Autocrine Regulation of Prostate-specific Antigen Gene Expression in a Human Prostatic Cancer (LNCaP) Subline

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ABSTRACT

Prostate-specific antigen (PSA), a Mr 34,000 serine protease, is recognized as a useful marker for the detection and prognosis of patients with prostate cancer. Although serum PSA is an excellent prognostic indicator, an increasing number of factors were found to regulate the PSA expression of prostatic cancer cells, which include androgenic steroids, the growth factors (GFs) and the extracellular matrix. The purpose of this study is to define a novel protein factor that may be responsible for regulating PSA expression by androgen-independent (AI) human prostate cancer cells. We have established a LNCaP subline (C4) from a parental LNCaP tumor grown in a castrated host. The C4 subline overexpressed PSA mRNA and protein. Serum-free conditioned medium (CM) isolated from the C4 subline is able to stimulate PSA gene expression in parental LNCaP cells in a concentration-dependent manner. This autocrine PSA-inducing activity was found to be organ specific because CMs from other fibroblast cell lines (such as bone, prostate, kidney, and lung fibroblasts) and the CMs from several prostatic carcinoma cell lines (such as parental LNCaP, PC-3, DU-145) and a bladder transitional carcinoma cell line (WH) fail to exhibit similar activity. The activity of the CM from the C4 subline cannot be substituted by GFs such as TGF-α, TGF-β, bFGF, HGF, KGF, or NGF; neuromedin (bombesin/GRP); secondary messenger analogue (dibutyryl cAMP); β2-adrenergic agonist (isoproterenol); or α1-adrenergic agonist (phenoxyhyline), indicating that the factor(s) may be a novel prostate-specific autocrine factor (PSAF). Both androgen and PSAF exhibit an additive effect on up-regulating PSA gene expression, suggesting that the signal transduction pathway elicited by PSAF may differ from that mediated by the androgen receptor. Further characterization of PSAF by heat, acid, and trypsin digestion revealed that the PSAF may be a protein factor with a unique amino acid composition. These observations suggest that a novel autocrine pathway mediated by PSAF may be responsible for the overexpression of PSA mRNA and protein in a human prostatic cancer cell line. The potential clinical significance of this factor will be discussed.

INTRODUCTION

PSA, produced exclusively by prostate epithelium, is a Mr 34,000 serine protease with chymotrypsin-, trypsin-, and esterase-like activities (1, 2). Because of its organ specificity, PSA has been used extensively as a serum marker for diagnosis and prognosis of prostate cancer and an immunohistochemical marker for the identification of prostatic tissues and cells in the pathological specimens. Several studies (3–9) indicated that PSA is not only expressed by normal but also by benign and malignant prostatic epithelial cells. More than 99% of metastatic prostate lesions were found to stain positively for PSA; however, serum PSA appears to be most useful in staging disease and in monitoring patients after definitive therapy. Kurijama et al. (10) and Killian et al. (11) demonstrated that serum PSA levels can be a useful marker for predicting the survival of patients who have stage D prostate cancer and who receive hormonal therapy. More recently, Stamey et al. (12) and Ercole et al. (13) evaluated the effect of androgen ablation therapy on serum PSA concentration in patients with previously untreated, stage D prostate cancer and have reached the same conclusion that patients in clinical remission after androgen ablation therapy can be identified by the low serum PSA levels, whereas patients with progressive disease after hormonal therapy will have markedly elevated serum PSA levels. These findings suggest that androgen deprivation therapy may have a direct impact on serum PSA concentration, and rebound of serum PSA levels during androgen deprivation may be due to a non-androgen factor(s)-mediated pathway. The implication of these observations will become clearer as detailed studies on PSA regulation become available.

To understand PSA regulation, a full-length cDNA clone (~1.9 kilobases) was sequenced (14, 15); sequence analysis (15, 16) revealed that PSA cDNA shares 82% homology with human glandular kallikrein cDNA (hGK-1). Further genomic DNA cloning indicated that these two genes localize at the same chromosome (i.e., 19q13) and that these two genes align in a head-to-tail orientation at a distance of only 12 kilobases (15, 17, 18). Several groups (19–23), including our laboratory, have shown that androgen is a strong inducer for the increase in the steady-state levels of PSA mRNA. Gleave et al. (24) recently suggested that several GFs may be involved in the PSA gene regulation. Obviously, PSA gene regulation warrants more intensive investigation in order to have a better understanding of the role of PSA in clinical application.

