Down-Regulation of Prostate-specific Antigen Expression by Finasteride through Inhibition of Complex Formation between Androgen Receptor and Steroid Receptor-binding Consensus in the Promoter of the PSA Gene in LNCaP Cells

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ABSTRACT

As a specific competitive inhibitor of 5a-reductase, an intracellular enzyme that converts testosterone to dihydrotestosterone, finasteride is being extensively used for the treatment of benign prostatic hyperplasia and in experimental settings for prostate cancer. In this study, we showed that finasteride markedly inhibited prostate-specific antigen (PSA) secretion and expression.

The promoter of the PSA gene contains several well-known cis-regulatory elements. Among them, steroid receptor-binding consensus (SRBC) has been identified as a functional androgen-responsive element. Our previous study showed that PSA was not only present in conditioned medium of the PSA-positive LNCaP cells but was also detectable in small amounts in PSA-negative cell lines, PC-3 and DU-145 (L. G. Wang et al., Oncol. Rep., 3: 911—917, 1996). A strong correlation between binding of nuclear factors to SRBC and the level of PSA present in the conditioned medium and cell extracts was found in these three cell lines, whereas no such correlation with binding was obtained using Sp1 oligonucleotide as a probe. Binding of LNCaP cell nuclear proteins to SRBC was diminished when the cells were exposed to 25 μM finasteride, at which concentration 50% of both PSA mRNA and protein were inhibited. As a major component of DNA-protein complexes, the level of androgen receptor was dramatically decreased in the cells treated with finasteride.

Our data indicate that inhibition of complex formation between SRBC and nuclear proteins due to the remarkable decrease in the level of androgen receptor plays a key role in the down-regulation of PSA gene expression by finasteride in LNCaP cells.

INTRODUCTION

As a specific competitive inhibitor of 5α-reductase, an intracellular enzyme that converts testosterone to dihydrotestosterone, finasteride is being extensively used for the treatment of BPH and in experimental settings for prostate cancer (1). Finasteride has no binding affinity for androgen receptor sites and possesses no androgenic, antiandrogenic, or other steroid hormone-related properties (1). However, we and others have shown that finasteride exhibits growth inhibitory effects on androgen-sensitive, PSA-positive LNCaP cells and, to a much lesser degree, on the androgen-independent and the PSA-negative cell lines: PC-3 and DU-145 in vitro (2, 3). To explore the possible mechanism by which finasteride inhibits prostate cancer cell growth, the effect of this agent on PSA expression was studied.

PSA is a glycoprotein with chymotrypsin-, trypsin-, and esterase-like activities (4, 5). Since it is produced almost exclusively by the epithelial cells of the prostate, PSA has been used extensively as a serum marker for diagnosis and prognosis of prostate cancer and as an immunohistochemical marker for the identification of prostatic tissues and cells in pathological specimens (6). Our previous study has shown that PSA is involved in the growth stimulation of the androgen-responsive LNCaP cells (7). The regulation of PSA gene expression by growth factors and hormones has been extensively studied (8—10). The promoter region of the PSA gene contains several well-known cis-regulatory elements, including a variant TATA box, a transcription factor Sp1-binding site, a SRBC, and a CACCC motif (11, 12). Among them, SRBC has been implicated as a functional androgen-responsive element (13). In this study, we showed that in LNCaP cells finasteride significantly inhibits PSA expression and that this down-regulation of PSA expression by finasteride correlates with the inhibition of complex formation between androgen receptors and SRBC. This decrease in complex formation can be accounted for by a dose-related decrease of the AR level in LNCaP cells after treatment with finasteride.

