Androgen-induced Inhibition of Cell Proliferation in an Androgen-insensitive Prostate Cancer Cell Line (PC-3) Transfected with a Human Androgen Receptor Complementary DNA

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ABSTRACT

A full length human androgen receptor complementary DNA was introduced into androgen receptor-negative PC-3 cells to determine if androgen sensitivity could be established in this cell line and to assess what influence, if any, androgen exposure would have on the growth of these cells. The androgen receptor complementary DNA was inserted into pSG5 in the region controlled by the SV40 promoter. This construct was cotransfected with pSRneo into PC-3 cells and stably transfected cells were selected and screened for the expression of the androgen receptor. Active expression of the receptor was demonstrated by Western blotting using a rabbit anti-androgen receptor antiserum and by [3H]methyltrienolone binding to cytosol extracts. Saturation ligand-binding analysis revealed the presence of a single class, high affinity (Kd = 0.122 nM) androgen-binding site in cytosol extracts of transfected cells but not in extracts from mock-transfected cells. In cells expressing the transfected androgen receptor, androgen decreased the proliferation rate and cloning efficiency and induced a more differentiated phenotype. These results demonstrate that PC-3 cells have retained the mechanisms required to respond to the activated androgen receptor and that the loss of androgen sensitivity in these cells is due to the lack of functional androgen receptor. This also provides a technique for determining whether androgen-resistant tumor cells contain functional androgen receptors or whether androgen sensitivity is due to abnormalities in downstream signaling pathways. The apparent androgen-induced decreased malignant state of these transfected cells suggests new directions for the treatment of prostate cancer.

INTRODUCTION

Prostate cancer is one of the leading causes of cancer-related deaths in North American males and continues to rise in incidence (1, 2). Early stage disease is treated by eliminating the actions of endogenous androgens, either by the administration of antiandrogens or by castration (3). Nevertheless, survival benefit with this treatment has yet to be definitively demonstrated (4). Unfortunately, most patients experience disease progression due to the development of an androgen-independent tumor (5).

The actions of androgens on target cells are initiated by the binding of steroid to a high-affinity intracellular protein or receptor. Upon binding steroid, the receptor undergoes transformation, resulting in the exposure of a DNA-binding region on the receptor molecule. The receptor-ligand complex is then able to interact with specific steroid response elements located on the chromatin, thereby initiating changes in the transcription rate of a number of specific genes. This eventually leads to the growth or differentiation of the target cell, dependent upon the differentiated state of the cell or on ancillary signals delivered through other receptors (6, 7). The emergence of androgen-independent neoplastic prostate tumors represents the proliferation of cells that do not require androgen stimulation for growth, with the majority of cells also exhibiting complete androgen unresponsiveness (5). The progression of the tumor to this state is not fully understood; however, in at least some cases, androgen independence has been associated with a lack or mutation of the androgen receptor (8–10).

It is unknown if cells forming the androgen-independent tumor stem from cells present in the primary tumor that were never responsive to androgen, that were once responsive to androgen and subsequently lost their ability to respond, or that have an altered androgen response. Three human prostate cancer cell lines have been established that do not depend on androgen for growth. Two of these lines, PC-3 and DU-145, are thought to lack expression of the androgen receptor and are completely unresponsive to androgen (10, 11). The third cell line, LNCaP, possesses a point mutation in the ligand-binding domain of the receptor gene (8, 9). As a result of this mutation, which reduces the ability of the receptor to discriminate between ligands, these cells respond with increased growth to a variety of steroids, including both androgens and antiandrogens (8, 9, 12, 13). Thus, unlike PC-3 and DU-145 cells, LNCaP cells are responsive to androgen.

In the present study, we examined the effect of introducing the functional androgen receptor into PC-3 cells to determine if androgen responsiveness could be established in this cell line and to assess what effect, if any, androgen exposure would have on the growth of these cells. Previous studies indicate that the androgen receptor is not expressed in PC-3 cells, despite the absence of major rearrangements of the receptor gene (11). Moreover, we have found that there are no mutations of the ligand-binding domain (exons E to H) of the androgen receptor gene in these cells, indicating that androgen binding assays should detect the receptor if present.