In this study, we defined and partially characterized a novel nonandrogen factor, PSAF, in up-regulating PSA expression by LNCaP subline derived from a LNCaP tumor maintained in a castrated host. Our data suggest that this factor may be produced by human prostatic cancer cells as they progressed from AD to AI status. Data from the characterization of PSAF indicate that this factor may be a unique prostate-specific protein factor, and its mechanism(s) of induction of PSA overexpression may differ from those mediated by androgen and known peptide GFs, as well as neurotransmitters. The possible clinical implications of this novel PSA regulatory pathway and its possible implication in AI prostate cancer patients will be discussed.

MATERIALS AND METHODS

Chemicals and Probes. RNAzol B was obtained from Biotex Laboratories, Inc. (Houston, TX). All restriction endonucleases, T4 polynucleotide kinase, and agarose were products of Bethesda Research Laboratories (Gaithersburg, MD). All radiolabeled nucleotides, Hyperfilm-MP, and random-primer labeling kit were purchased from Amersham Corp. (Chicago, IL). All of the peptide growth factors were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Tandem-E PSA, an immunoenzymetric kit for the quantitative measurement of PSA levels, was purchased from Hybritech, Inc. (San Diego, CA). The pancreatic trypsin inhibitor was obtained from Worthington Biochemical Corp. (Freehold, NJ). The PSA probe, a full-length cDNA clone, was obtained from Dr. D. Tindall (Mayo Clinic, Rochester, MN). PSA and hGK-1 sequence-specific oligonucleotides (25) were obtained from Dr. J. Trapman.
(Erasmus University, Rotterdam, the Netherlands). pODC10/2H (26), a human ODC cDNA probe, was a gift from Dr. O. A. Jänne (Rockefeller University, New York, NY).

Preparation of Conditioned Media from Cultured Cell Lines for the Induction of PSA Gene Expression. Conditioned media from various sources, as described previously (27), were collected and prepared by growing cells in 150-mm tissue culture dishes with T medium, 2% ECM (a serum-free defined media supplement; Celsi Co., Minnetonka, MN), and 2% fetal bovine serum until 60–70% confluent; washed with PBS, and changed to serum-free T medium containing 2% ECM. After 48 h, the CM was collected and filtered through a 0.2-μm filter and dialyzed with a 3500 molecular weight cut-off membrane (Spectrum Medical Industries, Inc., Los Angeles, CA) for 96 h at 4°C against distilled water containing 0.01 M phenylmethylsulfonyl fluoride; water was changed every 48 h. Samples were lyophilized and reconstituted to a 10-fold concentration with T medium, filtered, and diluted to the desired working concentration (0.1-2-fold) with T medium containing 2% ECM. The approximate protein concentration of the CM ranged from 0.91 to 1.3 mg/ml (control: T medium containing 2% ECM, 1.3 mg/ml) as determined by the Lowry method (28).

For an induction experiment, LNCaP cells (passage number ranging from 30 to 50) as indicator cells were cultured in 65-mm tissue culture dishes with T medium containing 2% ECM and 2% fetal bovine serum until 80% confluency was reached. These cells were washed with PBS twice and then shifted to serum-free T medium containing 2% ECM; these cells reached the 100% confluency 48 h after starvation. Different sources of CMs were added into these indicator cells for another 48-h incubation under the same serum-free condition, and then total cellular RNA was prepared 48 h after treatment and subjected to Northern blot analysis.

High-Molecular-Weight DNA Extraction and Southern Blot Analysis. High-molecular-weight DNA was purified using the procedure described by Davis et al. (29). An equal amount of DNA was digested with restriction endonucleases overnight at 37°C and then fractionated by electrophoresis in a 0.8% agarose gel containing IX TAE buffer (40 mM Tris-HCl, 18 mM NaCl, 2.0 mM EDTA) at 20 IBM sodium acetate, and 2 mMEDTA). After alkaline denaturation (30), DNA was transferred onto a Zetaprobe membrane (Bio-Rad, Richmond, CA). The membrane was baked for 2 h at 80°C, prehybridized in a hybridization buffer (10% dextran sulfate, 1% SDS, 1 mM NaCl, 20 μg/ml salmon sperm DNA) at 65°C, and then hybridized with 32P primer-labeled cDNA probe overnight at the same temperature. After hybridization, the membrane was washed in 2X SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) at room temperature for 20 min, and then the membrane was washed under high stringent conditions (0.5 × SSC, 1% SDS) at 65°C for 5 min. Autoradiography was performed using Hyperfilm-MP with the intensifying screen at −80°C.

