DHT and testosterone, but not DHEA or E₂, differentially modulate IGF-I, IGFBP-2, and IGFBP-3 in human prostatic stromal cells

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DHT and testosterone, but not DHEA or E₂, differentially modulate IGF-I, IGFBP-2, and IGFBP-3 in human prostatic stromal cells. Am J Physiol Endocrinol Metab 290: E952–E960, 2006. First published December 20, 2005; doi:10.1152/ajpendo.00451.2005.—Prostate cancer is one of the four most common cancers in the United States, affecting one of six men. Increased serum levels of androgens and IGF-I are associated with an augmented risk of prostate cancer. Dihydrotestosterone (DHT) and testosterone (T) stimulate prostate cancer cell growth, development, and function, whereas the effects of DHT and T in prostate stromal cells, and of dehydroepiandrosterone (DHEA) in prostate cancer or stromal cells, are uncertain. We investigated the actions of DHT, T, DHEA, and estradiol (E₂) on insulin-like growth factor (IGF)-I, IGF-II, IGF-I receptor (R), IGF-binding protein (IGFBP)-2, IGFBP-3, and IGFBP-5 in primary cultures of human prostatic stromal cells by assessing cell proliferation, mRNA expression, and protein secretion by MTT growth assay, quantitative real-time PCR, and ELISA, respectively. DHT and T each increased IGF-I (7-fold) and decreased IGFBP-3 (2-fold) mRNA expression and protein secretion in a dose- and time-dependent manner and increased IGFBP-2 (2-fold) mRNA in a dose- and time-dependent manner. DHEA and E₂ did not significantly alter these measures. Flutamide abolished the DHT-modulated increases in IGF-I and IGFBP-2, suggesting that the influences of DHT and T on these measures were androgen receptor mediated. None of the four steroids significantly affected IGF-IR, IGF-II, or IGFBP-5 mRNA levels or stromal cell proliferation. The effects of DHT on IGF-I, IGFBP-2, and IGFBP-3 were more pronounced in stromal cultures that did not express desmin. These data suggest that DHT and T promote prostate growth partly via modulation of the stromal cell IGF axis, with potential paracrine effects on prostate epithelial cells.

The prostate gland is an androgen-dependent tissue composed of secretory epithelial and stromal cells. Stromal cells, consisting primarily of smooth muscle cells and fibroblasts, contribute importantly to the development, differentiation, and maintenance of the normal prostate (9). Paracrine growth or inhibitory factors are produced by stromal cells in response to androgen exposure (12, 14, 35, 41, 51). These “andromedins” provide secondary regulatory signals to related epithelial cells in the prostate. Dysregulation of signaling between the stroma and epithelial cells is thought to contribute to prostate carcinogenesis (9).

Although many epidemiological studies do not support the concept that increased serum levels of testosterone (T) are associated with an increased risk of prostate cancer (17), androgens are essential for prostate development and carcinogenesis. Males who are castrated at early ages do not develop prostate cancer (40), and androgen ablation has been a standard treatment for this cancer (25) at least during the androgen-dependent stage. Androgen actions are mediated primarily via the androgen receptor (AR; see Ref. 6). Although T and dihydrotestosterone (DHT) are the two major endogenous androgens in men, DHT is the more active androgen in the prostate, where it is produced from T by 5α-reductase (21).

The adrenal steroids dehydroepiandrosterone (DHEA) and DHEA sulfate are the most abundant steroids in humans, and their levels decline markedly with aging (38). DHEA is widely available as a dietary supplement and is consumed by middle-aged and older individuals in the hope that it will retard aging by improving body composition, endocrine-metabolic, and immune functions (1). Because DHEA can serve as a precursor for the more potent androgens T and DHT, as well as estrogen, its effects on prostate health are of interest.

