Cancer **Prevention** Research

Endocrine-Immune-Paracrine Interactions in Prostate Cells as Targeted by Phytomedicines

Nora E. Gray, Xunxian Liu, Renee Choi, Marc R. Blackman and Julia T. Arnold

Abstract Dehydroepiandrosterone (DHEA) is used as a dietary supplement and can be metabolized to androgens and/or estrogens in the prostate. We investigated the hypothesis that DHEA metabolism may be increased in a reactive prostate stroma environment in the presence of proinflammatory cytokines such as transforming growth factor β1 (TGFβ1), and further, whether red clover extract, which contains a variety of compounds including isoflavones, can reverse this effect. LAPC-4 prostate cancer cells were grown in coculture with prostate stromal cells (6S) and treated with DHEA +/− TGFβ1 or interleukin-6. Prostate-specific antigen (PSA) expression and testosterone secretion in LAPC-4/6S cocultures were compared with those in monocultured epithelial and stromal cells by real-time PCR and/or ELISA. Combined administration of TGFβ1 + DHEA to cocultures increased PSA protein secretion two to four times, and PSA gene expression up to 50-fold. DHEA + TGFβ1 also increased coculture production of testosterone over DHEA treatment alone. Red clover isoflavone treatment led to a dose-dependent decrease in PSA protein and gene expression and testosterone metabolism induced by TGFβ1 + DHEA in prostate LAPC-4/6S cocultures. In this coculture model of endocrine-immune-paracrine interactions in the prostate, TGFβ1 greatly increased stromal-mediated DHEA effects on testosterone production and epithelial cell PSA production, whereas red clover isoflavones reversed these effects.

Dehydroepiandrosterone (DHEA) and its sulfated conjugate, DHEA-S, are present in adult men and women at plasma concentrations 100 to 500 times higher than those of testosterone and 1,000 to 10,000 times higher than those of estradiol (E_2; ref. 1). These levels decrease with age, prompting the use of DHEA as a self-prescribed dietary supplement for its alleged anabolic and antiaging effects, with unsubstantiated claims of beneficial effects as well as uncertain long-term safety (2).The controversy on whether DHEA may be cancer promoting or cancer preventing in humans continues to be debated (3). In *in vivo* studies in rodents, DHEA has been found to be an effective inhibitor of carcinogen-induced prostate cancers (4). In humans, DHEA can be metabolized to androgens and/or estrogens in the prostate (5), and thereby may affect prostate pathophysiology. Compared with serum levels of steroid hormones, the levels of intratissular androgens and estrogens metabolized from DHEA (5) are increasingly recognized as important targets of investigation. We hypothesize that DHEA metabolism may be altered from that in the normal prostate during the various stages of cancer progression.

Stromal cell activation is a critical step in the progression of cancers. Prostate stroma may become activated in response to the progression of the colocalized carcinoma (6) or by various stimuli from tissue injury including growth factors and other cytokines (7). Once activated, stromal cells often secrete larger amounts of growth factors and extracellular matrix components and remodeling enzymes, similar to a wound repair response, thereby creating a growth-promoting microenvironment that can alter epithelial function (8). Proinflammatory cytokines can modulate various cell functions in cancerous tissue and contribute to inducing stromal activation. Transforming growth factor β1 (TGFβ1), a proinflammatory cytokine that participates in many cellular processes such as growth, proliferation, differentiation, and apoptosis (9), is present in reactive stroma (10) and exerts multiple effects on carcinogenesis. In prostate cancer patients, TGFβ1 overproduction is associated with increased tumor grade, high vascularity, and the presence of metastases (9). Interleukin-6 (IL-6), another proinflammatory cytokine secreted by T cells and macrophages, stimulates immune response to trauma, especially tissue damage, leading to inflammation. IL-6 increases androgen responsiveness in prostate cancer cells *in vitro* (11). Other cytokines, IL-4 and IL-13, can increase the expression of steroid-metabolizing

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enzymes, potentially altering the metabolism of hormones, including DHEA (12). We propose that reactive prostate stroma modulates DHEA hormone metabolism.

Increased dietary isoflavone consumption is associated with a decreased risk of prostate cancer (13). Red clover (*Trifolium pretense*) is one source of isoflavones. The flowering tops of the red clover plant contain biochanin A, formononetin, daidzein, and genestein. Red clover is available as a dietary supplement, and standardized extracts are widely marketed to men as a treatment for symptoms of prostate enlargement. Red clover isoflavones inhibit the growth of prostate cancer cells (14), induce apoptosis in low- to moderate-grade prostate cancer (15), and inhibit 5α-reductase (16) and 17β-hydroxysteroid dehydrogenase (17), two enzymes involved in steroid metabolism.

The current study uses a coculture model of human prostatic stromal plus epithelial cells to simulate endocrine-immune-paracrine interactions in the prostate. Addition of the proinflammatory cytokines TGFβ1 and IL-6 facilitates investigations into mechanisms linking the immune, paracrine, and endocrine influences on cancer growth and progression, including metabolism of DHEA to testosterone and induction of the epithelial specific secretory product prostate-specific antigen (PSA) expression in prostate stromal plus epithelial cocultures. We hypothesized that combined cytokine + DHEA administration would increase PSA production and testosterone metabolism in the cocultures, and that the addition of red clover isoflavones would inhibit these cytokine + DHEA–mediated effects.

Materials and Methods

Cell culture

LAPC-4 cells were generously provided by Dr. Charles Sawyers (University of California at Los Angeles, Los Angeles, CA). Primary human prostate cancer–derived stromal cells were isolated from radical prostatectomy specimens (“6S”; kindly provided by Dr. John Isaacs, Johns Hopkins School of Medicine, Baltimore, MD) and have previously been described (18). Primary prostate stroma cells (PrSC) derived from normal prostate tissues were obtained from Cambrex-Clonetics. All cell types were grown in DMEM/F12 (1:1) medium (Invitrogen) with penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (292 μg/mL; Invitrogen), and 5% fetal bovine serum (HyClone Laboratories, Inc.) at 37°C in 5% CO_2 and propagated at 1:5 dilutions. Cells were kept as frozen stocks and used within seven passages after thawing.

Stromal cell TGFβ1 growth studies

6S stromal cells were seeded in triplicate onto 12-well plates at a density of 15,000 per well in “treatment media” consisting of Medium 199 (phenol red–free)/F12 phenol red–reduced media (Invitrogen; 1:1) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and 1% charcoal-dextran–treated fetal bovine serum (CDS; Hyclone Laboratories). Cultures were incubated overnight for 24 h and treated with TGFβ1 in concentrations ranging from 0.04 to 400 pmol/L. Cells were trypsinized and counted at day 0 and daily thereafter for 5 days, using a Coulter cell counter (Z1 Dual, Beckman Coulter). This study was repeated thrice, and the 6S stromal cells used were from passages 7 to 9.