As reported here, PC-3 cells transfected with the full length human androgen receptor cDNA [PC-3(T)] express significant amounts of the receptor and are androgen responsive as demonstrated by androgen-induced changes in cell morphology and proliferation. Surprisingly, exposure of these transfected cells to androgen resulted in decreased cell proliferation and a marked decrease in characteristics associated with the malignant state. These findings may have important implications for new directions in the future treatment of both androgen-dependent and -independent prostate cancer.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Reagents. PC-3 (10) and LNCaP (13) cell lines were obtained from American Type Culture Collection (Rockville, MD).

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Plasmid pSG5 was obtained from Stratagene (La Jolla, CA), and pMSG-CAT was obtained from Pharmacia P-L Biochemicals (Piscataway, NJ). 5α-DHT and triamcinolone acetonide were purchased from Sigma Chemical Co. (St. Louis, MO). [17α-Methyl-3H]methyltrienolone (R1818; specific activity, 87.0Ci/mmol) and radioinert R1818 were purchased from DuPont Canada (Mississauga, Ontario, Canada). FBS was purchased from Gibco-BRL (Burlington, Ontario, Canada) and stripped of steroids by charcoal adsorption. Briefly, dextran-coated charcoal was prepared by combining 2.0 g Norit A charcoal (dextran-coated) and stripped of steroids by charcoal adsorption. Briefly, dextran-coated charcoal was prepared by combining 2.0 g Norit A charcoal (dextran-coated) and stripped of steroids by charcoal adsorption.

**Construction of the Expression Plasmid, pSGAR.** The full length human androgen receptor cDNA clone, kindly provided by S. Liao (14), was digested with EcoRI and HindIII or with HindIII and BamHI resulting in a 2.3- and 1.3-kilobase fragment, respectively. Plasmid pSG5 was digested with EcoRI and BamHI, and the linearized plasmid DNA was ligated with the 2.3-kilobase EcoRI-HindIII and 1.3-kilobase HindIII-BamHI fragments. Proper ligation was confirmed by restriction mapping and reactivity with rabbit anti-androgen antibodies following expression (see below). The resultant construct contains the full length human androgen receptor cDNA (3.6 kilobases) under the control of the SV40 early promoter (Fig. 1).

**Transfection of PC-3 Cells.** PC-3 cells were transfected and assayed for both transient and stable expression of the androgen receptor. For transient expression, cells were transfected with plasmid by the calcium phosphate method (20 μg of each plasmid DNA/plate) (15). After transfection, the cells were exposed to glycerol shock (3.5 min) and then incubated overnight in RPMI 1640 supplemented with 10% FBS. The next morning, DHT was added to the incubation medium to a final concentration of 2.0 μg/ml (6.8 μM). The incubation was allowed to continue for an additional 48 h before harvesting the cells for CAT assay (see below). For stable transfectants, PC-3 cells were cotransfected with pSGAR and pSRneo (16) at a molar ratio of 10:1 (20 and 2 μg, respectively). The transfected cells were then incubated for 24 h in RPMI 1640 supplemented with 10% FBS. The cells were then incubated for 2 weeks in the same medium supplemented with Genticin (G418) to a final concentration of 800 μg/ml. The resulting G418-resistant cells were plated in 96-well plates at limiting dilution. Selected resistant single colonies were then examined for the expression of the androgen receptor by Western blotting and by ligand binding assay (see below).

**CAT Assay.** Transiently transfected PC-3 cells were harvested, washed twice with ice-cold PBS and resuspended in 75 ml 0.25 μTriton X-100 buffer (pH 7.5). The cells were lysed by sonication of the cell suspension 3 × 30 s. Distilled water (75 μl) was added to the suspension, and the mixture was sonicated for another 3 × 30 s. The suspension was then centrifuged for 20 min at 12,000 g, the resulting supernatant (cell lysate) was decanted and heated at 67°C for 10 min. For each assay, 15 μl cell lysate, 15 μl 1.0 μTriton X-100 buffer (pH 7.5), 2 μl 14C-acety-CoA (Amersham Co., Arlington Heights, IL), 50 μl chloramphenicol (34 mg/ml), and 85 μl distilled water were combined in scintillation vials and mixed well. Five ml of nonaqueous scintillation fluid (Beta-Max; ICN Biomedicals, Inc; Huntsville, AL) were added to the reaction mixture and radioactivity was determined in a Beckman SL1801 liquid scintillation spectrophotometer (17). Cpm were recorded at half-hour intervals until a plateau was established. Results are expressed as cpm/106 cells (final cell count) with baseline counts (determined at time = 0) subtracted.

