Androgen Receptor Gene Expression in Human Prostate Carcinoma Cell Lines

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ABSTRACT

Responses to androgen vary widely among prostate cancers and prostatic carcinoma cell lines. We have explored the basis for this heterogeneity by examining the levels of androgen receptor expression in a prostatic carcinoma cell line (LNCaP) that expresses the androgen receptor and two prostate carcinoma cell lines that do not contain detectable androgen receptor. We find that while the LNCaP cell line contains high levels of both the androgen receptor protein and mRNA, the receptor-negative cell lines DU-145 and PC-3 do not express androgen receptor protein as detected by immunoblotting or mRNA as detected by Northern analysis or S1 nuclease protection. These results indicate that the absence of androgen receptor expression in the androgen receptor-negative cell lines is caused by diminished androgen receptor mRNA levels. Genomic Southern analysis indicates that the differences in androgen receptor expression in each of these cell lines is not associated with detectable alterations in the structure of the androgen receptor gene.

INTRODUCTION

Many forms of prostate cancer are initially androgen dependent, but the response to androgen ablation is often transient (1). The mechanism of the transition from androgen-dependent to androgen-independent growth has been the subject of much interest. Studies of animal models of prostate carcinoma, particularly the Dunning rat prostatic adenocarcinoma have suggested that relapse following androgen ablation is due to the outgrowth of a population of androgen-independent carcinoma cells (2,3). The origin of these androgen-independent cells remains to be clarified. In the current studies, we sought to examine the nature of androgen responsiveness and androgen resistance by determining the nature and level of androgen receptor ligand binding, protein expression, and mRNA expression in the prostate carcinoma cell lines, LNCaP, PC-3, and DU-145.

MATERIALS AND METHODS

Cell Culture. The LNCaP (4), PC-3 (5), and DU-145 (6) cell lines were obtained from the American Type Culture Collection. The LNCaP cell line was maintained in RPMI containing 10% fetal bovine serum. The PC-3 cell line was cultured in HAM's nutrient mixture F-12 containing 7% fetal bovine serum. The DU-145 cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. In one experiment, LNCaP cells were exposed to the effects of exogenous steroids. In this experiment, the medium was removed 5 days after plating and replaced with medium containing 5% charcoal-stripped fetal bovine serum and either no added steroid, 10^{-4} M estradiol, or 10^{-4} M 5α-dihydrotestosterone. The incubations were continued for an additional 48 h, the cells were harvested, and RNA was isolated as described below.

DNA Preparations. Confluent monolayers of cells were scraped into ice cold PBS. The cell pellets were processed using a modification of a standard technique (7) as described previously (8). The genomic DNA preparations were dialyzed against 10 mM Tris-1 mM EDTA, pH 7.5.

RNA Preparations. Confluent cultures of cells were scraped into PBS. The cell pellets were solubilized in 10 volumes of guanidinium isothiocyanate and purified by centrifugation over a cushion of 5.7 M CsCl (9).

Radioligand Binding Assays. A whole cell monolayer binding assay similar to that described previously (10) was used to assay for specific 5α-dihydrotestosterone binding. Cultures of cells were grown to near confluence in 3-cm plates. Twenty-four h prior to performing the ligand binding assays, the medium was replaced with minimal essential medium containing 10% bovine serum albumin. To assay the level of specific 5α-dihydrotestosterone binding, the medium was removed and replaced with minimal essential medium containing 10% bovine serum albumin and [1,2,3,7,8,9-3H]-5α-dihydrotestosterone at concentrations ranging from 0.1 to 5.0 nM, in either the presence (i.e., nonspecific binding) or absence (i.e., total binding) of a 200-fold excess of unlabelled 5α-dihydrotestosterone. Specific 5α-dihydrotestosterone binding was calculated by subtraction of nonspecific from total binding. After incubation at 37°C for 1 h, the medium was aspirated, and the monolayers were washed twice with 5 ml of Tris-saline. The cells were dissociated by trypsinization and washed twice with 5 ml of ice cold Tris-saline. The cell pellets were resuspended in 1 ml of deionized water and disrupted by sonication. The quantity of cell-associated radioactivity was determined by scintillation counting. Cells grown in parallel and treated in an identical manner were solubilized and assayed for protein (11). The ligand binding data were analyzed according to the method of Scatchard (12).

