechanism of this has been associated with the AKT/glycogen synthase kinase 3β pathway, specifically through inhibition of GSK3β (9). Interestingly, GSK3β is also a downstream regulator of the Wnt/β-catenin pathway intricately associated with the phosphorylation of β-catenin. Multiple studies have shown

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the WNT pathway plays a role in the development and the progression of human prostate cancer (10-12). In short, in the absence of WNT signaling the pathway is constitutively down-regulated by a multiprotein destruction complex. β-Catenin is bound by axin, glycogen synthase kinase β (GSK3β), and other proteins and undergoes phosphorylation by GSK3β leading to recognition by the E3 ubiquitin ligase complex (βTrCP) and ubiquitin mediated degradation. In the presence of WNT ligand, frizzled binding causes disheveled (DSH) protein to dissociate glycogen synthase kinase (GSK) from the axin protein, thus blocking phosphorylation of β-catenin. The unphosphorylated β-catenin translocates to the nucleus, binding to T-cell factor (TCF) and leading to activation of TCF/lymphoid enhancer factor (LEF) gene targets (13, 14). High levels of β-catenin gene expression and accumulation in the cytoplasm/nucleus have been associated with advanced prostate cancer samples and the PC3 (invasive androgen independent) prostate cancer cell line (11, 14-16). In addition, detection of mutations in β-catenin has been reported in prostate cancer (17, 18).

Previously we have used cross species gene expression analysis to elucidate soy protein isolates affect on prostate cancer growth (19). One pathway that was modulated included the Wnt/β-catenin pathway. Here, we extend these initial findings to validate the role of the Wnt/β-catenin pathway on the soy protein and/or genistein effect on prostate cancer. This information will help provide a basis for developing a molecular explanation of the anticancer effect of soy protein.

Materials and Methods

Cell culture and soy or genistein treatment. Human and rat prostate cancer cell lines PC3 (human) and PAIII (rat) were selected for use in these studies. Soy protein isolates of the identical lot used in the published Lobund-Wistar rate studies were obtained from Dr. Morris Pollard (5, 20, 21). The isolates initially were obtained from Harlan...
TekLad Diets (Madison, WI, USA). In regards to prostate cancer cell lines the PAIII cell line was selected due to its origin and commonality to the Lobund-Wistar rat prostate cancer model (5, 20, 21). The PC3 cell line is an equivalent to PAIII in humans (androgen independent). The PC3 cell lines were received from Drs. Paul Lindholm and Andre Kadjacsy-Balla (MCW, Milwaukee, WI, USA) (PC3) and Dr. Morris Pollard and Mark Suckow (Notre Dame, South Bend, IN, USA) (PA-III). Genistein was obtained from Sigma (St. Louis, MO, USA) (MDL Number: MFCD00016952). These cells were cultured in RPMI supplemented with 10% fetal calf serum, 10 mM glutamine, and 10 mM sodium pyruvate, and passaged 1:8 or 1:10 when the cells reached 70-80% confluence with trypsin-EDTA passaged before use in the experimental protocols. Soy protein was solubulized in a 0.005% NaOH and water solution. After solublization and sterile filtering, the soy protein was added to the medium of the cancer cells. These cells were then treated with different log concentrations (from 10 to 1000 mg/ml) of the soy protein isolates or genistein and their growth rates were measured by growth curves in PAIII and PC3 cells (19). Based on these published growth studies, the concentration of 100 mg/ml soy protein isolates and 20 mM and 100 mM genistein were selected for this study.

Protein purification and Western blotting. Fifty micrograms of total protein extract from either soy, genistein, or untreated LNCAP and PC3 cells was loaded onto 10% Tris-HCL gels (Bio-Rad, Hercules, CA, USA) (MDL Number: MFCD00016952). These cells were cultured in RPMI supplemented with 10% fetal calf serum, 10 mM glutamine, and 10 mM sodium pyruvate, and passaged 1:8 or 1:10 when the cells reached 70-80% confluence with trypsin-EDTA passed before use in the experimental protocols. Soy protein was solubilized in a 0.005% NaOH and water solution. After solublization and sterile filtering, the soy protein was added to the medium of the cancer cells. These cells were then treated with different log concentrations (from 10 to 1000 mg/ml) of the soy protein isolates or genistein and their growth rates were measured by growth curves in PAIII and PC3 cells (19). Based on these published growth studies, the concentration of 100 mg/ml soy protein isolates and 20 mM and 100 mM genistein were selected for this study.