**Rabbit Anti-Human Androgen Receptor Antibodies.** An 820-bp-base pair Prl fragment and a 720-bp-base pair SacI fragment were purified from the human androgen receptor cDNA and were subcloned into pATH11 and pATH10 vectors, respectively, and then transformed into the Escherichia coli strain, AG1. The corresponding fusion proteins of Mr, 67,000 and M, 62,000 were purified by SDS-polyacrylamide gel electrophoresis. Rabbits were given injections of approximately 100 μg of the fusion protein in complete Freund's adjuvant and were then boosted every 5–6 weeks with 100 μg fusion protein in incomplete Freund's adjuvant. Two antiseras were generated: PAR-I, raised against trpE-PrI fragment fusion protein; and PAR-II, raised against trpE-SacI fragment fusion protein. Both antiseras reacted with androgen receptor-positive LNCaP cells, but not with androgen receptor negative PC-3 cells, in Western blots.

**Western Blotting.** Cells at 70–80% confluence were washed twice with ice-cold PBS, counted, and lysed with lysis buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 7 M urea) at a concentration of 107 cells/ml buffer. The sample was boiled for 10 min and then subjected to 5–7 passages through a 25-gauge needle. The samples were centrifuged at 12,000 g for 10 min and the precipitates were discarded. Fifty μl of the supernatant was then loaded onto a 7.5% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane at 30 V overnight at 4°C. The membrane was stained in Ponceau S solution (0.1% Ponceau S in 1% acetic acid) to establish equal loading and then blocked in TBS buffer (1 × PBS plus 0.02% Tween-20) containing 5% skim milk powder for 3–4 h. The membrane was probed with either the PAR-I or PAR-II anti-human androgen receptor antiserum. The blot was washed three times in TBS buffer and then incubated for 2 h with secondary antibody (alkaline phosphatase conjugated donkey anti-rabbit IgG antibodies purchased from Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in TBS containing 5% skim milk. The blot was then washed three times in TBS and incubated in reaction solution for 15–30 min until an optimal exposure was achieved. This solution contained 10 ml AP buffer [100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM MgCl2], 66 μl 5% nitroblue tetrazolium, and 33 μl 5% bromochloroindolyl...
phosphate. The blot was then washed again for 10-15 min in TBS buffer containing 0.1 M EDTA. Molecular weight markers were purchased from Gibco-BRL.

Saturation Ligand Binding. Transfected and mock-transfected PC-3 cells were grown in RPMI 1640 supplemented with 10% stripped fetal bovine serum. Confluent cells were harvested and washed with cold buffer TEGTMo (10 mM Tris, 1.5 mM disodium EDTA, 10% (v/v) glycerol, 12 mM monothioglycerol, 25 mM sodium molybdate (pH 7.4)). All subsequent procedures were conducted at 4°C. Cells were resuspended in 1.5 ml TEGTMo, homogenized using a Teflon pestle, and centrifuged at 104,000 g for 45 min. Aliquots (75 µl) of the resulting supernatant (cytosol) were incubated for 24 h at 4°C with 50 µl TEGTMo containing a range of [3H]R1881 concentrations. Parallel incubations were conducted in the presence of 1.0 µM DHT to determine nonspecific binding at each [3H]R1881 concentration. All incubates contained 1.0 µM triamcinolone acetonide to suppress binding of [3H]R1881 to the progestin receptor (18). Bound [3H]R1881 was separated from unbound by gel filtration on 7 × 32-mm Sephadex LH-20 columns at 4°C (19). Aliquots of incubate (100 µl) were loaded onto the columns and washed into the column bed with 100 µl TEGTMo. Thirty min after sample application, the macromolecular fraction containing the bound [3H]R1881 was eluted into scintillation vials with 400 µl TEGTMo. After overnight extraction into 5 ml Betacount (ICN) liquid scintillation fluid, radioactivity was quantified at 50% efficiency. A small aliquot (20 µl) of residual incubation mixture was taken at the end of the incubation period to determine the actual [3H]R1881 concentration. The range of concentrations was 0.07-6.0 nM. Cytosol protein content was determined by the dye-binding method of Bradford (20) with bovine serum albumin used as standard. Specific binding was calculated as the difference between total binding (measured in the absence of DHT) and nonspecific binding (measured in the presence of DHT). The resulting data were analyzed by the method of Scatchard using a computer-assisted nonlinear curve fitting method adapted to an IBM-PC microcomputer (21).