TGFβ1 + DHEA–induced effects on PSA and testosterone secretion in cocultures

LAPC-4 cancer epithelial cells were seeded in treatment media in triplicate onto Millipore PICM 12-mm inserts coated with a 1:10 dilution of Matrigel/H_2O, at a density of 5×10^5 cells per insert. Stromal cells (6S or PrSC) cells were seeded in treatment media in triplicate at 1×10^5 per well in 24-well plates. TGFβ1 was added to stromal cultures on the same day at 40 pmol/L to elicit the reactive stromal phenotype as previously reported (19). LAPC-4/6S coculture methods have previously been described (20). Epithelial and stromal cells were cultured separately in media containing 2% CDS for 3 d. Epithelial and stromal cultures were then combined in cocultures while monocultures remained separated. Cells in treatment media containing 1% CDS were treated with hormones; and then treated with ethanol control (<0.02%), 100 nmol/L DHEA +/− 40 pmol/L TGFβ1, or 10 nmol/L R1881; and allowed to coculture for 3 d. Media containing hormones were replaced and allowed to condition for 48 h. Conditioned media were collected from monocultures and cocultures (with media from epithelial and stromal compartments mixed together) and frozen at −80°C or assayed for PSA and testosterone by ELISA. Total PSA ELISA kits (DSLabs) were used to determine PSA concentrations as previously reported (21). Total testosterone was also measured with an ELISA kit (ALPCO). Each original triplicate experimental sample was assayed in duplicate. PSA and testosterone values were normalized to cell numbers as determined by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega) as previously reported (21).

TGFβ1 + DHEA–induced effects on PSA gene expression

Cocultures were prepared as above, with LAPC-4 seeded in triplicate in 30-mm Millipore inserts precoated with Matrigel film at a density of 2×10^6 per six-well plate and 6S cells plated at 5×10^5 per 60-mm dish, using treatment media as described above. After 3 d, cells were either combined in coculture or left in monoculture and treated with hormone treatments as above for 2 d, and then harvested to extract RNA. Methods for RNA extraction, reverse transcription, real-time PCR, and primers for ribosomal phosphoprotein PO and PSA were previously described (21).

Preparation of red clover isoflavones

Isoflavones present in red clover extract, biochanin A, formononetin, daidzein, and genistein (Sigma), were dissolved in DMSO and combined in the same proportions as the published formulation in the clinically used Promensil (Novogen; ref. 22). A 1-mL 22.6 mmol/L stock solution was prepared from the four isoflavones based on the following formulation: 61% biochanin A (15.3 mg/250 μL DMSO = 54 mmol/L), 20% formononetin (5 mg/250 μL DMSO = 18.6 mmol/L), 9% daidzein (2.2 mg/250 μL DMSO = 8.68 mmol/L), and 10% genistein (2.5 mg/250 μL DMSO = 9.25 mmol/L).

Effects of red clover on TGFβ1 + DHEA–mediated PSA and testosterone secretion

The same experimental procedure was used as above of the effects of TGFβ1 + DHEA on PSA and testosterone, but in addition to treatments with 100 nmol/L DHEA +/− 40 pmol/L TGFβ1 and 10 nmol/L R1881, cells were also treated with red clover isoflavones at 10, 30, 100, or 300 nmol/L; E_2 at 100 nmol/L; and ICI 182,780 (estrogen receptor antagonist) at 1 μmol/L.

Western blot analysis of stromal cell expression of AR and smooth muscle proteins

6S stromal cells were plated at a density of 5×10^5 per well on six-well plates, and 40 pmol/L TGFβ1 was added on the same day. Cells were then grown in treatment media containing 2% CDS for 2 d, treated with hormones (as above), and allowed to culture for 4 more days. Protein was extracted from cells and analyzed by Western blot for androgen receptor (AR), α-smooth muscle actin, desmin, smoothelin, vimentin, and glyceraldehyde-3-phosphate dehydrogenase as reported previously (18).

Statistical analysis

All data are expressed as the mean (±SE) derived from three replicates within each of three separate experiments. To delineate the effects of hormones or inhibitors, one-way ANOVA was done using the Tukey-Kramer honestly significant difference adjustment for multiple comparisons. An adjusted P value of 0.05 was considered significant. Probability designations are as follows: *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$, between hormone treatment; $^{+}$, $P = 0.05$; $^{++}$, $P = 0.01$; $^{+++}$, $P < 0.001$, within hormone treatments (monoculture versus coculture). In the red clover graphs: $^{\blacklozenge}$, $P = 0.05$, DHEA + TGFβ1 plus red clover treatment compared with DHEA + TGFβ1 alone.

Results

TGFβ1 effects on the growth of 6S cells

TGFβ1 effects on prostate stromal (6S) cell growth were tested using seven concentrations for 5 days. The growth curve showed increased 6S proliferation with lower doses, and growth arrest with higher doses (Fig. 1). With increasing concentrations, the morphology of the stromal cells became more myofibroblastic with prominent smooth-muscle–like actin fibers present (data not shown). Statistical analysis verified that from day 2 to day 5, 40 pmol/L and higher doses significantly inhibited 6S cell growth ($P = 0.01$), whereas growth stimulation by 10 pmol/L or lower doses was not significant (at both $P = 0.05$ and $P = 0.01$). The TGFβ1 concentration used in these experiments, 40 pmol/L, was 23.7% inhibitory by day 2 (the duration of the RNA experiment) and 40.7% inhibitory by day 5 (the usual duration of the ELISA experiment; $P < 0.01$).

DHEA-induced PSA protein expression in LAPC-4/6S or LAPC-4/PrSC cocultures was increased by TGFβ1 but not by IL-6

In recent studies, we reported that 6S stromal cells in coculture with LAPC-4 cells induce LAPC-4 PSA expression in the presence of DHEA, whereas LAPC-4 cells are unresponsive to DHEA in monocultures (20). This effect was replicated here, in that DHEA (100 nmol/L) increased LAPC-4/6S coculture PSA protein secretion up to 8.4 ng/mL/100,000 cells, whereas in LAPC-4 monocultures PSA levels were similar to control at 3.9 ng/mL/100,000 cells ($P = 0.03$; Fig. 2A). The addition of TGFβ1 to DHEA-treated cocultured cells significantly enhanced PSA secretion (20.0 ng/mL/100,000 cells; $P = 0.01$) to values similar to those induced by the positive control, the nonmetabolized androgen R1881 (10 nmol/L), which induced PSA secretion in LAPC-4 grown in monoculture (12.2 ng/mL/100,000 cells; $P = 0.05$) and coculture (22.0 ng/mL/100,000 cells; $P = 0.01$) conditions. The combination of DHEA + IL6 did not alter PSA secretion in cocultures beyond that observed after DHEA alone. Parallel studies were done with PrSC normal prostate stromal cells, which produced a similar pattern, where LAPC-4-PSA production was increased by hormone treatments to 10.5, 11.3, 17.5, and 16.3 ng/mL/100,000 cells in DHEA, DHEA + IL-6, DHEA + TGFβ, and R1881, respectively ($P > 0.05$). As in the 6S cocultures, the combination of DHEA + IL6 did not alter PSA secretion in PrSC cocultures beyond that observed after DHEA alone.