RNA Purification and Northern Blot Analysis. Total cellular RNAs were extracted from cells using the RNAzol B method, a single-step purification protocol described by Chomczynski and Sacchi (31). Equal amounts of RNAs were subjected to Northern blot analysis by electrophoresis on 0.8% agarose gel containing 1× TAN buffer (40 mM Tris-HCl, 18 mM NaCl, 20 mM sodium acetate, and 2 mM EDTA). After alkaline denaturation (30), DNA was transferred onto a Zetaprobe membrane (Bio-Rad, Richmond, CA). The membrane was baked for 2 h at 80°C, prehybridized in a hybridization buffer (10% dextran sulfate, 1% SDS, 1 mM NaCl, 20 μg/ml salmon sperm DNA) at 65°C, and then hybridized with 32P primer-labeled cDNA probe overnight at the same temperature. After hybridization, the membrane was washed in 2X SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) at room temperature for 20 min, and then the membrane was washed under high stringent conditions (0.5 × SSC, 1% SDS) at 65°C for 5 min. Autoradiography was performed using Hyperfilm-MP with the intensifying screen at −80°C.

RESULTS

Elevation of the Steady-State Levels of PSA mRNA in LNCap Sublines. Data from our recent studies (27) indicate that serum PSA in a murine model of human prostate cancer is influenced by androgen, GFs, and certain intrinsic tumor properties which are affected by the length of time that tumors are maintained in castrated male hosts. Immunohistochemical staining of tumor tissues revealed a reduced PSA staining in LNCap tumors from castrated hosts; but tissue PSA staining intensity increased when castrated hosts were treated with testosterone propionate or when tumors were maintained in castrated hosts for 3 to 4 weeks (21). Tissue PSA levels also correlated well with serum PSA levels (21). Data from Northern analysis (21) indicate that the steady-state levels of PSA mRNA decrease shortly after castration. However, the PSA mRNA levels rebound 2 weeks after castration, suggesting that overexpression of PSA mRNA and protein may be uncoupled from androgen control, a characteristic indicator of the progression of human prostate cancer toward its AI state. To define the deranged expression of PSA gene, we have derived LNCap sublines from tumors maintained either in intact (such as M line) or castrated hosts (such as C4 from 4-week postcastration; C5 from 5-week postcastration). Using a Sty fragment isolated from PSA cDNA probe as described by Qiu et al. (32), the variable elevated levels of PSA mRNA were detected in several LNCap sublines, including the M subline (Table 1). In Table 1, we also noted that the steady-state levels of PSA mRNA and protein do not correlate well in C5 cells, implying that the translational regulation of PSA gene in C5 cells may play a role in the increment of PSA secretion. However, the C4 subline exhibited the highest increment in PSA mRNA levels as compared to the parental LNCap cells (Table 1; Fig. 1A). Also, levels of PSA protein secretion in the medium by the C4 subline were elevated accordingly (Table 1). Since PSA cDNA shares 82% sequence homology with hGK-1 cDNA (15, 16), it is crucial to rule out the possibility that most of the signal in the C4 subline detected by PSA cDNA probe reflects the PSA mRNA levels. Using both the PSA and hGK-1 sequence-specific oligonucleotide probe (Fig. 1B), the overexpression of PSA mRNA can be excluded from the human glandular kallikrein mRNA.

Analysis of PSA Gene in Both Parental LNCap Cells and Their Sublines. To explore the possible molecular mechanism(s) associated with the overexpression of PSA mRNA in the C4 subline, we examined both the amplification and methylation status of PSA gene from different LNCap sublines. Southern analysis data (Fig. 2A) indicated that HindIII digestion generates a single PSA gene fragment (i.e., 7.1 kilobases) as described previously by Riege et al. (18). This band was detected in various LNCap sublines and was retained at the same intensity. Furthermore, using several different restriction enzymes, no amplification of PSA gene can be found in the C4 subline in comparison to the PSA gene in the parental LNCap cells (Fig. 2B). An overall DNA methylation pattern of PSA gene was determined using methylation-sensitive restriction enzymes such as HpaII and MspI. As shown in Fig. 2B, the DNA methylation pattern of the PSA gene in various LNCap sublines remained unaltered. Furthermore, methylation pattern changes cannot be detected using other methylation-sensitive restriction enzymes such as HhaI (Cfo I), Smal, and XhoI (data not shown).