Northern Analysis. RNA samples were denatured in buffer containing 2.2 M formaldehyde and 50% formamide, loaded onto 1% agarose gels, cast, and run in buffer containing 2.2 M formaldehyde (13). After running, the gels were washed briefly in 50 mM NaOH and 10 mM NaCl and transferred to Zetaprobe nylon membrane (Bio-Rad, Richmond, CA) by capillary blotting. Following transfer, the RNA was covalently cross-linked to the membrane with UV light (Stratallinker, Stratagene, San Diego, CA). The membranes were prehybridized for 2 h in 10 mM EDTA-0.5 M NaH2PO4 (pH 7.2) and 7% SDS at 68°C. Hybridization was performed in the same buffer containing probe labeled with [α-32P]dCTP using the random hexamer method of Feinberg and Vogelstein (14). After hybridization, the membranes were washed at 60°C in 40 mM NaH2PO4 (pH 7.2), 1 mM EDTA, and SDS (5% SDS in the first wash and 1% SDS in the final wash).

Southern Analysis. Samples of genomic DNA were digested with the restriction endonuclease EcoRI, electrophoresed on 0.8% agarose gels, and transferred and cross-linked to a nylon Zetaprobe membrane. Following prehybridization at 68°C for 2 h in 7% SDS, 1 mM EDTA, and 0.5 M NaH2PO4 (pH 7.2), the filters were hybridized in another aliquot of the same buffer containing probes specific for each exon of the androgen receptor gene (8) labeled with [α-32P]dCTP using the random priming method (14). Probe concentration in all cases was approximately 1 × 10^6 cpm/ml and the probe specific activity was >5 × 10^6 cpm/μg DNA. Following hybridization, the filters were washed in 0.1 × standard saline-citrate-0.1% SDS at 68°C and autoradiographed at -80°C.

RNA Probe Synthesis. Two segments of the androgen receptor gene (see below) were subcloned into the plasmid vector, BSKM13+ (Stratagene, 5-694), the Medical Life and Health Insurance Medical Research Fund, the Welch Foundation (1-1090), a grant from the Perot Family Foundation, a grant from the American Cancer Society (IN-142), and Grant DK03892 from the NIH. M. J. M. is a Culppeper medical scholar. W. D. T. was a recipient of a C. J. Martin Fellowship from the National Health and Medical Research Council of Australia.

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2. The abbreviations used are: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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tagene Cloning Systems, La Jolla, CA) and used to synthesize continuously labeled antisense RNA probes for \( S_1 \) nuclelease protection assays. Region I was a 530-base pair \( PstI-SmaI \) restriction endonuclease fragment derived from the 5' untranslated region of the androgen receptor gene. Region II consisted of a 713-base pair \( HindIII-EcoRI \) restriction endonuclease fragment derived from the coding region (nucleotides 1850 to 2563) of the human androgen receptor complementary DNA (15). Following linearization with an appropriate restriction endonuclease (\( HindIII \) for probe I, \( BamHI \) for probe II), \( in vitro \) transcription was performed using either the \( T7 \) polymerase (probe II) or the \( T3 \) polymerase (probe I), as described by the manufacturer, in a reaction containing 0.4 mM concentrations each of unlabeled CTP, ATP, and GTP and 12 \( \mu \)M [\( \alpha-^{32}P \)]UTP (final specific activity, 400 Ci/mmol; Amersham Corp.). Following synthesis, the DNA template was digested with RNase-free DNase (RIQ DNASE; Promega Biotech) and the unincorporated nucleotides were removed by ammonium acetate precipitation.

Preparation of a Synthetic “Sense” Androgen Receptor mRNA and the Standard Curve. To allow the precise quantitation of the androgen receptor mRNA present in RNA samples, synthetic sense RNA transcripts were prepared using as template the plasmid containing region II from the coding region of the androgen receptor (see above). In these transcription reactions, the plasmid was linearized instead with the restriction endonucleases EcoRI and the \( T7 \) RNA polymerase was used, leading to the synthesis of RNA transcripts of the same polarity as the native androgen receptor RNA. \( In vitro \) transcription with the \( T7 \) RNA polymerase was carried out as described for the antisense RNA probe synthesis, except that the specific activity of the sense standard was 2 orders of magnitude less than that used for the synthesis of the antisense RNA probe. After removal of the unincorporated nucleotides, the amount of purified sense RNA used in the standard curve was inferred from the nucleotide sequence of the RNA probe and the specific activity of the radiolabeled UTP (2.5 Ci/mmol).