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Figure 2. Prostate cancer tissue stained by immunohistochemistry for the GSK-3 protein under ×400 magnification at 4 different zoom levels. The darker brown stain is indicates a positive result.
Blots were probed with one of the following primary antibodies at a dilution of 1:50,000: Fzd-3, Dvl-1 (3F12), GSK-3 (H-76), GAPDH, cyclin-D1 (DCS-6), cyclin-D2 (C-17), axin (H-98), ICAT, C-JUN (H-79), β-catenin (E-5) and Wnt-4. All primary antibodies were obtained from Lifespan Biosciences (Seattle, WA, USA), except Fzd3 was obtained from SantaCruz Biotechnologies (Santa Cruz, CA, USA). Secondary horseradish peroxidase (HRP) (Bio-Rad) conjugates were used at a dilution of 1:250,000. All rinses were carried out in 0.3% PBS-Tween. Blots were visualized by autoradiography using ECL Advance reagents (Amersham Biosciences) according to the manufacturer’s instruction. Autoradiographs were then scanned and the results quantitated for graphical presentation.

Immunohistochemistry. After institutional review board approval tissue microarrays were prepared from archived paraffin blocks from 288 radical prostatectomy cases present at the Medical College of Wisconsin (Milwaukee, WI, USA). For each case 0.6 mm cores of tumor were isolated and 5 μm slides were stored for immunohistochemistry. Four representative prostate cancer tissue slides were deparaffinized by washing three times in xylene for 5 min each. The tissue was re-hydrated by successively washing twice in 100% ethanol, 95%, and distilled water for 5 min each. Antigen retrieval was performed either by autoclaving for 20 minutes in Dako Target Retrieval Solution (Dako, Carpinteria, CA, USA) followed by a cooling for 20 min at room temperature (DSV, ICAT, β-catenin, axin), or with proteinase K (10 μg/ml) for 30 min at room temperature (GSK3, FZD3). Antigen retrieval was not performed for the Wnt4 antibody. Slides were rinsed with phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide for thirty minutes at room temperature. Slides were again rinsed with PBS and tissues were blocked with fetal bovine serum (FBS) for 1 hour at room temperature. Primary antibodies were diluted 1:10 with DAKO Background Reducing Solution and incubated on tissue slides overnight at 4°C. Primary antibodies were shaken from the slides and slides were washed with 5 quick rinses with PBS followed by a 5 minutes rinse. Biotinylated secondary antibodies (Bio-Rad) were diluted 1:50 in DAKO Background Reducing Solution (Dako) and incubated with their appropriate primary antibodies for 2 hours at room temperature.

Figure 3. Prostate cancer tissue stained by immunohistochemistry for the β-catenin protein under ×400 magnification at 4 different zoom levels. The darker brown stain indicates a positive result.
Slides were rinsed as described for primary antibodies. Strept-Avidin HRP was diluted 1:100 with PBS and incubated with each of the slides at room temperature for one hour. HRP was removed from the slides by shaking and slides were rinsed for 15 minutes in PBS. Diaminobenzidin (DAB) solution (Immunovision Technologies, Burlingame, CA, USA) was added to each tissue sample and allowed to incubate for 3 minutes. Slides were rinsed in running water to stop the enzymatic reaction. Slides were counterstained by incubating with hematoxylin (EMD Chemicals, Gibbstown, NJ, USA) for 3 minutes followed by a 5 minutes rinse in running water. Tissues were preserved using Vectamount® (Vector Laboratories, Burlingame, CA, USA).