Cell Proliferation Rate. Cells (8 × 10⁴) were plated in 2.0 ml of RPMI 1640 supplemented with 10% stripped FBS in 24-well plates. Where indicated, DHT was added to give a final concentration of 2 µg/ml medium (6.8 µM). At 4 h and at the indicated times, cells were trypsinized and counted. The number of cells present in each of the plates after 4 h of incubation was determined to establish that an equal number of cells were present in all wells (∼10,000). All values represent the mean number of cells ± SEM of three replications.

Colony Assay. Cells grown to 80-90% confluence were harvested and washed with serum-free RPMI 1640. The cells were resuspended in medium to achieve 10,000 cells/ml. To this volume, 2.3 ml methylcellulose and 1.0 ml stripped FBS were added giving a final volume of 5.0 ml. Where indicated, DHT was added to give a final concentration of 6.8 µM. After incubation at 37°C for 30-50 min, 1 ml of sample was transferred to 30-mm tissue culture plates and incubated for 10 days. The number of colonies formed was then calculated with the criterion that a colony consisted of 40 or more cells. Assays were performed in quadruplicate for each sample.

Apoptosis Assay. PC-3 and PC-3(T) cells were grown in RPMI 1640 supplemented with 10% stripped FBS for 7 days in the presence of DHT (6.8 µM). The cells were then split and divided into three treatment groups: RPMI 1640 + 10% FBS alone; + DHT; + ionomycin; or + combined DHT and ionomycin. Cells assigned DHT treatment were placed in medium (RPMI 1640 + 10% stripped FBS) containing 2 µg DHT/ml while cells assigned to ionomycin were placed in medium without DHT. After 48 h of incubation, ionomycin treatment was administered to the designated cells and the incubation was continued overnight. The final concentration of ionomycin used was 2 µg/ml. Cells were then harvested, washed twice with PBS, and lysed by adding 200 µl lysis buffer [5 mM guanidine thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, and 20 mM dithiothreitol (pH 7.0)]/5 × 10⁴ cells. Cellular DNA was precipitated with ethyl alcohol and electrophoresed on 2.5% agarose gel. CTLI-2 cells undergoing apoptosis were used as a positive control.

RESULTS

Androgen Responsiveness of Transfected PC-3 Cells. To first determine if PC-3 cells could express the androgen receptor following transfection with the cDNA sequence, PC-3 cells were cotransfected with pSGAR, which contained the full length human androgen receptor CDNA, and pMSG-CAT, which contained the CAT reporter gene transcribed from mouse mammary tumor virus long terminal repeat. This reporter gene is steroid hormone responsive in the presence of a functional steroid hormone receptor (22). The cotransfectants were grown in RPMI 1640 containing 10% stripped FBS in the presence or absence of 2 µg DHT/ml (6.8 µM). Forty-eight h after the addition of DHT, cells were harvested and assayed for CAT activity. For control comparisons, mock-transfected PC-3 cells (CK) and PC-3 cells transfected with pMSG-CAT alone, or cotransfected with pSG5CAT and pSG5 or pSR,neo, were included.

In the absence of DHT, CAT activity of the pSGAR-pMSG-CAT cotransfectant was not different from that of the control transfecnts. However, with DHT exposure, CAT activity in the pSGAR-pMSG-CAT cotransfectant was increased more than 4-fold when compared to transfecnts that lacked the pSGAR construct (Fig. 2). These results demonstrated that PC-3 cells transfected with the pSGAR construct express the androgen receptor in sufficient quantities to stimulate the expression of a reporter gene.