The insulin-like growth factor (IGF) axis, which includes IGF-I and IGF-II, two cell membrane receptors (IGF-IR and IGF-IRR), six binding proteins (IGFBPs 1–6), and several IGFBP proteases, contributes to the growth and function of almost every organ in the body (11, 20). IGFs are potent mitogenic and anti-apoptotic molecules that modulate epithelial cell proliferation in several organs, including the prostate, breast, lung, and colon (39, 42). IGFBPs modulate the interactions of IGFs with their receptors; IGF-IR, a tyrosine kinase receptor, is particularly relevant to cancer studies (26). Although IGFs and IGFBPs are synthesized primarily in the liver, they are also produced locally by most tissues, where they act in an autocrine or paracrine manner (32). High serum levels of IGF-I are associated with an increased risk of prostate cancer (5, 7, 23, 36, 44), and decreased serum levels of IGFBP-3 are found in prostate cancer patients and in those with metastatic disease (7).

The interrelationships among prostate stroma, testicular and adrenal androgens, and the IGF axis in the pathogenesis of prostate cancer are not well defined. Thus we evaluated the effects of DHT, T, DHEA, and estradiol (E₂) on components of the IGF axis in primary cultures of human prostatic stromal cells.

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MATERIALS AND METHODS

Human prostate stromal cell isolation. Four different human primary prostatic stromal cultures, termed 5S, 6S, 9S, and 12S, were generated from radical prostatectomy specimens derived from four patients, ages 52–56, with Gleason Grade 6 (5S, 9S, and 12S) or 7 (6S) prostate cancer, as described previously (18), and kindly provided by Dr. John Isaacs, Johns Hopkins Medical Institutions. Tissue for 5S and 6S cells was taken from a portion of the cancerous prostate that housed benign prostate hyperplasia (BPH) lesions, whereas the 9S and 12S cells were derived from peripheral regions of the prostate specimens where no tumors were palpable. Prostate tissue obtained at surgery was collected in isolation media consisting of DMEM with 10% FBS, penicillin (200 U/ml), and streptomycin (200 μg/ml; Invitrogen) in 20 ml isolation medium (as above) for 2.5 h at 37°C on a shaking rotor. The tissue digest was vigorously pipetted and epithelial clumps settled from stromal cells for 15 min without centrifugation. The stromal cells present in supernatant were removed and plated on 100-mm plastic culture dishes. The tissue digest was again vigorously pipetted to release more stromal cells. Any stromal cells remaining with the epithelial clumps were further separated by selective adherence to plastic tissue culture dishes for 1 h. Stromal cell populations were routinely >98% pure, as assessed by phase microscopy and by immunohistochemistry using antibodies for cytokeratins and vimentin (data not shown). Promoting stromal cell growth and inhibiting epithelial cell outgrowths were achieved by continued culture in serum-containing media (DMEM-F12 plus 5% FBS). In addition, a normal primary prostatic stromal culture, PRSC, derived from the cadaver of a 55-yr-old man with no known prostate disease, was purchased from Cambrex (Walkersville, MD).

5S and 6S cell pellets were trypsinized and seeded in growth medium consisting of DMEM-F12 (1:1) medium (Invitrogen) with penicillin (200 U/ml), streptomycin (100 μg/ml; Invitrogen), and L-glutamine (292 μg/ml; Invitrogen), and FBS (5%; HyClone Laboratories, Logan, UT) at 37°C in 5% CO2 and 10% C02. CellTiter 96 Assay (MTT assay; Promega), as previously described (2). The assay measures the cellular conversion of a tetrazolium salt to a formazan product, which can be detected spectrophotometrically, and provides a relative estimate of cell growth. Stromal cells were seeded in 96-well plates precoated with Matrigel matrix film at a density of 3,000 cells/well in Medium 199-F12 (1:1) phenol red-reduced media supplemented with 2% CDS for 2 days. Hormones were added at various doses (DHEA, DHT, T, and E2; Sigma), and the final CDS concentration was 1%. Cell growth was assessed after exposure to steroid hormone concentrations from 0.1 to 10,000 nM vs. control for 5 days in the dose-response experiments and 100 nM hormone vs. control for 1–9 days for the time course experiments. The absorbance at 570 nm was measured with the SpectraMax Plus (Molecular Devices, Sunnyvale, CA) and analyzed using SoftMax Pro software. Eight replicates per condition were assayed, and data averaged from three separate experiments are presented.