TGFβ1 effects on DHEA-induced PSA gene expression in LAPC-4/6S cocultures

DHEA increased LAPC-4 PSA gene expression in LAPC-4/6S cocultures (18.1-fold) as compared with the effect in LAPC-4 cells in monoculture (6.2-fold; $P < 0.01$; Fig. 2B). Addition of TGFβ1 further enhanced DHEA-induced PSA gene expression in LAPC-4/6S cocultures (385-fold; $P = 0.01$). In comparison, R1881 increased PSA expression in both monocultures (196-fold; $P < 0.001$) and cocultures (292-fold; $P < 0.0015$).

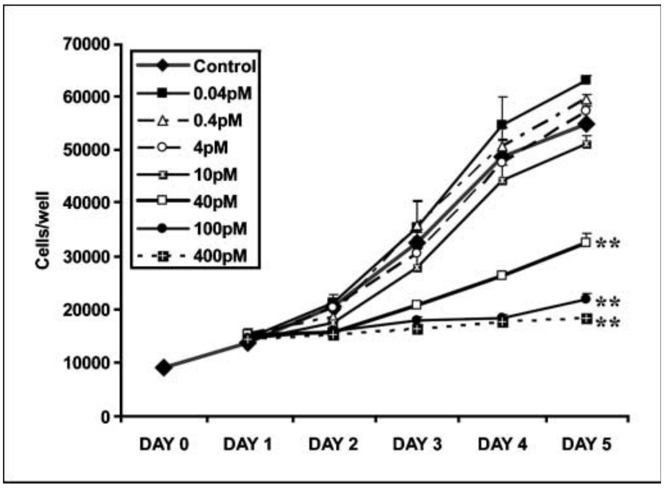

Fig. 1. Effects of TGFβ1 on proliferation of 6S prostate stromal cells. 6S stromal cells were plated in triplicate on 12-well plates at 15,000 per well. Cultures were incubated for 24 h and treated with various concentrations of TGFβ1. Cells were trypsinized and counted at day 0 and daily thereafter for 5 d. *Points*, mean of three separate experiments; *bars*, SE. **, $P = 0.01$.

Testosterone secretion in DHEA-treated LAPC-4/6S cocultures was increased by TGFβ1 but not by IL-6

In conditioned medium from hormone-treated cultures, a significant increase in testosterone concentrations was detected in cocultures (LAPC-4/6S coculture) treated with 100 nmol/L DHEA (13.6 pg/mL/100,000 cells; $P = 0.05$) as compared with similarly treated LAPC-4 or 6S cells in monoculture (7.8 for 6S monoculture and 8.0 for LAPC-4 monoculture; Fig. 3). With the addition of TGFβ1, DHEA treatment increased 6S monoculture stromal cell testosterone production to 14.6 pg/mL/100,000 cells ($P < 0.01$). In LAPC-4/6S cocultures, TGFβ1 + DHEA treatment produced even greater concentrations of testosterone (24.7 pg/mL/100,000 cells; $P < 0.01$) than after DHEA treatment alone. TGFβ1 + DHEA induced higher testosterone concentrations than did IL-6 + DHEA in both 6S monocultures and cocultures (8.2 and 15.6 pg/mL/100,000 cells, respectively; $P < 0.01$). Testosterone was increased with IL-6 + DHEA in 6S cocultures over 6S monocultures ($P = 0.05$) but the effect was not statistically different than in cocultures treated with DHEA alone. Control-treated monocultures of LAPC-4 cells secreted lower amounts of testosterone than under all other treatment conditions ($P = 0.05$). However, all values for LAPC-4 monoculture were less than or not different from the testosterone concentrations found in R1881-treated cells and were likely attributable to background in the assay.

Red clover isoflavones decreased TGFβ1 + DHEA–induced PSA protein levels and gene expression in LAPC-4/6S cocultures

A range of concentrations for the red clover isoflavones mixture was pretested for potential toxicity and found to be nontoxic up to 1 μmol/L for LAPC-4 cells and 10 μmol/L for 6S cells (data not shown). The dose of 100 nmol/L red clover isoflavones was chosen because it was nontoxic and is similar to circulating concentrations of these isoflavones measured in patients treated with the red clover isoflavones (23). As before, DHEA treatment of LAPC-4 cells increased PSA protein secretion more in the presence of stromal cells (6S coculture) versus monocultures (2.11 versus 1.01 ng/mL/100,000 cells; $P = 0.01$; Fig. 4A). TGFβ1 augmented DHEA-induced PSA protein secretion in cocultures (13.06 ng/mL/100,000 cells; $P < 0.01$). The addition of 100 nmol/L red clover isoflavones to the TGFβ1 + DHEA combination inhibited TGFβ1 + DHEA induction of PSA in cocultures, with resulting concentrations similar to those after DHEA treatment alone (4.11 ng/mL/100,000 cells). E_2 (100 nmol/L) also inhibited TGFβ1 + DHEA–induced PSA expression in LAPC-4 cocultures (2.10 ng/mL/100,000 cells), whereas blocking estrogen receptors (ERα and ERβ) with the antagonist ICI 182,780 (100 nmol/L) did not reverse the red clover inhibition (3.47 ng/mL/100,000 cells). R1881 induced high levels of PSA in both monoculture and coculture (13.13 and 13.23 ng/mL/100,000 cells, respectively; $P < 0.01$). Addition of TGFβ1 alone induced no discernable amounts of PSA in LAPC-4 cells in monocultures or cocultures. Red clover alone induced no PSA, and TGFβ1 +/− red clover did not alter R1881-induced PSA expression (data not shown).