Expression of the Androgen Receptor in Stable Transfectants. Stably transfected PC-3 cells were obtained by cotransfecting cells with pSGAR and pSR,neo. Neomycin-resistant cells were selected by incubation with 800 µg G418/ml and the resulting stable neomycin-resistant clones were selected for further study. As indicated by Western blotting with rabbit anti-human androgen receptor polyclonal antibodies (Fig. 3, PAR-I; PAR-II, not shown), a protein band with a molecular weight of 110,000 was present in the transfected cells (Fig. 3, Lane 2). This protein was the same size as the androgen receptor from LNCaP cells (Fig. 3, Lane 1) and was absent in ordinary PC-3 cells (Fig. 3, Lane 3). The level of the androgen receptor expressed in transfected PC-3 cells was apparently lower than that expressed in LNCaP cells as indicated by the intensity of the protein band.

Saturation Binding Analysis. The expression of an androgen receptor capable of binding androgens was demonstrated in stably transfected PC-3 cells by a binding assay using the synthetic androgen [3H]R1881 as ligand. PC-3(T) and PC-3 cells grown in the absence of androgen stimulation (RPMI 1640 + 10% stripped FBS) were homogenized and cytosol extracts were obtained. In the absence of bound ligand, steroid hormone receptors are recovered in the cytosol fraction of homogenized tissues or cells. Cytosols were incubated at 4°C for 24 h with a range of [3H]R1881 concentrations. Because R1881 also binds to the progestin receptor, triamcinolone acetonide was added to all incubates to eliminate possible detection of the
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Fig. 3. Expression of the androgen receptor in stable PC-3 transfectants as evidenced by Western blotting. LNCaP (Lane 1), PC-3(T) (Lane 2) and mock-transfected PC-3 (Lane 3) cell lysates were electrophoresed in 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked and probed with rabbit anti-human androgen receptor serum (PAR-1; 1:1000 dilution) in TBS buffer containing 5% skim milk powder. The band representing the androgen receptor was detected at M, 110,000. KD, molecular weight in thousands.

Progestin receptor (18). To assess nonspecific binding, parallel incubations with an excess of radioinert R1881 were included.

A single class high affinity androgen binding component was present in PC-3(T) cells (Fig 4, B and C). In contrast, no specific [3H]R1881 binding was detected in cytosol extracts of mock-transfected PC-3 cells (Fig. 4A; note the differences in Y axis scales between A and B). The apparent Kd value obtained for the androgen-binding site present in PC-3(T) cytosol extract was 0.122 nM, which is comparable with values reported for normal and malignant human prostate tissue (0.46 and 0.50 nM, respectively; Ref. 23).

Morphological Changes in Transfected PC-3 Cells. The morphology of the stable-transfected PC-3(T) cells was dramatically altered as compared with mock-transfected PC-3 cells (Fig. 5, A and C). Transfected cells were longer and more epithelial-like in appearance than the mock-transfected cells. Following DHT treatment, these changes were more marked, with longer cell processes being formed (Fig. 5, C and D). As expected, DHT treatment of mock-transfected cells was without any noticeable effect (Fig. 5, A and B).

Changes in Proliferation Rate. The effect of androgen exposure on the proliferation rate of PC-3(T) cells was examined by placing PC-3(T) cells in medium containing 2 μg DHT/ml (6.8 μM) or in steroid-free medium. Mock-transfected PC-3 cells were run in parallel as a control. At 4, 48, and 72 h of incubation, cells were trypsinized and counted to assess cell proliferation. The 4-h time point was included to confirm that an equal number of cells was present in the wells at the beginning of the experiment. In the absence of androgen,
PC-3(T) cells were found to proliferate slightly slower than the mock-transfected cells (Fig. 6). DHT treatment of mock-transfected cells did not influence the growth rate; however, in PC-3(T) cells, DHT treatment resulted in a more than 50% decrease in cell proliferation at 72 h of incubation. To assess the effect of different concentrations of DHT on PC-3(T) cell proliferation, PC-3(T) cells were plated (20,000 cells/well in 24-well plates) in medium containing a range of DHT concentrations (0.1 nM to 10 μM). Cells were trypsinized and counted after 48 or 72 h of incubation. Only slight differences in cell number were present after 48 h of incubation with DHT; however, the presence of DHT at concentrations as low as 0.1 nM was found to inhibit cell proliferation by more than 40% after 72 h of incubation (Fig. 7).