RNA extraction and real-time quantitative RT-PCR. RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer’s manual. The resulting RNA pellet was extracted a second time with TRizol reagent to increase RNA purity. RNA was quantified spectrophotometrically at 260 nm. cDNA synthesis and real-time RT-PCR were performed as described by Latil et al. (31) with some modifications. Briefly, RNA was reverse transcribed with Superscript III RNase H+ RT from Invitrogen, according to the manufacturer’s manual, with 10 units of RNasin RNase inhibitor (Promega), 50 units of Superscript III, 150 ng of random hexamer (Amerham Bio-sciences), and 1.5 μg of total RNA. PCR was performed using a SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA) and quantitated by an ABI Prism 7900 Sequence Detection System (Applied Biosystems) according to the manufacturer’s manual. RNA purity was assessed using controls for cDNA synthesis made with no RT, ensuring no DNA contamination. The size of the real-time PCR products was verified by electrophoresis on 4% agarose gels. In addition, dissociation curve analysis was also performed after each PCR reaction to ensure that a single product and no primer-dimers were present. Relative mRNA expression level was calculated using the ΔΔCT method, where the calibrator was the RPLPO gene. RPLPO encodes human acidic ribosomal phosphoprotein P0 and was used as the endogenous RNA control to correct for the amount of total RNA used in each PCR reaction. All of the primers have been described previously (2).

ELISAs. Secreted IGF-I was quantified using acid ethanol extraction (10) and the Quantikine human IGF-I kit (R&D Systems, Minneapolis, MN). Conditioned medium (600 μl) collected as described above was lyophilized to a dry pellet. Water (20 μl) was added to resuspend the pellet. Eighty microliters of 12.5% 2 N formic acid-87.5% ethanol was added and incubated for 30 min at room temperature to free IGF-I from the complexes with IGFBPs. The solution was centrifuged, and the supernatant was neutralized by adding 0.4 volume of 0.855 M Tris base. Seven volumes of 1 mg/ml BSA with 0.02% w/v p-mercaptoethanol, and 0.02% phenol red were added; 50 μl of the prepared sample were used in each well of the Quantikine kit as instructed in the manual. Conditioned medium was assayed for secreted IGFBP-3 using the IGFBP-3 ELISA kit from Diagnostic Systems Laboratories (Webster, TX) as described by the manufacturer.

Western blotting. Cultured cells in growth medium were collected by trypsinization. The number of cells was quantitated by a Z1 Coulter Particle Counter (Beckman Coulter, Miami, FL). Cells (1 × 106) were lysed and resuspended in SDS loading buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 200 mM β-mercaptoethanol, and 0.02% SDS). All of the proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the antibodies to the core proteins of IGFBPs.
were then washed four times in TBST and incubated with species-appropriate horseradish peroxidase-conjugated secondary antibodies in TBST containing 5% fat-free milk. After being washed four times in TBST, the membranes were visualized using the ECL SuperSignal West Pico Substrate (Pierce, Rockford, IL). Film images were digitally captured using a Kodak Image Station (Perkin-Elmer, Boston, MA).

Statistical analysis. Results are presented as mean values ± SE of three independent experiments, each performed with three to eight replicates. The statistical analysis was performed using the SAS (Statistical Analysis System; SAS Institute, Cary, NC) or JMP (SAS institute) statistical package. The distributional assumption of normality was checked using normal probability plots and the Shapiro-Wilk’s test and a log_{10} transformation applied when that assumption was not met. Results of quantitative real-time RT-PCR experiments are presented as degrees of changes of transcripts derived from hormone-treated vs. untreated cells after normalization for the reference control gene RPLPO. ANOVA was performed using Ct and the degree of change as dependent variables for the analysis of the real-time RT-PCR. The Tukey-Kramer procedure was used to test for multiple comparisons. An adjusted P value of 0.05 was considered significant.

RESULTS

Hormone effects on stromal proliferation. Administration of DHT, T, DHEA, and E2 did not significantly affect the growth of prostate stromal (6S) cells. Both dose-response and time course studies (Fig. 1) revealed hormone-induced proliferation patterns similar to controls, except for an 11–27% inhibition (P < 0.0001) observed at the highest dose (10 μM) of hormone (Fig. 1A).