MATERIALS AND METHODS

Reagents. Finasteride was a gift from the Merck Research Laboratory through the courtesy of Dr. G. J. Gormley. PSA antibody was purchased from the DAKO Corporation (Carpinteria, CA). Antibody against AR, corresponding to amino acids 900—919 mapping at the carboxyl terminus of the AR of human origin, anti-rabbit IgG-heraldeshed peroxidase, and protein agarse A plus G were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Tandem-E PSA, an immunoenzymetic kit for the quantitative measurement of PSA levels, was obtained from HybriTech, Inc. (San Diego, CA). A Western blotting detection kit and [γ-32P]ATP were purchased from Amersham (Arlington Heights, IL). Reagents for SDS-PAGE and protein determination were obtained from Bio-Rad (Richmond, CA). RPMI 1640 and FBS were obtained from Life Technologies, Inc. (Gaithersburg, MD). Other chemicals for this study were purchased from Sigma Chemical Company (St. Louis, MO). Oligonucleotides containing the Sp1 site CAGGCGAGGGCCGGATCTCT (12) and SRBC GCAGAACAGCAAGTGCTAGC in the promoter of the PSA gene (12, 14) were synthesized by Dr. R. Pergolizzi in the Biopolymer Facility of our hospital.

Cell Culture. The prostatic carcinoma cell lines LNCaP, PC-3, and DU-145 were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured under conditions as described previously (7).

Determination of PSA Secretion. The level of the PSA secreted into the medium was determined using a Tandem-E PSA immunoenzymetic kit according to the instructions provided by the manufacturer and by Western blotting as described below.

Western Blotting of Cellular Proteins and Medium. LNCaP cells at 65—75% confluence in RPMI 1640 containing 10% FBS were harvested and washed with cold PBS once. Total cellular proteins were extracted as described previously (15). One hundred μg of cellular extracts or 50 μl of culture medium from LNCaP cells, treated with various concentrations of finasteride, were separated by 4% /10% stack SDS-PAGE, electrotransferred to nitrocellulose filters, and immunoblotted with antibodies against PSA and/or AR, respectively. Quantitation by densitometry of the X-ray films was done using...
an Imaging Densitometer Model GS-700 (Bio-Rad Laboratories, Hercules, CA).

Northern Blotting Assay. LNCaP cells grown to 65—75% confluence in RPMI 1640 containing 10% FBS were treated with varying concentrations of finasteride for 72 h. The cells were harvested and washed with cold PBS, and total RNA was extracted using the guanidinium/phenol method as described previously (15, 16). RNA was quantitated spectrophotometrically, and 20 μg aliquots were fractionated on 1.2% agarose-formaldehyde gels and transferred to Zeta- Probe Blotting Membrane (Bio-Rad). The double-stranded PSA-specific oligonucleotide probe (5'-AGCCFAGAGAAGGCTGTGAGCCAAGGAGGGAGGGTCTFCC1TFGGCATGGGATGGGGATGAAGTAAGGAGAAGGGACT-3') described by Young et al. (8), 5' end labeled, was used for hybridization as described previously (15). Radioautographs were analyzed using an Imaging Densitometer Model GS-700 (Bio-Rad) and normalized to 28S RNA.

MGSA. MGSA was performed as described previously (15). Briefly, LNCaP cells grown to 65—75% confluence in RPMI 1640 containing 10% FBS were harvested and washed once with cold PBS, and nuclear proteins were extracted in the presence of protease inhibitors, including 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μg/ml aprotinin. The proteins were divided into aliquots and stored at —20°C until use.

Five μg of nuclear protein were reacted for 30 min at room temperature with the 32P-labeled Sp1 or SRBC probe in binding buffer composed of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5% (v/v) glycerol, and 2.0 μg of poly(dI)/(dC) in a final volume of 20 μl. For identification of specific proteins involved in the formation of DNA-protein complexes, the proteins were immunoprecipitated with the antibody at 4°C overnight prior to the binding assay. Controls were performed under the same conditions without any antibody or with histone H1 antibody. The reaction mixtures were then subjected to 8% low ionic strength PAGE [6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1.0 mM EDTA]. The binding complexes were visualized by exposing the dried gel to X-ray film at —70°C overnight.

Statistics. All data of PSA secretion determined using the Tandem-E PSA immunoenzymetric kit were expressed as the mean ± SD. Significant differences among the means were determined using Fisher's exact test, and P < 0.05 was used to identify significance.