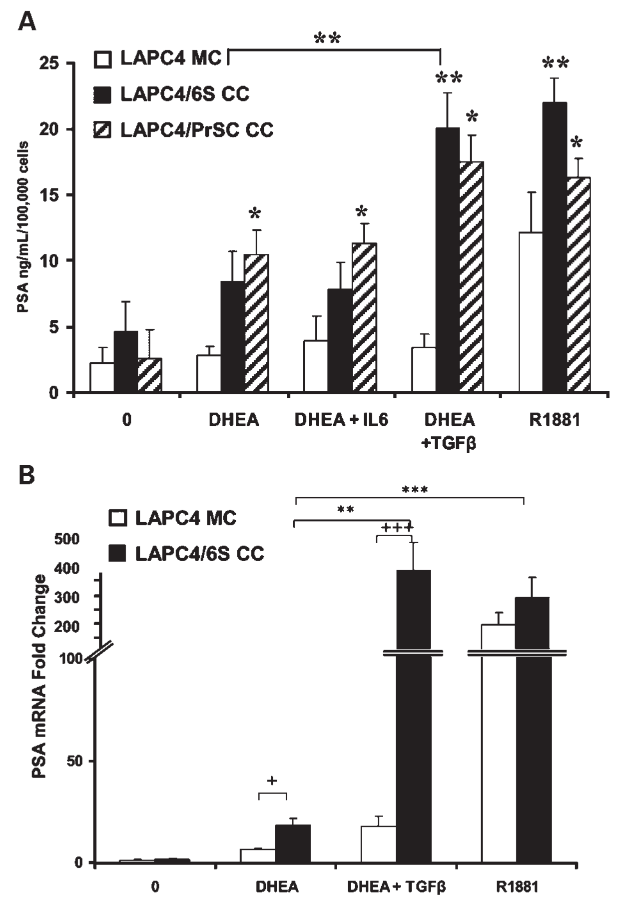

Fig. 2. TGFβ1 augmented DHEA-induced PSA protein production and gene expression in LAPC-4/6S or LAPC-4/PrSC cocultures. *A*, LAPC-4 cells were seeded in treatment media in triplicate onto 30-mm inserts coated with a film of Matrigel at a density of 5×10^5 per well. Stromal cells (6S or PrSC) were seeded in triplicate at 1×10^5 per well in 24-well plates. TGFβ1 was added to stromal cultures on the same day at 40 pmol/L to elicit a reactive stromal phenotype. Cocultures were combined after 2 d while monocultures remained separated; hormones were added; and cells allowed to coculture for 3 d. Media containing hormones were replaced and allowed to condition for 48 h. Conditioned media were assayed for PSA by ELISA. Each original triplicate experimental sample was assayed in duplicate. PSA values were normalized to cell numbers as determined by the modified MTT assay. *B*, cocultures were prepared with LAPC-4 seeded in treatment media in triplicate in 30-mm inserts precoated with Matrigel film at a density of 2×10^6 per six-well plate and 6S cells plated at 5×10^5 per 60-mm dish, using treatment media as described (see Materials and Methods). After 3 d, cells were either combined in coculture or left in monoculture and treated with hormone treatments for 2 d, and then harvested to extract RNA. PSA gene expression was assayed by real-time PCR and normalized to ribosomal phosphoprotein PO expression. *Columns*, mean from three separate experiments; *bars*, SE. *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$, within monoculture or coculture. $^+$, $P = 0.05$; $^{+++}$, $P < 0.001$, between monoculture and coculture.

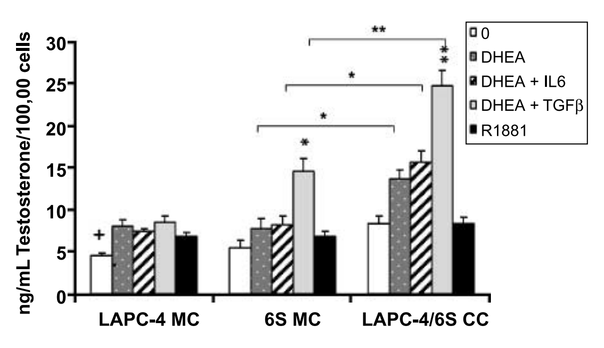

Fig. 3. Effects of DHEA, TGFβ1, and IL-6 on testosterone secretion in cocultured LAPC-4/6S cells. LAPC-4 cells were seeded in treatment media in triplicate onto 30-mm inserts coated with a film of Matrigel at a density of 5×10^5 per insert. Stromal cells (6S) were seeded in triplicate at 1×10^5 per well in 24-well plates. TGFβ1 was added to stromal cultures on the same day at 40 pmol/L to stimulate a reactive stromal phenotype. Cocultures were combined after 2 d while monocultures remained separated; hormones were added; and cells allowed to coculture for 3 d. Media containing hormones were replaced and allowed to condition for 48 h. Conditioned media were assayed for testosterone by ELISA. Each original triplicate experimental sample was assayed in duplicate. Testosterone values were normalized to cell numbers as determined by the modified MTT assay. *Columns*, mean from three separate experiments; *bars*, SE. *, $P = 0.05$; **, $P = 0.01$, within monoculture or coculture. $^+$, $P = 0.05$; $^{+++}$, $P < 0.001$, between monoculture and coculture.

PSA gene expression in LAPC-4 cocultures was again found to be significantly increased 146-fold by TGFβ1 + DHEA as compared with the 40-fold increase by DHEA alone ($P = 0.05$; Fig. 4B). Red clover inhibited TGFβ1 + DHEA induction of PSA mRNA in both monocultured (LAPC-4 monoculture) and cocultured LAPC-4 cells (6S coculture) by 24- and 41-fold, respectively. Addition of 100 nmol/L E_2 also inhibited PSA gene expression in both monoculture (90% inhibition to 7.93-fold) and coculture (83% inhibition to 25.76-fold), whereas blocking ERβ or ERα with ICI 182,780 did not inhibit the red clover effect in either monoculture or coculture (15.72- and 45.11-fold, respectively). R1881 induced high levels of PSA gene expression in both monoculture (192-fold; $P < 0.01$) and coculture (233-fold; $P = 0.01$).

Changes in testosterone secretion in the presence of red clover

Testosterone concentrations were measured in conditioned media of stromal 6S monocultures and LAPC-4/6S cocultures.

DHEA-treated cocultures exhibited greater increases in testosterone (24.04 pg/mL/100,000 cells) than did DHEA-treated 6S monocultures (11.23 pg/mL/100,000 cells; Fig. 4C; $P < 0.01$). TGFβ1 augmented DHEA metabolism to testosterone in cocultures (58.24 pg/mL/100,000 cells; $P < 0.01$). DHEA-treated 6S cells in monoculture also exhibited increased testosterone secretion (33 ng/mL; $P < 0.05$). Red clover inhibited the TGFβ1 + DHEA–induced testosterone secretion in cocultures (14.51 pg/mL/100,000 cells, $P = 0.05$) to values similar to those after DHEA treatment alone. Blocking ERβ or ERα with ICI 182,780 did not block the inhibitory effect on testosterone secretion of red clover (22.46 pg/mL/100,000 cells). E_2 did not significantly alter testosterone secretion in TGFβ1 + DHEA–treated cultures (56.22 pg/mL/100,000 cells). Addition of TGFβ1 alone or R1881 alone or red clover isoflavone treatment alone elicited little or no change in testosterone secretion (data not shown). Cultures treated with R1881 + TGFβ1 or with R1881 + TGFβ1 + red clover showed no significant changes in testosterone secretion (data not shown) as is expected because R1881 is not metabolizable to testosterone. Additionally, parallel ELISA assays for E_2 from the same samples showed no secretion of E_2 by the cultures, and E_2 was present only in those samples treated with additional E_2 (data not shown).