Modulation of the IGF axis by DHT, T, DHEA, and E2. To assess the possible effects of DHT, T, DHEA, and E2 on the IGF axis, dose-response and time course experiments were performed to quantify mRNA expression of IGF-I, IGF-II, IGF-IR, IGFBPs-2, 3, and 5, and protein expression of IGF-I and IGFBP-3 in vehicle control- vs. hormone-treated primary stromal cells from the 6S stromal cell cultures. DHT and T administration increased IGF-I mRNA and protein expression in a dose- and time-dependent manner (Fig. 2, A–D). After 4

Fig. 1. 6S stromal cell proliferation after treatment with dihydrotestosterone (DHT), testosterone (T), dehydroepiandrosterone (DHEA), and estradiol (E2). Cell proliferation was measured by the MTT assay (see MATERIALS AND METHODS). Top: dose-response study. OD, optical density. Bottom: time response study. Graphs illustrate mean values ± SE averaged from 3 experiments.

Fig. 2. Insulin-like growth factor (IGF)-I expression in 6S primary prostate stromal cells treated with DHT, T, DHEA, and E2. Dose (A and B) and time (C and D) responses of IGF-I mRNA and protein expression measured by real-time RT-PCR and ELISA. Graphs illustrate mean values ± SE averaged from 3 experiments. *P < 0.05 and **P < 0.0001.
days of treatment, DHT increased IGF-I mRNA at all doses tested (0.1 nM-10 μM; \( P < 0.0001 \)), whereas T increased IGF-I mRNA beginning at a dose of 1 nM (\( P < 0.0001 \)). IGF-I mRNA increased to a greater extent after treatment with DHT vs. T (\( P < 0.0001 \); Fig. 2A). In contrast, the effects of DHT and T on IGF-I protein did not differ significantly after 6 days of treatment (Fig. 2B); both DHT and T treatments maximally increased IGF-I protein secretion in the medium eightfold at 10 μM (\( P < 0.0001 \)) compared with control.

In time-response experiments, 100 nM DHT treatment also increased IGF-I mRNA more than did T (\( P = 0.001 \); Fig. 2C). Additionally, DHT treatment increased IGF-I mRNA at day 1 (\( P = 0.016 \)) and continuously to day 7 (\( P < 0.0001 \)), whereas T treatment increased IGF-I mRNA beginning at day 3 of treatment (\( P < 0.0001 \)). There was a general increased trend in IGF-I protein secretion after treatment with DHT and T at 100 nM for 7 days (Fig. 2D); both DHT and T treatments maximally increased IGF-I protein secretion in the medium eightfold at 10 μM (\( P < 0.0001 \)) compared with control.

DHEA treatment exerted no significant effects on IGF-I mRNA in dose-response or time course studies, except at day 7 with 100 nM DHEA treatment when a 1.7 ± 0.2-fold increase in mRNA level was observed compared with control (\( P = 0.03 \); Fig. 2A-D). This slight increase in IGF-I mRNA from DHEA treatment was threefold less than the increase in IGF-I mRNA after DHT and T treatment (\( P < 0.0001 \)) and did not differ significantly from the effect of E2 treatment. Moreover, DHEA treatment did not change IGF-I protein level (data not shown), and E2 did not significantly affect IGF-I mRNA level in any dose-response or time course study (Fig. 2, A-D).

IGFBP-2 mRNA levels were increased by DHT to 2 ± 0.2- and 1.87 ± 0.08-fold at 1,000 and 10,000 nM, respectively (\( P = 0.01 \)), in the dose-response study (Fig. 3A) and were increased in the time course study by day 3 of the treatment (\( P = 0.0008 \) for DHT and \( P = 0.036 \) for T) and maximally at 2.3 ± 0.1-fold for DHT and 2.0 ± 0.2-fold for T at day 7 (\( P < 0.0001 \) for both T and DHT; Fig. 3B). The effects of DHT and T treatment on IGFBP-2 mRNA expression did not differ significantly. DHEA and E2 did not significantly affect levels of IGFBP-2 mRNA (Fig. 3A and B).