RESULTS

Inhibition of PSA Secretion and Expression. Incubation of LNCaP cells with various concentrations of finasteride for 48 h caused a marked decrease of PSA level in the medium as determined immu-
Correlation of PSA Secretion/Expression with the Binding of Nuclear Extracts to the SRBC in the PSA Promoter. As observed earlier, PSA protein was found not only in the medium conditioned by an androgen-responsive prostate cancer cell line LNCaP but also, to a much lesser extent, in the medium conditioned by the androgen-independent cell lines, PC-3 and DU-145 (7). Previous evidence demonstrated that the transcription regulation of the PSA gene by androgens took place via the SRBC present in the promoter region of the PSA gene (17). In view of this observation, we examined whether a correlation existed between the binding of nuclear proteins to SRBC or the Sp1 that are present in the promoter region of the PSA gene and the PSA gene expression. As shown in Fig. 5, left panel, the difference in the level of PSA present in the conditioned media was found to correlate with the difference in the level of the binding of nuclear proteins to the SRBC. No such binding complex formation was observed when the Sp1 oligonucleotide was reacted in an identical manner (Fig. 5, right panel). The specificity of the complex formed between SRBC

n enzymatically. As shown in Fig. 1, this inhibition of the PSA secretion by finasteride was found to be dose (Fig. 1A) and time (Fig. 1B) dependent. At lower concentrations of the drug (25 μM), the amount of PSA in the medium was decreased by approximately 56% without significant decrease of the cell viability. This result was consistent with the result obtained with Western blotting shown in Fig. 2A. However, no such decrease of the secretion of the IGFBP-2 was observed (Fig. 2B). The decrease of the PSA secretion into the medium after treatment of LNCaP cells with finasteride paralleled the protein level present in the total cellular extracts as measured by Western blotting (Fig. 3A). Again, no such decrease was found when the IGFBP-2 antibody was used in the assay (Fig. 3B). These results indicate that the decreased PSA protein secretion may result from the inhibition by finasteride of the protein expression in the cells, and that this inhibition was a specific event.

To understand whether the decrease of PSA protein is due to the changes of rates of the transcription or the translation of the protein, the mRNA of PSA was determined in LNCaP cells after treatment with finasteride. As shown in Fig. 4, the very pronounced decrease in the level of PSA mRNA was observed in a dose-dependent manner after exposure of LNCaP cells to finasteride for 72 h that accurately paralleled the decrease in the amount of PSA protein. This result suggests that the regulation of PSA gene expression by finasteride was, at least in part, in the transcriptional level.

Correlation of PSA Secretion/Expression with the Binding of Nuclear Extracts to the SRBC in the PSA Promoter. As observed earlier, PSA protein was found not only in the medium conditioned by an androgen-responsive prostate cancer cell line LNCaP but also, to a much lesser extent, in the medium conditioned by the androgen-independent cell lines, PC-3 and DU-145 (7). Previous evidence demonstrated that the transcription regulation of the PSA gene by androgens took place via the SRBC present in the promoter region of the PSA gene (17). In view of this observation, we examined whether a correlation existed between the binding of nuclear proteins to SRBC or the Sp1 that are present in the promoter region of the PSA gene and the PSA gene expression. As shown in Fig. 5, left panel, the difference in the level of PSA present in the conditioned media was found to correlate with the difference in the level of the binding of nuclear proteins to the SRBC. No such binding complex formation was observed when the Sp1 oligonucleotide was reacted in an identical manner (Fig. 5, right panel). The specificity of the complex formed between SRBC

Fig. 3. Levels of PSA and IGFBP-2 in total cell extracts. LNCaP cells grown exponentially were exposed to 25 or 100 μM finasteride in regular RPMI 1640 containing 10% FBS for 24, 48, or 72 h, and the cells were harvested, washed once with ice-cold PBS, and the total cell proteins were extracted with PBS/DTDS containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 μg leupeptin. One hundred μg of proteins were subjected to 10% SDS-PAGE and the proteins in the gel were electrotransferred to nitrocellulose membranes and immunblotted with PSA (A) and IGFBP-2 (B) antibody, respectively.