Red clover isoflavone inhibition of PSA and testosterone metabolism was dose responsive

Increasing doses of red clover isoflavones (from 0 to 300 nmol/L) resulted in progressive inhibition of the TGFβ1-augmented increase in DHEA-induced PSA secretion (Fig. 5A; 13.40 ng/mL/100,000 cells; $P < 0.01$) in LAPC-4/6S cocultures, with significant reductions seen at concentrations of 30, 100, and 300 nmol/L red clover (at 38%, 65%, and 95% inhibition to 8.3, 4.7, and 0.71 ng/mL/100,000 cells, respectively; $P = 0.05$). LAPC-4 cells cocultured with 6S cells exhibited significantly greater PSA production than did those in monoculture under all DHEA-treated conditions ($P < 0.01$). Cultures treated with R1881 + TGFβ1 or with R1881 + TGFβ1 + red clover showed no change in PSA secretion (data not shown).

Likewise, increasing doses of red clover isoflavones (from 0 to 300 nmol/L) resulted in greater inhibition of the DHEA + TGFβ1–augmented increase ($P < 0.01$) in testosterone secretion (116.78 pg/mL/100,000 cells) in 6S cells in cocultures, with significant ($P = 0.01$) reductions observed at concentrations of 30, 100, and 300 nmol/L, with 51%, 64%, and 81% inhibition to 58, 42, and 23 pg/mL, respectively (Fig. 5B; $P = 0.01$). Red clover also inhibited testosterone secretion in 6S monocultures where significant reductions of TGFβ1 + DHEA–induced testosterone secretion (96.33 pg/mL/100,000 cells) occurred at concentrations of 30, 100, and 300 nmol/L, with 46%, 65%, and 82% inhibition to 52, 33, and 17 pg/mL, respectively (Fig. 5B; $P = 0.05$).

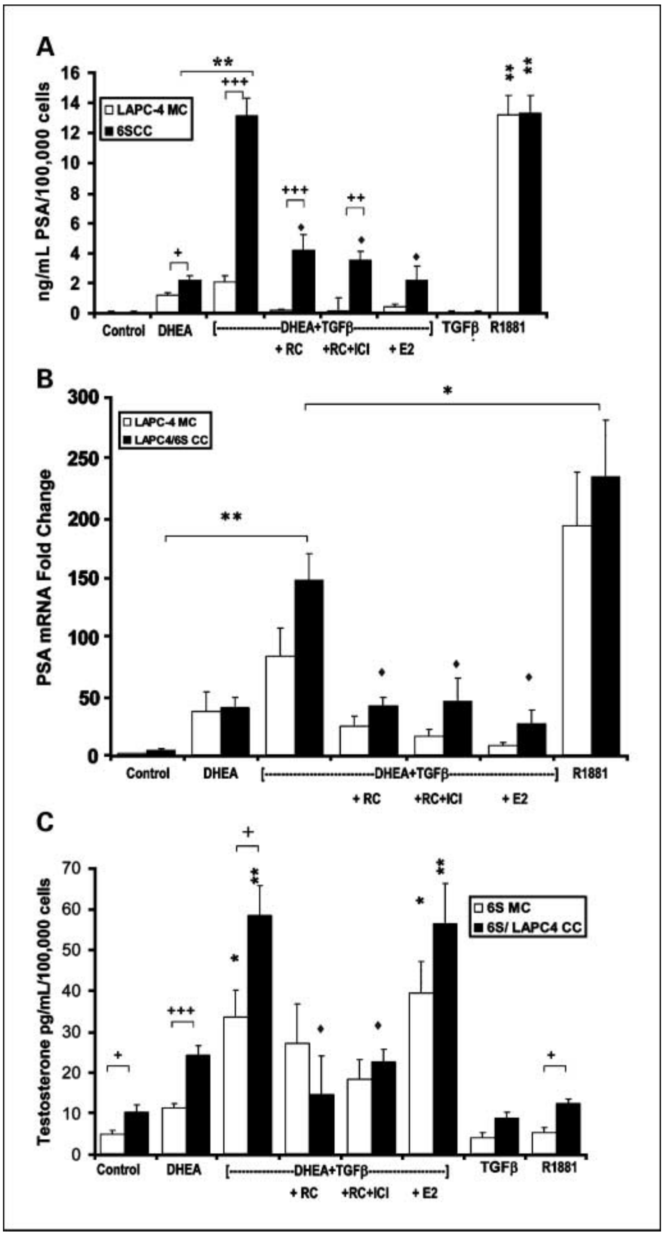

Fig. 4. Red clover effects on TGFβ1 + DHEA–stimulated LAPC-4/6S cocultures. *A*, LAPC-4 PSA production. The same experimental procedure was used as in the PSA ELISA experiment depicted in Fig. 2A, but in addition to hormone treatments of 100 nmol/L DHEA +/− 40 pmol/L TGFβ1, cells were also treated with DHEA/TGFβ1 + 100 nmol/L red clover (*RC*) isoflavones; DHEA/TGFβ1 + red clover plus 100 nmol/L ICI 182,780 (*ICI*; estrogen receptor antagonist); DHEA/TGFβ1 + 100 nmol/L E_2; TGFβ1 alone; or 10 nmol/L R1881. *Columns*, average from three separate experiments; *bars*, SE. *B*, LAPC-4 PSA gene expression. LAPC-4 cells were plated in triplicate in monoculture and in coculture with 6S stromal cells, as described in Fig. 2B. Stromal cells were pretreated with 40 pmol/L TGFβ1 for 3 d, then cultures were combined and treated with 100 nmol/L DHEA +/− 40 pmol/L TGFβ1; DHEA/TGFβ1 + 100 nmol/L red clover isoflavones; DHEA/TGFβ1 + red clover + 1 μmol/L ICI 182,780 (estrogen receptor antagonist); DHEA/TGFβ1 + 100 nmol/L E_2; or 10 nmol/L R1881 for 48 h. RNA was extracted and cDNA was reverse transcribed and probed by real-time PCR for PSA expression, standardized to ribosomal phosphoprotein PO expression. *Columns*, mean from three experiments; *bars*, SE. *C*, stromal testosterone secretion in cocultured LAPC-4/6S cells. Testosterone concentrations were determined in conditioned media from stromal cell monocultures, compared with cocultures from the same experiments illustrated in Fig. 3. Hormone treatments include 100 nmol/L DHEA +/− 40 pmol/L TGFβ1; DHEA/TGFβ1 + 100 nmol/L red clover isoflavones; DHEA/TGFβ1 + red clover plus 100 nmol/L ICI 182,780; DHEA/TGFβ1 + 100 nmol/L E_2; TGFβ1 alone; or 10 nmol/L R1881. *Columns*, mean from three experiments; *bars*, SE. *, $P = 0.05$; **, $P = 0.01$; $^{+}$, $P = 0.05$; $^{++}$, $P = 0.01$; $^{+++}$, $P = 0.001$; $^{\blacklozenge}$, $P = 0.05$, compared with DHEA + TGFβ1 alone.