\( S_1 \) Nuclease RNA Protection Assay. \( S_1 \) nuclelease protection assays were performed using a protocol similar to that of Burke (16). Approximately \( 8 \times 10^6 \) cpm of the purified uniformly labeled antisense RNA probe was co precipitated with 25 \( \mu \)g (receptor-positive) or 40 \( \mu \)g (receptor-negative) RNA samples, rinsed with ethanol, and dried. The samples were resuspended in 25 \( \mu \)l of a solution containing 0.4 M NaCl, 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.5), 1 mM EDTA, and 64% deionized formamide, denatured at 80°C for 5 min, and incubated for 16 h at 52°C. After hybridization, 300 \( \mu \)l of ice cold 5% glycerol, 50 mM Tris (pH 4.5), 1 M NaCl, 25 mM MgCl\(_2\), 30 mM sodium phosphate (pH 6.8), 0.8 mM sodium deoxycholate, and 5% (v/v) glycerol were added. Nucleic digestion was performed with 4000 units of \( S_1 \) nuclease (Boehringer Mannheim, Indianapolis, IN) at 42°C for 1 h. Digestion was terminated by the addition of 15 \( \mu \)l of buffer containing 0.5 M Tris (pH 8.0), 25 mM EDTA, and 5 \( \mu \)g of tRNA. The samples were extracted with phenol/chloroform (1:1, v/v), precipitated at \(-20°C\) following addition of 1 ml of chilled ethanol, and then resuspended and precipitated twice with ammonium acetate. After the final precipitation, the samples were washed in ethanol, dried, resuspended in 90% (v/v) formamide, electrophoresed on a denaturing 5% polyacrylamide gel containing 8 M urea, and visualized by autoradiography.

In order to quantitate the absolute levels of androgen receptor mRNA present in these samples, in each experiment we included a standard curve that used known amounts of synthetic androgen receptor RNA transcripts of the same polarity as native androgen receptor mRNA. This synthetic mRNA was identical in structure but opposite in polarity to the labeled probe (probe II) employed in the quantitation assays (see above). Standard samples that ranged from 0.005 to 0.1 fmol of synthetic sense androgen receptor mRNA were included in the quantitation experiments. Standard samples were hybridized with labeled probe, digested with \( S_1 \) nuclease, and analyzed as above. The autoradiographs of both the standard and the experimental samples were scanned using a Molecular Dynamics Computing Densitometer (Model 300A; Sunnyvale, CA), and the values obtained (arbitrary units) were plotted as a function of the amount of synthetic sense androgen receptor mRNA. Absolute levels of androgen receptor mRNA were inferred by comparison of the experimental values to the values obtained for the standard curve.

Western Analysis. Confluent monolayers of cells were scraped into ice cold PBS and pelleted by centrifugation. Pellets were washed twice with 10 volumes of PBS, resuspended in 10 volumes of electrophoresis loading buffer \( [70 \text{ mM SDS-100 mM dithiothreitol-10} \% \text{ glycerol-0.6 mM bromphenol blue-80 mM Tris, pH 6.9}] \) and stored at \(-20°C\). Prior to electrophoresis samples were thawed, resuspended by sonication, heated in a boiling water bath for 3 min, diluted in loading buffer to the desired protein concentration, sonicated again to produce a homogeneous solution, and boiled again for 3 min. The final protein concentration was determined by a modification of the method of Lowry et al. (11) which included the initial precipitation of protein with 10% (w/v) trichloroacetic acid in the presence of 0.8 mM sodium deoxycholate. Bovine serum albumin was used as the protein standard.

Electrophoresis was carried out in 7.5% polyacrylamide gels containing 3.5 mM SDS according to the method of Laemmli (17) using 100 \( \mu \)l of sample in each lane. Ten \( \mu \)l of Rainbow \( ^{14}C \)-methylated protein molecular weight markers (CFA.756; Amersham, Arlington Heights, IL) were mixed with 90 \( \mu \)l of loading buffer and applied to one lane of each gel. Following electrophoresis, protein bands were transferred to nitrocellulose and analyzed by immunoblotting as described by Harris et al. (18) using affinity-purified rabbit antibodies that recognize the amino (NH\(_2\)) terminus of the androgen receptor. Blots were incubated with \( ^{125}I \)-labeled goat anti-rabbit IgG (F(ab')\(_2\)) (New England Nuclear Research Products, Boston, MA) at \( 5 \times 10^5 \) cpm/ml to detect anti-NH\(_2\)-terminal antibody binding. Autoradiograms were produced by exposing Kodak XAR2 film to immunoblots for 65 h at \(-70°C\) with one intensifying screen.