siRNA and BrdU labeling. PC3 cells were grown with and without soy isolate for 48 hours. Cells were then transfected with GSK3, FZD3, and Wnt4 siRNAs (Ambion, Austin, TX, USA) using Lipofectamine (Invitrogen, Carlsbad, CA, USA) using the 24 well plate format according to the manufacturer’s recommendation. Briefly, 20 pg of each siRNA and 1 μl of Lipofectamine were separately diluted in 50 μl of RPMI 1640 serum free medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated at room temperature for 5 minutes. The siRNA and Lipofectamine were then combined, incubated for 20 minutes at room temperature, and added to the cells. Cells were then allowed to grow an additional 48 hours. BrdU was added to the cells at a concentration of 20 μM and incubated an additional 2 hours. BrdU incorporation was detected using an anti-BrdU antibody (BD Biosciences, San Jose, CA, USA), followed by an HRP labeled secondary (Jackson ImmunoResearch, West Grove, PA, USA), and DAB solution (Immunovision Technologies). Cellular proliferation was measured by positive BrdU staining as measured by positive nuclear counting in 200 cells. Experiments were run three independent times and results were analyzed as mean plus standard deviation of nuclear staining.

Results

Immunohistochemistry, Western blotting, and siRNA techniques were utilized to confirm the presence and activity of the Wnt/β-catenin pathway in the presence of soy protein. Immunohistochemistry was used to visualize the presence and subcellular localization of the stained proteins in prostate tumors. Frizzled-3, GSK-3 and β-catenin showed positive staining that demonstrated their presence in prostate tumors (Figures 1-3). Immunohistochemistry revealed localization of the frizzled-3 protein of the cell membrane, GSK-3 in the cytoplasm, while β-catenin was seen in all three compartments (membrane, cytoplasm, and nucleus). No significant differences in staining were noted with respect to tumor Gleason grade or patient outcomes data (data not shown). Western blotting using the human PC3 prostate cancer cell line treated with either soy isolate or genistein confirmed the presence of the Wnt pathway proteins and demonstrated expression changes when compared to untreated controls. The evaluated proteins include: disheveled, frizzled-3, Wnt-4, cyclin D2, axin, β-catenin, C-JUN, GSK-3, GAPDH, and cylin D1 (Figure 4). Similar data were obtained for human prostate cancer LNCaP cells.

The effects of soy lead to decreased Wnt-4 expression with no change in frizzled-3 protein levels, mimicking the RNA expression data. In addition, there appears to be a mild decrease in β-catenin protein levels with soy treatment. No changes in axin or GSK3B were noted with soy treatment. To evaluate the functional role of the Wnt pathway on soy-based proliferation changes, we performed inhibitory studies in human PC3 prostate cancer cells using siRNA to either Wnt-4, frizzled-3 or GSK3B (Figure 5). Untreated PC3 cells or PC3 cells treated with soy isolate or soy isolate with siRNA for either Wnt-4, Frizzled-3 or GSK3B were analyzed for changes in cellular proliferation using BrdU nuclear labeling (Figure 5). Treatment with soy alone demonstrated a decrease in proliferation as noted by decreased BrdU incorporation compared to control (no soy) cells.
cells with Wnt-4 siRNA inhibition demonstrated a larger decrease in proliferation compared to both soy-treated and control cells. Soy-treated cells on Frizzled-3 siRNA treatment revealed no change in proliferation compared to those with soy treatment alone, and in both proliferation was lower than that of control cells. Of note, soy-treated cells treated with siRNA to GSK3B demonstrated an increase in proliferation as measured by BrdU incorporation that returned to control levels.

Discussion

Wnt signaling pathway controls the regulation of β-catenin, which facilitates cell proliferation through regulated gene transcription. Changes in the Wnt pathway have been seen in prostate cancer in particular through the identification of β-catenin mutations (10-12, 17, 18). More recent studies suggest that involvement of the Wnt/β-catenin pathway may be an indicator of more aggressive prostate cancer (14, 22). In particular the PC3 (invasive androgen-independent) prostate cancer cell line was used to correlate with previous studies showing high levels of β-catenin gene expression and accumulation in the cytoplasm/nucleus (11, 14-16). Lu et al. noted higher levels of Wnt/β-catenin pathway signaling in advanced prostate cancer cell lines such as PC3. They also used celecoxib, sulindac sulfide, and nitric oxide-aspirin to decrease Wnt/β-catenin signaling; however, prior to our work no studies have been performed using whole soy protein and genistein (16,19).