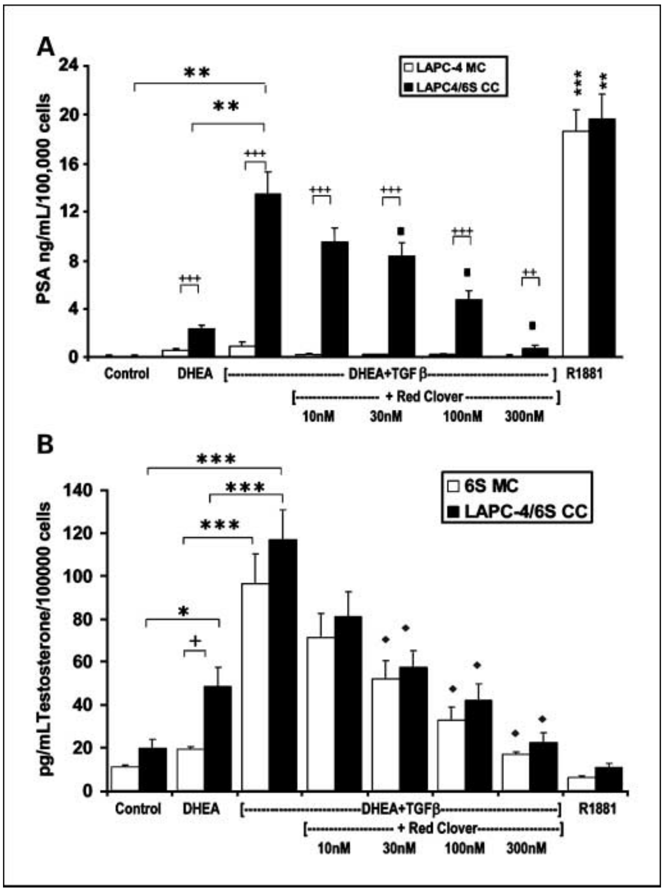

Fig. 5. Dose-responsive effects of red clover isoflavones to inhibit DHEA + TGFβ1–induced PSA expression in LAPC-4 cell monocultures and cocultures and testosterone metabolism in 6S stromal cell monocultures and cocultures. *A*, the same experimental procedure for PSA ELISA was used as in Figs. 2 and 4, but in addition to hormone treatments of 100 nmol/L DHEA +/− 40 pmol/L TGFβ1 and 10 nmol/L R1881, cells were also treated with DHEA + TGFβ1 + red clover isoflavones at 10, 30, 100, or 300 nmol/L. *B*, testosterone concentrations were determined in conditioned media from stromal cell monocultures, compared with cocultures from the same experiments represented in *A*. Hormone treatments included 100 nmol/L DHEA +/− 40 pmol/L TGFβ1; DHEA/TGFβ1 + red clover isoflavones at 10, 30, 100, or 300 nmol/L; and 10 nmol/L R1881. *Columns*, mean from three experiments; *bars*, SE. *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$; $^{+}$, $P = 0.05$; $^{++}$, $P = 0.01$; $^{+++}$, $P = 0.001$; $^{\blacklozenge}$, $P = 0.05$, compared with DHEA + TGFβ1 alone.

TGFβ1 + DHEA effects on stromal cell expression of AR and smooth muscle cell markers in 6S cells

Western blot analysis revealed that the combination of TGFβ1 + DHEA increased AR expression in 6S cells similar to that in cells treated with R1881 (Fig. 6). Red clover decreased the AR expression induced by DHEA + TGFβ1. All treatments including TGFβ1 increased α-smooth muscle actin. Desmin, smoothelin, and vimentin expression were unaffected by any of the treatments.

Discussion

In this coculture model of endocrine (DHEA)-immune (TGFβ1)-paracrine (stromal-epithelial) interactions in the prostate, the addition of TGFβ1 to DHEA-treated prostate stromal plus epithelial cells reproduced a reactive stromal microenvironment and significantly increased the androgenicity of both cell types, as measured by increased PSA (epithelial) and testosterone (stromal) production. These results expand our previous study showing that DHEA-treated LAPC-4 cells do not secrete PSA in the absence of prostate stromal cells. Our developing hypothesis is that the presence of DHEA in the prostate may be benign in normal prostate tissues, as represented by epithelial cells cocultured with stromal cells and treated with DHEA only, but may promote more androgenic effects in a reactive stromal microenvironment as represented by the addition of TGFβ to DHEA-treated cocultures. We investigated whether red clover isoflavones might reverse these TGFβ1-mediated effects and found that they may be beneficial in inhibition of androgenic effects in the prostate tissue microenvironment.

The results of this study suggest the involvement of at least three factors in the effects of TGFβ1 + DHEA–treated stromal cells on epithelial cells: (*a*) induction of a reactive stromal phenotype by TGFβ1; (*b*) increase in steroid (DHEA) metabolism; and (*c*) production of secondary paracrine mediators to promote the changes in expression of PSA and testosterone in prostate cocultures.

Alterations in stromal phenotype have been reported in many types of human cancer (24). Modified or activated prostate stromal cells have myofibroblastic characteristics, including increased levels of smooth muscle α actin (6). TGFβ1 and other proinflammatory cytokines mediate the reactive stromal response and promote a wound-repair–type reactive myofibroblast phenotype in prostate cancer (8, 10, 25, 26). It has been suggested that ~20% of human cancers are associated with chronic infection or inflammation (27). Such lesions have been characterized in the prostate as proliferative inflammatory atrophy and illustrate the association between inflammation and unusually high proliferation (28). In addition to contributions from immune cells, TGFβ1 is also overexpressed in prostatic intraepithelial neoplasia and prostate cancer cells, and may induce adjacent stroma to become reactive (8). By adding proinflammatory cytokines such as TGFβ1 or IL-6 to a coculture model of stromal plus epithelial cells from the prostate microenvironment, we aimed to mimic the increased levels of cytokines and characteristics of reactive stroma, such as are present in proliferative inflammatory atrophy, prostatic intraepithelial neoplasia, and prostate cancer.