Changes in Clonogenicity. Original PC-3 cells are anchorage independent and can form colonies in semisolid methylcellulose (10). To determine if colony-forming ability is altered in PC-3(T) cells exposed to androgen, PC-3 and PC-3(T) cells were incubated with methylcellulose in steroid-free medium or in medium containing 6.8 μM DHT. In the absence of DHT, PC-3(T) cells showed essentially the same level of colony formation as did mock-transfected cells. Exposure to DHT did not influence the ability of mock-transfected PC-3 cells to form colonies; however, markedly fewer PC-3(T) cell colonies were formed in the presence of DHT (Fig. 8). Thus in both anchorage-dependent and independent assays, PC-3(T) cells demonstrated a marked growth decrease in the presence of exogenous androgen.

Apoptosis Assay. In ordinary androgen-sensitive prostate cancer cells, exposure to DHT results in a marked increase in cell proliferation whereas removal of the DHT results in decreased proliferation and programmed cell death or apoptosis (24–26). Apoptosis results from DNA fragmentation caused by the activation of calcium-depen-
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Fig. 7. Effect of different DHT concentrations on the proliferation of PC-3(T) cells. PC-3(T) cells (20 × 10⁴) were grown in RPMI-1640 medium + 10% stripped FBS in 24-well plates in the presence or absence of DHT in concentrations ranging from 0.1 nm to 10 μM. At 48 and 72 h of incubation with DHT, cells were trypsinized and counted. Bars, mean ± SEM of 3 observations/group.

Fig. 8. Effect of DHT on the colony formation of PC-3(T) cells. PC-3(T) and mock-transfected PC-3 cells (10,000) were incubated at 37°C for 30-50 min with RPMI 1640, stripped FBS, and methylcellulose in the presence or absence of 6.8 μM DHT. Cells were then transferred to 30-mm culture plates and allowed to incubate for 10 days. The number of colonies formed that consisted of 40 or more cells was then determined. Bars, mean ± SEM of 4 observations/group.

Fig. 9. Lack of androgen-induced apoptosis in PC-3(T) cells exposed to DHT. PC-3(T) cells grown in interleukin 2-free medium were included. In these cells, depletion of interleukin 2 triggers the apoptosis process (29). As shown in Fig. 9, Lane C, a distinct DNA ladder was observed in these cells. These results indicate that the inhibition of cell proliferation in PC-3(T) cells was not attributable to androgen-induced programmed cell death.

DISCUSSION

A major challenge for the successful treatment of androgen-responsive neoplastic prostate tumors is to arrest or prevent the progression of androgen-independent as well as androgen-dependent neoplastic growth. An improved understanding of the cell types present in these tumors and possible hormonal regulation of growth of these cells might reveal important new directions for the treatment of prostate cancer. In the present study, we have examined the effect of conferring androgen sensitivity to an androgen-insensitive human prostate cancer cell line by the introduction of a full length human androgen receptor cDNA.

Imortalized steroid-sensitive cancer cells, including LNCaP cells, as well as normal androgen receptor-containing prostate cells typically respond to steroid hormone treatment with increased cell proliferation (10, 12). Our results show that the opposite effect occurs in androgen receptor-negative prostate cancer cells that are given the ability to recognize androgen. The proliferation of PC-3(T) cells was found to decrease by more than 50% after 72 h of incubation with exogenous androgen. Similarly, the ability of PC-3(T) cells to form colonies was decreased by exogenous androgen.

Kushner et al. (30) have reported that in estrogen receptor-transfected hamster ovarian cells that overexpress the estrogen receptor, estrogen treatment induces cell lysis, presumably by inducing apoptosis. Our data indicate that a different mechanism was responsible for the decreased proliferation of PC-3(T) cells. The androgen receptor gene transfected into PC-3 cells in this study was not overexpressed despite being controlled by the SV40 promoter. Western blot analysis indicated that the amount of receptor produced in these cells was lower than that produced by androgen-responsive LNCaP cells. Similarly, ligand saturation binding analysis indicated a level of approximately 80 fmol of receptor/mg protein, which is lower than that reported for PC-82 human prostate androgen-dependent cells (120 fmol/mg protein) (31) and higher than that reported for human ventral prostate tissue (26 fmol/mg protein) (23). The low level of androgen receptor expression in PC-3(T) cells may be a consequence of selection against transfectant cell lines expressing high levels of androgen receptor by growth inhibition even in the absence of exogenous androgen. Moreover, the results obtained in our apoptosis experiment indicate that the inhibitory effect of DHT on PC-3(T) cell growth cannot be attributed to androgen-directed programmed cell death.