DHT and T treatment tended to decrease IGFBP-3 mRNA expression in a dose-dependent manner, with significant effects observed only at the highest dose tested (10 μM, \( P = 0.0035 \) and 0.032 for DHT and T, respectively). Similarly, DHT and T treatment reduced IGFBP-3 mRNA expression by 2 ± 0.04-fold after 4 days of treatment (Fig. 4A). In contrast to mRNA expression, DHT and T significantly decreased IGFBP-3 protein secretion at all doses tested (0.1 nM-10 μM, \( P < 0.0001 \); Fig. 4B), with maximal reduction to 50% of control at 10 μM, similar to the decrease seen in IGFBP-3 mRNA expression.

Fig. 3. IGF-binding protein (IGFBP)-2 expression in 6S primary prostate stromal cells treated with DHT, T, DHEA, and E2. Dose (A) and time (B) responses of IGFBP-2 mRNA expression measured by real-time RT-PCR. Graphs illustrate mean values ± SE averaged from 3 experiments. + \( P < 0.05 \), # \( P < 0.001 \), and * \( P < 0.0001 \).

Fig. 4. IGFBP-3 expression in 6S primary prostate stromal cells treated with DHT, T, DHEA, and E2. Dose (A and B) and time (C and D) responses of IGFBP-3 mRNA and protein expression measured by real-time RT-PCR and ELISA. Graphs illustrate mean values ± SE averaged from 3 experiments. + \( P < 0.05 \) and * \( P < 0.0001 \).
The effects of DHT and T on IGFBP-3 mRNA and protein expression did not differ significantly from one another.

In time course studies, 100 nM DHT decreased IGFBP-3 mRNA at days 3, 5, and 7 ($P < 0.023$, $P < 0.0001$, respectively), whereas 100 nM T decreased IGFBP-3 by 50% at days 5 and 7 ($P < 0.02$ and $P < 0.0001$, respectively; Fig. 4C). A similar pattern was observed in the time course study of IGFBP-3 protein secretion (Fig. 4D). DHEA treatment did not significantly affect IGFBP-3 mRNA expression but decreased IGFBP-3 protein secretion by 20% ($P < 0.0001$) at the highest dose tested (10 μM) and by 12% ($P < 0.0001$) after 7 days of treatment with 100 nM. The effect of DHEA on IGFBP-3 protein secretion was less than those of DHT or T ($P < 0.0001$). E2 did not significantly affect IGFBP-3 mRNA expression (Fig. 4A-D). There were no significant effects of DHT, T, DHEA, or E2 on IGF-II, IGF-IR, and IGFBP-5 mRNA (data not shown).

Effects of flutamide and Casodex on DHT-modulated IGF-I, IGFBP-2, and IGFBP-3. The AR antagonists flutamide and Casodex were employed to determine if modulation of IGF-I, IGFBP-2, and IGFBP-3 expression by androgens was mediated by the AR. Both flutamide and Casodex decreased cell growth in a dose-dependent manner (Fig. 5). Flutamide treatment for 5 days decreased cell growth 10% at 10 μM and 30% by 30 μM ($P < 0.0001$), whereas Casodex reduced cell growth by 20% at 10 μM and 40% at 30 μM ($P < 0.0001$). The effects of Casodex to inhibit cell growth were greater than those of flutamide. Flutamide and Casodex both diminished DHT-stimulated IGF-I mRNA expression in a dose-dependent manner (Fig. 6A). Cotreatment with vehicle control or 10 nM of DHT plus AR blockers at different concentrations (0, 0.5, 1, 5, 10, and 30 μM flutamide or 0, 0.5, 1, 5, and 10 μM Casodex) for 5 days elicited a dose-dependent diminution in IGF-I mRNA expression. The 5.5-fold increase in IGF-I mRNA stimulated by 10 nM DHT was diminished to 3.5- or 3-fold by 10 μM flutamide ($P < 0.0001$) or 10 μM Casodex ($P < 0.0001$) treatment, respectively, and further reduced to 2-fold, the same level as vehicle control, after 30 μM flutamide cotreatment. In the absence of DHT, Casodex (5 μM), but not flutamide treatment alone, led to a slight (1.7-fold) but significant ($P < 0.0001$) increase in IGF-I mRNA. This finding differs from the previously reported pure anti-androgenic effect of Casodex compared with flutamide (16). There was a nonsignificant increase in IGF-I mRNA expression with flutamide plus vehi-