In the LAPC-4/6S cocultures, TGFβ + DHEA induced significantly more PSA protein and gene expression than did DHEA alone, whereas IL-6 + DHEA did not produce similar additive effects on PSA. Both LAPC-4 and 6S cells contain TGFβ receptors I, II, and III (data not shown). Parallel LAPC-4/PrSC cocultures produced similar results. TGFβ1 + DHEA also resulted in higher metabolism to testosterone in both monocultured and cocultured stromal cells, whereas IL-6 + DHEA treatment failed to induce a similar, significant increase in testosterone metabolism in either cocultures or stromal monocultures. IL-6 also did not produce the reactive stroma cytoskeletal morphology observed after TGFβ1 treatment (data not shown). IL-6 induces hydroxysteroid dehydrogenase enzymes (12) and would be expected to increase DHEA metabolism in this model. Clinically, both IL-6 and TGFβ1 are elevated in patients with prostate metastases and both have been found to be correlated with increased serum PSA concentrations (29). TGFβ1-treated stroma may produce distinct paracrine factors that contribute to the effect on epithelial cells. Because we found no significant additional responsivity with the IL-6 treatment, we focused our experiments on the effects of DHEA + TGFβ1 treatments.

TGFβ1 stimulated the growth of prostate stromal cells at lower doses (0.001-0.01 ng/mL) and inhibited the growth of prostate stromal cells and promoted differentiation into smooth muscle actin structures at higher doses (0.1-1.0 ng/mL; ref. 30). We conducted growth experiments with varying doses of TGFβ1 to confirm that the 6S primary prostate stromal cells also respond as above, and to evaluate the stimulatory or inhibitory effect of 40 pmol/L of TGFβ1 on 6S cell growth at the time points used for PSA gene and protein expression (days 2 and 5, respectively). In the growth experiments, a concentration of 40 pmol/L TGFβ1 used in subsequent experiments led to results between those of the control and the highest, growth static dose, 400 pmol/L. The 40 pmol/L dosage significantly inhibited 6S stromal cell growth with a trend similar to that reported (30). The increased stromal testosterone secretion or stromal-induced PSA secretion and gene expression in epithelial cells by addition of TGFβ1 + DHEA (40 pmol/L) was associated with a decreased, but not an increased, number of 6S stromal cells. In addition, addition of DHEA to 40 pmol/L TGFβ1 did not affect the growth compared with 40 pmol/L TGFβ1 alone (data not shown), confirming our prior results of no change in stromal cell growth in the presence of DHEA or its metabolites (18).

Conditioned medium from TGFβ1 + DHEA–treated LAPC-4/6S cocultures contained increased PSA concentrations as well as enhanced metabolism of DHEA to testosterone. DHEA can be metabolized to testosterone and dihydrotestosterone via several enzymatic steps, including actions of 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and 5α-reductase. TGFβ1 decreases 3β-hydroxysteroid dehydrogenase in adrenocortical cells (31) and 17β-hydroxysteroid dehydrogenase in breast cancer cells (32). To our knowledge, there are no reports of TGFβ1 effects on prostatic metabolism of DHEA to testosterone. TGFβ1 can decrease the activity of CYP7B, which metabolizes DHEA to 7α-OH-DHEA, a ligand for ERβ (33), as measured in inflammatory tissues. TGFβ1 modulation of the DHEA metabolic pathway may alter the balance of androgenic and estrogenic ligands affecting the growth and function of the prostate.

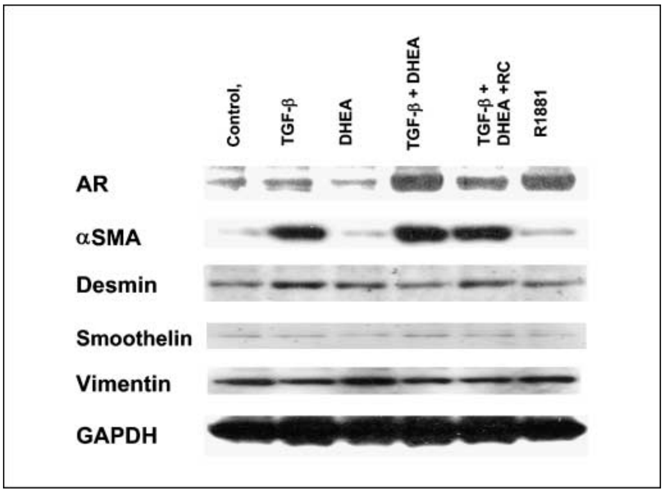

Fig. 6. Expression of AR and stromal cytoskeletal protein in 6S cells treated with TGFβ1 + DHEA and red clover. 6S prostate stromal cells were plated at a density of 5 × 10^5 per well on six-well plates. TGFβ1 (40 pmol/L) was added on the same day as when cells were plated. Cells were treated with 100 nmol/L DHEA +/− 40 pmol/L TGFβ1, DHEA/TGFβ1 + 100 nmol/L red clover isoflavones, and 10 nmol/L R1881, and allowed to culture for 4 d. Protein was extracted from cells and analyzed by Western blot for AR, α-smooth muscle actin (*αSMA*), desmin, smoothelin, vimentin, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Representative blot of three separate experiments.

PSA measurement in this coculture model is used as a relative biomarker of androgenic activity. This is not to be confused with the diagnostic use of PSA in clinical settings. The expression is dependent on cell culture conditions that can be variable. TGFβ1 + DHEA seems to have effects on the LAPC-4 cell PSA gene expression even in monocultures as there was an increase in gene, but not protein, expression of PSA (Figs. 2B and 4B). TGFβ1 may promote PSA gene expression via up-regulation of LAPC-4 steroid-metabolizing enzymes, increasing the presence of androgenic metabolites of DHEA or Smad3 interactions with the AR-induced PSA expression (34). However, as Fig. 3 indicates, there was no increase of testosterone secretion in TGFβ1 + DHEA–treated LAPC-4 monocultures. Understanding the basis for the discrepancies between TGFβ1-induced PSA gene and protein expression in LAPC-4 monocultures will require further study.

As was the case with PSA production, TGFβ1 + DHEA administration resulted in a greater increase in testosterone metabolism over 6S cells in monoculture and coculture treated with DHEA alone. Additionally, increased testosterone concentrations were found in cocultures versus stromal monocultures. Several possibilities may be that LAPC-4 cells provide paracrine reciprocal contributions to the stromal metabolism and/or stromal cells or TGFβ1 induce LAPC-4 metabolic enzymes.

This coculture model has potential value for identifying natural or synthetic agents that may modulate endocrine-immune-paracrine interactions in the prostate. To this end, we treated cells with red clover isoflavones, which were combined in the same proportions as in commercially available preparations. Red clover isoflavone administration significantly inhibited TGFβ1 + DHEA induction of expression of PSA and testosterone in a dose-dependent manner at final concentrations similar to those achieved clinically (30-300 nmol/L; refs. 23, 35).