Even in the absence of exogenous DHT exposure, PC-3(T) cells grew slightly slower than mock-transfected PC-3 cells. Although we used stripped FBS in our culture medium, it remains possible that the presence of the androgen receptor might have allowed PC-3(T) cells to respond to low levels of androgen remaining after charcoal stripping. Marked effects on PC-3(T) cell proliferation were apparent at
DHT concentrations as low as 0.1 nM. The presence of residual androgen following charcoal stripping might also explain the morphological distinctions observed between PC-3(T) cells and mock-transfected PC-3 cells. Transfected cells were more elongated in shape with cellular processes present. These properties were increased with DHT treatment only in the transfected cell line, suggesting that DHT exposure enhanced a differentiated state in these cells.

The androgen-induced changes in morphology, growth, and colony formation all suggest that establishing androgen responsiveness in PC-3 cells results in a decreased malignant state of the cell. These data are consistent with those obtained in similar studies on estrogen-regulated growth of estrogen-insensitive breast cancer cells. Jiang and Jordan (32) recently introduced the human estrogen receptor gene and selected mutations into estrogen-receptor negative breast cancer cells and noted the effect of estrogen on cell growth. As with our transfected cells, exposure to steroid resulted in a marked decrease in proliferation. The extension of these findings to a human prostate cancer cell line raises the possibility that this may be a general property of steroid-insensitive neoplastic cells found in steroid target organs.

At present, the mechanism underlying the progression of steroid-independent neoplastic growth in patients undergoing androgen withdrawal is unknown. Steroid-insensitive cells may represent a subset of cells derived from the primary tumor that have lost their sensitivity to steroid or that have never expressed the steroid receptor and thrive once the steroid responsive cells are inhibited. In any event, the data from the present study and that by Jiang and Jordan (32) indicate that these cells may possess functional steroid receptor elements for active steroid receptor recognition. Clearly, in our study, different DNA sequences are being regulated or are regulated in a different manner in PC-3(T) cells than in ordinary androgen receptor-positive prostate cancer cells (33). Further comparisons of these cells are required to identify factors contributing to the differences in steroid-influenced cell growth. One possible factor is the EGF receptor. Androgen exposure is known to increase EGF receptors in LNCaP cells, and this increased sensitivity to EGF may be a crucial step in the mitogenic action of androgens on these cells (34). Recent work by Sehgal et al. (35) indicates that androgen exposure also increases the amount of amphiregulin, a high affinity EGF receptor agonist, produced in LNCaP cells. Thus, androgen may increase the autocrine action of EGF by increasing the sensitivity of the cell to EGF while also increasing the production of the ligand for the receptor. It would be of interest to determine what effect androgen exposure has on these parameters in PC-3(T) cells.

It is tempting to speculate on the impact these findings may have on the future direction of prostatic cancer treatment. Current treatment of early stage neoplastic prostate growth which involves the removal or inhibition of androgen action often results in a limited response. This treatment is effective in halting the proliferation of cells that are androgen dependent. However, due to the heterogeneity of cell types present in the tumor, cells that lack the androgen receptor, exemplified by PC-3 and DU-145 cells, as well as cells that contain the androgen receptor but are not dependent upon androgen for growth, exemplified by LNCaP cells, can continue to proliferate. If an additional cell type is present, one similar to the PC-3(T) cell, then androgen removal or androgen receptor blockade could actually enhance the proliferation of this cell type in the tumor. The results presented in this study also suggest that insertion of an active form of the androgen receptor into androgen receptor-negative prostate cancer cells might endogenous androgens to retard tumor progression. Further investigation with PC-3(T) cells used as a model system should reveal why these cells respond to androgen with slowed growth and may shape future directions for prostate cancer treatment.

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