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Fig. 5. Effect of flutamide (Flut) and Casodex (CS) on cell growth in 6S primary prostate stromal cells. Dose response of flutamide and Casodex on cell growth in the presence or absence of 10 nM DHT, as measured by MTT. Graphs illustrate mean values ± SE averaged from 3 experiments. *$P < 0.0001$.

Fig. 6. Effect of flutamide and Casodex on DHT-modulated IGF-I, IGFBP-2, and IGFBP-3 in 6S primary prostate stromal cells. Dose response of flutamide and Casodex on mRNA and protein expression of IGF-I (A and B), mRNA expression of IGFBP-2 expressed as degree of change relative to control (C), and mRNA expressed as degree of change relative to control and protein expression of IGFBP-3 (D and E) in the presence or absence of 10 nM DHT. Symbols in D are the same as for A. F: Casodex effect at 10 μM on IGFBP-3 protein secretion with or without 10 nM DHT measured by ELISA. Graphs illustrate mean values ± SE averaged from 3 experiments. *$P < 0.0001$ comparing flutamide or casodex treatment with vehicle control in the no-DHT treatment group only.
creased from 10 to 30 M. A parallel pattern was observed with flutamide effect on DHT-modulated IGF-I protein (Fig. 6B). A concentration of 30 M flutamide was needed to abolish the threefold DHT-mediated increase in IGF-I protein. A similar result was observed with 10 M Casodex on IGF-I protein (data not shown).

Flutamide and Casodex exerted opposite effects on IGFBP-2 mRNA expression (Fig. 6C). Flutamide led to a dose-dependent reduction in the DHT-modulated increase in IGFBP-2 expression, and abolished the DHT-mediated effect at 30 M, while having no significant effect in control cells. In contrast, Casodex increased IGFBP-2 mRNA in a dose-dependent manner in control-treated cells (to a maximum of 2-fold at 10 M, P < 0.0001) and in DHT-treated samples (to a maximum of 2.8-fold at 10 M, P < 0.0001).

Casodex decreased IGFBP-3 mRNA levels in a dose-dependent manner (Fig. 6D). Flutamide exerted similar, but lesser, effects on IGFBP-3 expression compared with Casodex. Hydroxyflutamide has been reported to act as an agonist (49) at 10 M or higher in the absence of androgen. Flutamide decreased IGFBP-3 mRNA by 40% at 5 M and by 45% at 10 M, the same level achieved with 10 nM DHT treatment. Flutamide decreased IGFBP-3 protein secretion at all concentrations of flutamide tested (Fig. 6E), with a 10% reduction at 1 M, 20% reduction at 10 M, and a 34% reduction at 30 M. Cotreatment with flutamide or Casodex plus 10 nM DHT did not result in more reduction in the IGFBP-3 mRNA or protein compared with 10 nM DHT treatment alone (Fig. 4, A and B). Treatment with Casodex alone at 10 M also decreased IGFBP-3 protein level by 54 percent (P < 0.0001), 34% more than occurred using the same dose of flutamide (Fig. 6F).