Recently, we have identified changes in the Wnt/β-catenin pathway in prostate cancer and its response to soy protein/genistein treatments through genomics and cross species analysis (19). In order to find specific genes that play a significant role in the clinical effects of soy on prostate cancer, we combined data across species and focused on associated gene expression changes. In our previous study, we described 11 genes in the Wnt pathway that were differentially regulated by both soy and genistein (19). Therefore, the soy effect on the Wnt/β-catenin pathway is largely explained by genistein. This confirms previous studies that have suggested that genistein plays a major role in the overall effect of soy protein diets on prostate cancer (6-8). In these studies, we suggested that soy treatment lead to a shutdown of Wnt signaling by decreased Wnt-4 levels, which failed to allow frizzled-3 to inhibit GSK3B, leading to decreased levels of β-catenin and loss of TCF inhibition of transcription. Interestingly, an additional soy-specific effector was found to be up-regulated in human cells. Beta catenin inhibitor protein 1 (ICAT) is a direct inhibitor of beta catenin binding to intranuclear targets. The finding that ICAT may be an additional factor in effect of soy on prostate cancer in addition to genistein alone is worthy of further evaluation. The majority of changes in the Wnt/β-catenin pathway components can be seen on treatment with soy protein isolate.

Here we have extended these genomics-based findings by demonstrating the presence of components of the Wnt/β-catenin pathway in prostate cancer cells and cell lines using immunohistochemistry and Western blotting. The Wnt/β-catenin pathway has also been shown to be important in prostate cancer using immunohistochemistry in previous studies (11,15). We have confirmed the presence of this pathway in human prostate cancer samples (Figures 1-3). These proteins are expressed with respect to soy treatment as demonstrated by Western blotting with and without soy treatment in the PC3 prostate cancer cell line. In particular,
soy treatment led to decreased Wnt-4 protein and increased levels of frizzled-3 receptor, mimicking the genomics results (19). In addition, there was a mild decrease in β-catenin with soy treatments. The presence of both axin and GSK3B was also confirmed in the PC3 cells lines with soy treatment. These data suggest that soy does indeed control the Wnt pathway, likely through some aspect of the GSK3 protein regulation. This conclusion is strengthened by the siRNA inhibitor studies demonstrating abrogation of soy-based decreases in prostate cancer, BrdU incorporation, and cellular proliferation by GSK3 inhibition. Using siRNA, the blockade of GSK-3 restores soy-treated cellular proliferation to control levels. This finding suggests that soy inhibition requires GSK-3 activity in prostate cancer. The inhibition of frizzled-3 with siRNA results in no changes in soy-based decreased cell proliferation, as its effect is mediated and limited by the presence of its activating ligand, Wnt-4. The inhibition of Wnt-4 with siRNA has a limited effect on soy based suppression of prostate cancer cell proliferation, possibly due to modulation of the low remaining levels of Wnt-4 expression. Taken together, these data suggest that the Wnt signaling pathway in prostate cancer is present and affected by soy treatment, and that the major effect of soy treatment on prostate cancer appears to occur through GSK-3 activity.

Experiments in rat models and associative studies in humans show that the effect of soy in certain prostate tumors is substantial. The mechanism of this significant reduction in tumorigenesis is most likely multifactorial, yet the Wnt signaling pathway may provide a crucial mechanism pending additional investigation. The Wnt pathway has been studied in multiple different types of cancer and developmental systems and continues to be an interesting and influential pathway in the development of cancer. This pathway has shown to be differentially regulated when using dietary manipulation, especially with soy protein. Herein, we describe evidence that the interaction in which soy protein inhibition of the Wnt/β-catenin pathway may be through GSK3 activation, causing increased phosphorylation and degradation of the cell growth promoter β-catenin. This mechanism is similar to the main proposed mechanism for the effect of genistein on prostate tumors, a result that would be expected as the genomic results for soy and genistein demonstrated a high degree of overlap. With further investigation of the Wnt pathway, it may prove to be one component of a multitargeted molecular chemotherapeutic regimen. As earlier studies have related changes in the Wnt/β-catenin pathway to aggressive prostate cancer, the current studies may be used to assist in further clinical trials including low-grade prostate cancer, but also as an addition to chemotherapy in late-stage advanced prostate cancer (13-15, 22).

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