Correlation of PSA Secretion/Expression with the Binding of Nuclear Extracts to the SRBC in the PSA Promoter. As observed earlier, PSA protein was found not only in the medium conditioned by an androgen-responsive prostate cancer cell line LNCaP but also, to a much lesser extent, in the medium conditioned by the androgen-independent cell lines, PC-3 and DU-145 (7). Previous evidence demonstrated that the transcription regulation of the PSA gene by androgens took place via the SRBC present in the promoter region of the PSA gene (17). In view of this observation, we examined whether a correlation existed between the binding of nuclear proteins to SRBC or the Sp1 that are present in the promoter region of the PSA gene and the PSA gene expression. As shown in Fig. 5, left panel, the difference in the level of PSA present in the conditioned media was found to correlate with the difference in the level of the binding of nuclear proteins to the SRBC. No such binding complex formation was observed when the Sp1 oligonucleotide was reacted in an identical manner (Fig. 5, right panel). The specificity of the complex formed between SRBC

Fig. 4. Extent of PSA gene expression in LNCaP cells as a function of dosage of finasteride. LNCaP cells grown at 65%–75% confluence in RPMI 1640 containing 10% FBS were exposed to indicated concentrations of finasteride for 72 h, and the cells were harvested, washed once with ice-cold PBS, and total RNA was extracted. The PSA mRNA was then detected with Northern blotting using 5' end-labeled PSA-specific oligonucleotide as the probe. Top panel, PSA mRNA measured by Northern blotting; middle panel, total RNA; and bottom panel, density of the plots of PSA mRNA determined from X-ray film by an Imaging Densitometer Model GS-700. Data are the means of two separate experiments scored by the densitometer and normalized to 28S RNA.

Fig. 5. Levels of nuclear binding to the SRBC or Sp1. A, left panel, nuclear proteins extracted from the LNCaP cells treated with 25, 50, or 100 μM finasteride as described in Fig. 3 were analyzed by Western blotting with the specific antisera to the SRBC. B, right panel, nuclear proteins extracted from the LNCaP cells treated with 25, 50, or 100 μM finasteride as described in Fig. 3 were analyzed by Western blotting with the specific antisera to the Sp1.
Fig. 5. Binding of nuclear proteins extracts from the androgen-sensitive cell line LNCaP and androgen-independent cell lines PC-3 and DU-145 to SRBC. Five μg of nuclear cell extracts were reacted with 32P end-labeled SRBC oligonucleotide: 5'−GCAGAACAGCAGTGTAGC-3' (left panel) or Sp1 oligonucleotide 5'−CAGGGGCGGAGGCGGAGATC-3' (right panel) in binding buffer containing 2 μg/ml poly(dC)/(d) for 30 min. The reaction mixtures were then subjected to an 8% natural polyacrylamide gel, and the binding complexes were detected by exposing the dried gel to X-ray film at −70°C overnight.

Fig. 6. Determination of the specificity of the binding complexes formed between SRBC and nuclear proteins. Five μg of nuclear proteins extracted from normal cultured LNCaP cells reacted with 32P end-labeled SRBC oligonucleotide in the presence of cold homologous (SRBC, left panel) or nonhomologous unlabeled Sp1 oligonucleotide (Sp1, right panel) for 30 min at 37°C. The reaction mixtures were subjected to an 8% natural polyacrylamide gel. The binding complexes were detected by exposing the dried gel to X-ray film at −70°C overnight.

and nuclear factors obtained from LNCaP cells was further established by competition analysis using a homologous unlabeled SRBC oligonucleotide (Fig. 6, left panel) or the nonhomologous unlabeled Sp1 oligonucleotide (Fig. 6, right panel). The presence of unlabeled homologous SRBC decreased the level of complexes in a concentration-dependent manner. In contrast, the extent of complexes formed was not altered in the presence of high concentrations of Sp1 oligonucleotide.
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were made from LNCaP cells treated with various concentrations of finasteride for 72 h. As shown in Fig. 7, the levels of binding complexes were markedly decreased when the nuclear proteins prepared from LNCaP cells were treated with 25 μM or higher concentrations of finasteride. The absence of a dose-response effect regarding the binding complexes may be due to the fact that the inhibition of finasteride at 25 μM on the PSA expression has almost reached the peak level. This result parallels the PSA level present in the cells treated with the same concentrations of the drug.