Comparative modulation by DHT of IGF-I, IGFBP-2, and IGFBP-3 in prostate primary stromal cell cultures. We examined whether androgen modulation of IGF-I, IGFBP-2, and IGFBP-3 in 6S primary stromal cells also occurred in other primary prostatic stromal cell cultures. As shown in Fig. 7A, 100 nM DHT increased IGF-I mRNA after 5 days of treatment in the 6S (7-fold, P < 0.0001), 9S (3-fold, P < 0.0001), and 12S (2.1-fold, P = 0.0006) cultures but not in the 5S and PRSC cultures. DHT treatment (100 nM) increased IGFBP-2 mRNA in 6S (2.2-fold, P < 0.0001), 9S (1.5-fold, P < 0.0001), and PRSC (1.2-fold, P < 0.0001), but not in 12S or 5S, cultures (Fig. 7B). DHT stimulation of both IGF-I (P < 0.0001) and IGFBP-2 (P < 0.0001) mRNA was greater in 6S vs. 9S cultures. DHT (100 nM) decreased IGFBP-3 mRNA expression in 6S (0.53-fold, P < 0.0001) and 9S (0.65-fold, P < 0.0001) cultures, with no significant effect on 12S, 5S, or PRSC cultures (Fig. 7C). To further investigate the possible reasons for different responses among the stromal cell lots, we evaluated several stromal cell differentiation markers by immunoblot (Fig. 8), including AR, vimentin, smooth muscle α-actin (SMA), and desmin. No differences in AR protein expression were evident among the different stromal cultures. All the stromal cells, 6S, 9S, 12S, 5S, and PRSC, expressed similar levels of vimentin, whereas 6S expressed much less SMA compared with the other stromal cultures; moreover, only 5S and PRSC expressed desmin (Fig. 8).

To our knowledge, this is the first report evaluating the effects of DHT, T, DHEA, and E2 on components of the IGF axis, including IGF-I, IGF-II, IGF-IR, IGFBP-2, IGFBP-3, and IGFBP-5, in primary cultures of human prostatic stromal cells derived from patients with prostate cancer. Our findings are consistent with a paracrine role of stromal cells in prostate tissue in that steroid hormones exerted no detectable effects on stromal cell growth, whereas they did modulate the IGF axis, which is important in epithelial cell functioning. These data build on previous work demonstrating responses to these same hormones in human prostate LNCaP cancer (epithelial) cells (2).

In human primary prostate stromal cell cultures, administration of DHT and T, but not of DHEA or E2, modulated IGF-I, IGFBP-2, and IGFBP-3 gene and protein expression. DHT modulation of IGF-I and IGFBP-2 was mediated via the AR, as assessed by blocking the AR with Casodex and flutamide. Both flutamide and, to a greater extent, Casodex exerted partial AR agonistic effects in decreasing IGFBP-3 mRNA and protein, consistent with their stimulatory effects on IGF-I and IGFBP-2; however, whether the downregulation of IGFBP-3 expression by androgen was mediated through AR remains to be determined. Moreover, the effects of flutamide or Casodex treatment on IGFBP-3 expression paralleled their effects on cell growth, suggesting that IGFBP-3 expression was closely linked to stromal cell growth. The agonistic effect of Casodex on IGFBP-2 mRNA expression in this study was consistent...
with reported Casodex effects on AR harboring mutation at position 741 (22); however, it is unlikely that the stromal cells used in these experiments contain an AR with the same mutation because both flutamide and Casodex were able to block the DHT-induced IGF-I mRNA expression. Whether differences in androgen-responsive units in IGF-I and IGFBP-2 promoter sequences account for some of the differential effects of Casodex on these genes remains to be elucidated.

DHEA and E2 did not significantly affect IGF axis gene and protein expression in stromal cells in this study, in contrast to their recently reported actions on human LNCaP prostate cancer cells (2), wherein they increased cell proliferation, Prostate-specific antigen production, and modulation of the IGF axis, albeit with effects that were reduced and delayed compared with those elicited by DHT and T. The responses to DHEA and E2 in LNCaP cells may have been due in part to the known mutation in the LNCaP cell AR (T877A), which allows for binding of DHEA and E2 and AR transactivation (8, 52). Additionally, DHEA and E2 effects in LNCaP cells may have resulted from cellular metabolism of DHEA to more potent androgenic or estrogenic metabolites, with consequent binding to AR or estrogen receptor-β (4, 19, 29). Prostate stromal cells possess a wild-type AR that is not activated directly by DHEA or E2, but they do harbor the steroidogenic enzymes hydroxysteroid dehydrogenases 3β-HSD, 17β-HSD type 5, and 5α-reductase, which allow for metabolism of DHEA to T and DHT (3, 13). Further studies are warranted to determine whether, and to what extent, DHEA is metabolized by prostate stromal cells.