Anti-NH\(_2\)-terminal antibodies were produced by immunizing rabbit U402 with the carrier protein keyhole limpet hemocyanin coupled to a synthetic 21-amino acid peptide corresponding to the NH\(_2\)-terminal amino acid sequence of human and rat androgen receptor protein, purified on a peptide affinity chromatography column, and characterized as described previously (19). Affinity-purified antibodies were diluted 1:30 in BLOTTO and used at a final concentration of 33 \( \mu \)g protein/ml as determined by the method of Lowry et al. (11).

RESULTS

PC-3 and DU-145 Cell Lines Express No Detectable Dihydrotestosterone Binding. To establish the level of androgen receptor in the prostate carcinoma cell lines, we examined the levels of specific 5\( \alpha \)-dihydrotestosterone binding sites present in the LNCaP, PC-3, and DU-145 cell lines (Table 1). The LNCaP cell line expresses high levels of androgen receptor, while the DU-145 and PC-3 cell lines express no detectable specific 5\( \alpha \)-dihydrotestosterone binding. Scatchard analysis of these ligand binding data indicate that androgen receptor in the LNCaP cell line binds 5\( \alpha \)-dihydrotestosterone with an apparent \( K_d \) of 0.7 nM.

LNCaP Cell Line Expresses High Levels of the Androgen Receptor Protein. We next examined the levels of immunoreactive androgen receptor protein. The ligand binding assays were performed in monolayer cultures of the prostate carcinoma cell lines. The number of specific 5\( \alpha \)-dihydrotestosterone binding sites and the apparent \( K_d \) of the androgen receptor were derived by Scatchard analysis of the binding data as described in "Materials and Methods." Specific 5\( \alpha \)-dihydrotestosterone binding represented over 90% of the total binding detected at saturation in the LNCaP cell monolayers. By contrast, no specific binding was detected above background in the DU-145 and PC-3 cell lines. NA, not applicable.
active androgen receptor protein expressed by three of the prostate carcinoma cell lines using polyclonal antipeptide antibodies directed at the NH2-terminus of the receptor protein (19). The result of such an experiment is shown in Fig. 1. Immunoreactive androgen receptor protein with an apparent molecular weight of approximately 110,000 is clearly present in the lane loaded with as little as 10 ng of protein from LNCaP cells. No such band is observed in lanes loaded with 110 ng of protein from DU-145 cells or 120 ng of protein from PC-3 cells, even when the time of exposure is increased from 65 h (Fig. 1) to 1 week (data not shown). Taken together, these data demonstrate that the DU-145 and PC-3 cell lines do not express detectable androgen receptor mRNA. The inclusion in these samples of synthetic sense androgen receptor mRNA and for RNA samples prepared from the DU-145 and PC-3 cell lines. Interestingly, in the same experiment we observed that the levels of androgen receptor RNA in LNCaP cells appear to diminish in response to both estradiol and 5a-dihydrotestosterone.

This qualitative analysis has been rendered more quantitative by the use of an S_{i} nuclease protection assay for androgen receptor mRNA. The probes for these experiments were derived from two different regions of the androgen receptor gene. The results of one such experiment and a schematic diagram of the two probes used are shown in Fig. 3. The results of these assays demonstrate that the DU-145 and PC-3 cell lines do not express detectable androgen receptor mRNA. The inclusion in these experiments of a standard curve constructed using known amounts of synthetic sense androgen receptor mRNA transcribed in vitro allows the estimation of the quantity of androgen receptor mRNA present in these samples to be inferred. A diagramatic comparison of the values obtained in this manner for synthetic sense androgen receptor mRNA and for RNA samples prepared from the LNCaP prostate carcinoma cell line (designated A), for PC-3 and DU-145 prostate carcinoma cell lines (designated C), and for a strain of normal control genital skin fibroblasts (designated B) is shown in Fig. 4. From these data we estimate that the LNCaP cells and the control genital skin fibroblasts express approximately 25.6 and 1.4 amol of androgen receptor mRNA per μg of total cellular RNA, respectively. The PC-3 and DU-145 cells express less than 0.1 amol of androgen receptor mRNA per μg of total RNA, which...
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represents the limit of detection of the assay. This value is at least 200-fold lower than the level of androgen receptor mRNA expression in the LNCaP cell line and less than one-tenth that found in the control (704) genital skin fibroblast cultures which express low levels of androgen receptor mRNA and protein. Similar values were obtained in experiments using probe I.