Fig. 8 shows the effect of androgen antibody on the formation of the binding complexes and the effect of finasteride on the level of AR in the cells. Complexes were significantly diminished when the nuclear extracts were pre-precipitated with AR antibody, whereas no changes were observed when the histone H1 antibody proteins were used (Fig. 8A). This finding provides additional evidence that the AR plays a key role in the formation of the binding complexes and confirms the previous mechanism as outlined by Luke and Coffey (17). After treatment with various concentrations of finasteride, the AR levels in the cell extracts of LNCaP cells were remarkably decreased in a dose-dependent manner (Fig. 8B). As documented in the Fig. 8C, following the treatment of LNCaP cells with 25 μM finasteride, AR levels in cellular extracts were decreased to approximately 50% of baseline without significant cytotoxicity.

DISCUSSION

Finasteride, an effective 5α-reductase inhibitor, has been introduced to clinicians for treatment of BPH and for trials for the treatment of PC (18, 19). The effects of the drug on serum PSA in men with stage D PC have been described earlier (20).

Previous observation showed that besides the importance of the PSA as a serum marker for both BPH and PC, PSA itself plays a role in the growth stimulation of the androgen-responsive prostate cancer cell line, LNCaP cells (7). In this study, we provide evidence that finasteride markedly inhibits both PSA secretion and expression in LNCaP cells. Thus, the down-regulation of finasteride on PSA and possibly other AR target gene expressions may imply an additional mechanism by which this drug decreases prostate volume.

Effects of steroids on target cells are mediated by their respective receptors. A number of reports have shown that antagonists of steroid
receptors inhibit binding of receptors to target DNA (21, 22). The promoter of the PSA gene contains several well-known cis-regulatory elements. Among them, SRBC has been implicated as a functional androgen-responsive element (13). A core sequence for the androgen-response element has been found to be GGA/TACAnnn-TGGTCT (23). PSA was not only present in conditioned medium from the PSA-positive cell line LNCaP cells, but was also detectable in small amounts in the PSA-negative cell lines PC-3 and DU-145 (7). A strong correlation between binding of nuclear factors to SRBC and the level of PSA present in the cell extracts was found in those cell lines, whereas no such correlation with binding was obtained using Sp1 oligonucleotide as a probe. Binding of LNCaP cell nuclear proteins to SRBC was diminished when the cells were exposed to 25 μM finasteride, at which concentration over 50% of the PSA expression was inhibited. These results imply that the down-regulation of PSA expression by finasteride is through the inhibition of complex formation of nuclear proteins with SRBC in the promoter region of the PSA gene in these cells.

The AR is a member of a steroid superfamily of ligand-inducible intracellular regulators that activate or repress transcription of target genes (24, 25). Several genes have been demonstrated to be regulated by androgen in LNCaP cells primarily at the level of transcription initiation presumably via AR, including the human kallikrein-related PSA and hGK-1 genes (17, 26). ARs are structurally and functionally organized into domains that mediate hormone binding, nuclear translocation, dimerization, DNA binding, and transcriptional activation (27–30). In the present study, using AR antibody to remove AR from nuclear extracts followed by MGSa, the binding of nuclear proteins to SRBC decreased to undetectable levels. Our result provides additional evidence that AR is a major component of SRBC-protein complexes. This finding paralleled the observed decrease of the AR level in the cells treated with finasteride. Thus, lack of formation of SRBC-protein complexes due to the decrease in the level of AR may play a key role in the down-regulation of PSA gene expression by finasteride.

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*Cancer Res* 1997;57:714-719.

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