Of interest are the noted variations in degree of response among the different lots of primary stromal cells. DHT administration consistently modulated IGF-I, IGFBP-2, and IGFBP-3 gene and protein expression only in 6S and 9S stromal cell cultures, with no apparent effects in 5S cultures. All stromal cultures expressed similar levels of AR protein; therefore, the differences in responsivity of the IGF system to DHT observed in 6S and 9S vs. 5S stromal cultures do not appear to be explained by alterations in AR expression. Cytokeratin 18 expression was minimal to nonexistent in the stromal cell cultures we examined, suggesting that epithelial contamination was very low, and did not explain the observed difference in androgen sensitivity.

Prostatic stroma can be characterized by three different types of cells based on the stromal cell differentiation markers vimentin, SMA, and desmin. Expression of vimentin without additional smooth muscle markers suggested a fibroblast-like phenotype (47). Coexpression of vimentin and SMA, without that of late-stage smooth muscle markers, such as desmin, calponin, or myosin, was consistent with a myofibroblast phenotype (46), whereas expression of vimentin, SMA, and late-stage smooth muscle marker was characteristic of a smooth muscle phenotype (47). On the basis of the expression of these markers as detected by Western blots, the 6S cultures could be classified primarily as fibroblasts; 9S and 12S cultures could be classified as a mix of fibroblasts and myofibroblasts with little or no smooth muscle cells; and 5S and PRSC cultures would likely be made up of smooth muscle cells, myofibroblasts, and possibly some fibroblasts.

Our data suggest that androgenic modulation of IGF-I, IGFBP-2, and IGFBP-3 occurs mainly in stromal cells with a phenotype characteristic of fibroblastic or myofibroblastic cells. Neither DHT, T, DHEA, nor E2 significantly affected IGF-I receptor, IGF-II, or IGFBP-5 mRNA levels or stromal cell proliferation. Previous studies have reported a 30–50% increase in cell growth after T and DHT administration to prostate stromal cells derived from BPH tissue (24, 28); however, the effects of androgens or estrogens on cell growth of stromal cells derived from normal or malignant prostate tissue have not been clearly defined.

Prostate adenocarcinoma derives from cancer of the epithelial cells, and the pivotal role(s) of stromal cells in prostate carcinogenesis via stromal-epithelial interaction is well accepted (9, 37, 45). Several growth factor systems have been implicated as the mediators of prostate stromal-epithelial interactions, including epidermal growth factor, transforming growth factor-α and -β, fibroblast growth factor family, vascular endothelial growth factor, nerve growth factor, platelet-derived growth factor, hepatocyte growth factor, and IGFs (50). IGF-I has been demonstrated to be an important factor in the initiation and progression of primary prostate cancer in the transgenic adenocarcinoma of mouse prostate (TRAMP) mouse and noble rat models, in response to treatment with T and E2 (27, 48). IGFBP-3 induces apoptosis, both by decreasing the bioavailability of IGF-I and by IGF-independent mechanisms (15, 34). IGFBP-2 was overexpressed in the setting of prostate intraepithelial neoplasia, and overexpression of IGFBP-2 has been associated with a malignant invasive phenotype (43). Additionally, IGFBP-2 was shown to be overexpressed and to promote cell invasion in ovarian cancer (33).

In the current study, we found that DHT, which is important for prostate cancer cell survival, elicits dose- and time-dependent changes in gene and/or protein expression of IGF-I, IGFBP-2, and IGFBP-3 in primary cultures of human prostatic stromal cells and that modulation of the IGF axis varies with stromal cell phenotype. Further studies are warranted in a larger number of different primary stromal cell cultures derived from normal, prostatic, and neoplastic prostate glands to determine the exact relationship of stromal cell phenotype in promoting DHT-induced IGF expression. The current findings suggest that DHT and T promote prostate cancer growth in part via modulation of the stromal cell IGF axis, with consequent
paracrine effects on prostate cancer epithelial cells. Taken together with recent findings in prostate cancer epithelial cells (2), these data provide a basis for further studies investigating stromal and epithelial cell communications in response to these hormones.

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REFERENCES