Historically, red clover has been used in patients with cancer and various respiratory problems, and is currently given to manage menopausal symptoms as well as symptoms of prostate enlargement.[1] The safety and bioavailability of red clover extract have been evaluated to some extent. For example, 40 mg red clover isoflavones taken twice daily for 2 weeks was well tolerated and produced plasma concentrations of isoflavones similar to those seen in populations consuming high dietary amounts of isoflavones (35); moreover, it was readily absorbed by the prostate (23). *In vivo* studies in mice fed a diet supplemented with 5% red clover isoflavones for 14 months reported significantly reduced expression of TGFβ1 in prostatic epithelium (36), suggesting that red clover isoflavones may modulate cytokine expression.

Red clover effects in the LAPC-4/6S cocultures could be mediated by the phytoestrogens contained in the mixture,

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[1] http://nccam.nih.gov/health/redclover/

including genistein and daidzein. Genistein comprises 10% of the red clover formulation (22) and is a potent antiandrogen, able to block >70% of dihydrotestosterone-induced PSA production *in vitro* (37). To compare red clover effects with those of a pure estrogen, E_2 was added to TGFβ1 + DHEA–treated LAPC-4/6S cocultures. E_2 decreased PSA protein and gene expression but exerted no significant effect on testosterone metabolism. The addition of ICI 182,780, an estrogen receptor antagonist, did not reverse the inhibitory effect of red clover on TGFβ1 + DHEA–stimulated PSA secretion or gene expression and testosterone metabolism. These results suggest that the red clover effects we observed are not mediated by epithelial ERβ or stromal ERα.

The red clover effects on inhibiting PSA in prostate cell cocultures reported herein are seemingly inconsistent with the results of a clinical study that found no change in serum PSA concentrations in men who consumed 160 mg of red clover daily before radical prostatectomy (15). The use of PSA as a marker for androgenicity in *in vitro* studies should be distinguished from the use of circulating PSA concentrations as a diagnostic tool for prostate cancer detection or management; it also reflects the difference between cellular expression of PSA versus leakage into the circulatory system.

Two possible mechanisms may explain the observed effects of red clover. Red clover inhibited the TGFβ1 + DHEA–induced increase of AR expression in 6S cells, supporting the idea that red clover might be modulating androgen activity in prostate cells. In addition, red clover may affect one or more of the enzymes involved in DHEA metabolism, as it decreased production of the DHEA metabolite, testosterone. Biochanin A, the predominant isoflavone in red clover, has been shown to modulate 17β-hydroxysteroid dehydrogenase type 5 in transfected bacteria (17), and genistein and biochanin A are potent inhibitors of 5α-reductase and 17β-hydroxysteroid dehydrogenase activity in genital skin fibroblasts (16). Whereas it is premature to speculate that these enzymes are also modulated in prostate stromal and epithelial cells, the fact that they can be altered by isoflavones makes them attractive candidates for future study. Additional animal and clinical studies are absolutely necessary to determine the usefulness of red clover extracts as safe or effective for human use.

We consistently observed morphologic alterations in 6S cells following treatment with TGFβ1. TGFβ1-treated cells seemed to be more confluent, but were actually larger in size, and displayed an increase in cytoskeletal fibers (data not shown), which was confirmed by Western blotting, which revealed increased expression of α-smooth muscle actin, a hallmark of the activated phenotype (8), under all TGFβ1 treated conditions. Treatment with TGFβ1 + DHEA resulted in increased expression of AR protein. A complex cross talk exists between androgen and TGFβ1 signaling where interactions can regulate activation of rat prostate stromal cells (38). This is consistent with our finding that AR expression was up-regulated in 6S cells only with combined administration of TGFβ1 + DHEA and not with TGFβ1 alone. α-Smooth muscle actin protein expression was also increased in all stromal cells treated with TGFβ1, independent of DHEA, as found in rat prostate stromal cells treated with TGFβ1 +/− dihydrotestosterone (38).

The stromal 6S cells used in this study have previously been characterized to have more reactive phenotype (18) with an increased ability to secrete insulin-like growth factor I in response to dihydrotestosterone, compared with other stromal cell lots from cancer or normal prostate. When compared with normal primary stromal cells, DHEA-treated stromal cell lots derived from cancer tissues showed increased ability to induce PSA expression in cocultures (20). The 6S cells in this study display increased "reactivity" with the addition of TGFβ1. This increase in reactivity was also found in parallel coculture studies done with PrSC normal prostate stromal cells, which produced a similar increase in TGFβ1 + DHEA–induced LAPC-4/PrSC PSA and testosterone production over amounts induced by DHEA alone. The comparison between 6S or PrSC without and with TGFβ1 provides a better experimental representation of normal versus reactive stroma, respectively, than the comparison of untreated primary normal versus cancer-associated stromal cells.

The reported increased PSA production in TGFβ1 + DHEA–treated cocultures plus the significantly greater metabolism to testosterone observed in stromal cells treated with TGFβ1 + DHEA versus DHEA alone suggests that reactive stroma, as modeled by the addition of TGFβ1, responds differently to DHEA than does normal stroma, further supporting the hypothesis that the effects of DHEA in the prostate depend on the prostate microenvironment. Further characterization of TGFβ1-treated stromal cells is needed to identify other factors involved in promoting DHEA metabolism; identify secreted paracrine factors that augment PSA production; and determine whether altered metabolism of DHEA may occur in various prostate cancer lesions *in vivo* and, if so, its role in prostate pathology.

As is the case with any *in vitro* experiment, a coculture model is highly artificial and a highly variable technique. Variability was controlled through intraexperimental and interexperimental replicates, as is evidenced by the small SE on the graphs as well as the consistency of effect pattern through out different experiments. The coculture model is useful in that it enables us to reproduce some of the variations in prostate cancer microenvironment in a controlled manner. However, there are a multitude of other *in vivo* and *in vitro* factors that undoubtedly play a role in carcinogenesis at the level of an organism, and thus, our ability to extrapolate potential clinical significance from the present data is limited. Nonetheless, stromal paracrine factors and steroid-metabolizing enzymes that are identified to play a role in the effects described in this report will be further characterized in additional *in vitro* and *in situ* studies, and may offer insights to better inform the design of clinical and translational investigations.

In summary, we report that the addition of TGFβ1 + DHEA to the stromal-epithelial cocultures increases PSA protein secretion and gene expression over DHEA treatment alone while also enhancing the metabolism of DHEA to testosterone. Addition of TGFβ1 serves as a stimulation of the reactive prostate stroma associated with the cancer tissue microenvironment. These results suggest that in cancer tissues compared with normal prostate, there may be a promotion of metabolism of DHEA to androgenic ligands and a production of stromal paracrine factors resulting in increased PSA and testosterone production. Administration of red clover isoflavones decreased TGFβ1 + DHEA–mediated PSA protein and gene expression and testosterone metabolism in LAPC-4/6S cocultures in a dose-dependent manner. This coculture model of endocrine-immune-paracrine interactions in the prostate provides a possible tool for identification of natural products or traditional medicines with multiple mechanisms that may prevent cancer progression by participating in stromal-epithelial cell interactions, such as by altering paracrine hormonal signals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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