Androgen Receptor Gene in the LNCaP, DU-145, and PC-3 Cell Lines Is without Large Scale Rearrangements. The virtual absence of androgen receptor mRNA expression in the DU-145 and PC-3 cell lines raised the question as to whether the androgen receptor gene in these cell lines had undergone an alteration that had rendered it nonfunctional. This possibility was addressed by analyzing the androgen receptor gene structure in the three prostate carcinoma cell lines and in normal diploid fibroblasts by genomic Southern analysis (Fig. 5). All eight coding exons of the androgen receptor gene (8, 20) are present in each of the cell lines, and no large deletions or rearrangements of the androgen receptor gene have occurred.

DISCUSSION

While it is believed that the majority of prostate cancer is derived from the androgen-dependent prostatic epithelium, these tumors vary widely both in histological appearance and biological behavior from patient to patient. Furthermore, the responses of patients to various forms of endocrine therapy are not uniform (1).

Studies of the heterogeneity of prostate carcinoma and the hormonal responsiveness of this tumor have been most extensively performed in the rat Dunning model of prostate carcinoma (2, 21). These studies have suggested that the progression from an androgen-sensitive to an androgen-independent state is associated with the outgrowth of androgen-insensitive cells from an initially heterogeneous mixture of tumor cells. Interestingly, studies recently reported by Quarmby et al. (22) suggest that this progression is associated with a change in the level of expression of the androgen receptor during the progression from the androgen-sensitive to the androgen-insensitive stages of the Dunning tumor.

The available human prostate carcinoma cell lines also comprise a wide spectrum, ranging from the LNCaP cell line which is androgen responsive and which expresses high levels of androgen receptor to the PC-3 and DU-145 cell lines which are...
not androgen responsive and do not express measurable androgen receptor. We have explored this heterogeneity by examining the expression of the androgen receptor in these three continuous human prostate carcinoma cell lines. Our studies indicate that the high level of specific 5a-dihydrotestosterone binding presented in the LNCaP cell line correlates with the presence of an androgen receptor protein with an apparent molecular weight of approximately 110,000, similar to that observed in the prostate and other target tissues (23, 24). These experiments also demonstrate that the absence of detectable androgen binding in the PC-3 and DU-145 cell lines is associated with the absence of detectable normal or mutant androgen receptor protein using antibodies directed at an epitope located at the amino terminus of the androgen receptor protein.

Our Western analyses were limited, however, both by their sensitivity and by the nature of the assay, which detects only a limited number of epitopes within the androgen receptor protein. To circumvent these shortcomings we extended these findings and examined androgen receptor mRNA expression within these cell lines by Northern and S1 nuclease protection analysis using probes designed to detect two distinct regions of the androgen receptor mRNA molecule. Using these assays we demonstrated that RNA samples prepared from the LNCaP cell line contained high levels of androgen receptor mRNA. Furthermore, the mobilities of the androgen receptor mRNA species visualized in these samples, approximately 7 and 10 kilobases, are similar to those observed in preparations from other human tissues.3 However, using these same assays, we were unable to detect androgen receptor mRNA in the PC-3 or DU-145 cell lines. This appears to represent a complete absence of androgen receptor mRNA, inasmuch as our methods would permit the detection of androgen receptor mRNA at a level one-tenth that found in fibroblasts, which express only low levels of androgen receptor mRNA and protein.

The virtual absence of detectable androgen receptor mRNA in the PC-3 and DU-145 cell lines does not appear to be correlated with discernible alterations within the androgen receptor gene itself because no gross deletions or rearrangements are detectable by genomic Southern blotting. While this does not exclude the possibility that subtle mutations such as point mutations might be present within the androgen receptor gene of the PC-3 and DU-145 cell lines, it would appear that the androgen receptor gene is inactive in these receptor-negative prostate carcinoma cell lines.

While the relevance of these studies to the progression of human prostate cancer is not clear, the derivation and androgen-independent growth of the receptor-negative prostate carcinoma cell lines used in these studies invite comparison to androgen-independent prostate carcinoma. Further analysis of the mechanisms resulting in the absence of androgen receptor expression in the PC-3 and DU-145 cell lines should provide insight into the factors controlling androgen receptor expression and may identify processes that lead to the development of androgen-independent human prostate cancers.

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