Induction of PSA Gene Expression in Parental LNCap Cell by Conditioned Media from C4 Subline. As shown in Fig. 3A, an approximately 5- to 10-fold increase in the steady-state levels of PSA mRNA was detected in the parental LNCap cells by incubating with a 2-fold concentrated CM from the C4 subline. The induction by C4 CM on PSA gene expression was found to be concentration dependent (Fig. 3A) and exhibits cell type specificity. For example, whereas the CM from the M line showed only marginal PSA-inducing activity and

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<sup>a</sup> PSA levels were determined by the TANDEM-E method from Hybritech, Inc.
<sup>b</sup> PSA mRNA level in the parental LNCap cell line is defined as 1.0, based on the densitometrical quantification data.
the variable levels of PSA mRNA induction may be due to experimental variation (Fig. 3A), the CM from the parental LNCaP cells was totally inactive (Fig. 6). CMs prepared from other prostate cancer cell lines (such as PC-3 and DU-145) and a human bladder transitional carcinoma cell line such as WH cell line failed to exhibit similar PSA-inducing activity (data not shown). Unlike the increase in PSA mRNA levels, the expression of other androgen-regulated genes (such as ODC) was not affected by the CM from the LNCaP sublines (Fig. 3A), suggesting that the active component in the CM may be a unique factor(s) dissimilar from androgen. Previously, we have demonstrated that the CM from several fibroblast cell lines, including prostate (NbF-1 and rUGM), bone (MS), kidney (normal rat kidney), and lung (CCD16), fail to increase the steady-state levels of PSA mRNA. These data suggest that the expression of PSAF by the LNCaP subline may be organ-specific and thus could possibly represent a unique factor secreted by AI tumor.

Effect of Hormone, GFs, Neuropeptides, α1- and β2-Adrenergic Agonists on PSA Gene Expression in Parental LNCaP and Its Subline. As shown in Fig. 4(A and B), the addition of dihydrotestosterone is able to further increase steady-state levels of PSA mRNA of LNCaP cells in vitro (24, 27). Therefore, it is possible that the CM from these cells may also be able to induce PSA gene expression. However, as shown in Fig. 3B, CM from various fibroblast cell lines, including prostate (NbF-1 and rUGM), bone (MS), kidney (normal rat kidney), and lung (CCD16), fail to increase the steady-state levels of PSA mRNA. These data suggest that the expression of PSAF by the LNCaP subline may be organ-specific and thus could possibly represent a unique factor secreted by AI tumor.

Fig. 1. Elevation of PSA mRNA in parental LNCaP cells and their sublines. Sublines of LNCaP were derived as described above, and cells were maintained in serum-free T media containing 2% TCM supplement 24 h before harvest. Twenty µg of total cellular RNA were subjected to Northern analysis using random primer-labeled SsrI fragment (ca. 400 base pairs) isolated from PSA cDNA probe (A) or end-labeled PSA sequences-specific oligonucleotide probe (B). Sequence-specific oligonucleotide probes for PSA and hGK-1 were obtained from Dr. Trapman (25).

Fig. 2. Analysis of PSA gene in LNCaP cells and their sublines. High-molecular-weight DNAs were isolated from each subline of LNCaP as described above. Fifteen µg of total DNA were digested with either HindIII (A) or HpaII and MspI (B) and subjected to Southern analysis.

Fig. 3. The effect of PSA gene expression in parental LNCaP cells in the presence of CM from various sources. The parental LNCaP cells grown in the same conditions as in Fig. 1 were incubated with CM based on similar protein content (control cells were incubated with T medium containing 2% TCM and 2% fetal bovine serum). Forty-eight h after treatment, total cellular RNA was prepared and 20 µg of total cellular RNA were subjected to Northern analysis. A. protein content of CM is 1.3 mg/ml from M subline (IX) or is 1.0 mg/ml C4 subline (VI); B. protein content of CMs from each fibroblast line, ranging from 1.2 to 1.0 mg/ml. NRK, normal rat kidney fibroblastic cell line; rUGM, urogenital sinus mesenchymal cell line derived from fetal Noble rat; NbF-1, ventral prostate fibroblastic cell line derived from adult Noble rat; MS, human bone fibroblastic cell line derived from osteogenic sarcoma; CCD16, human lung fibroblastic cell line derived from normal adult; 3T3, embryonic mouse fibroblastic cell line (NIH-3T3). Note: the autoradiograph in B was exposed for a longer period than the one in A to determine if fibroblast CM stimulates PSA gene expression in parental LNCaP cells.
in both parental LNCaP and C4 subline cells. On the other hand, both C4 CM and dihydrotestosterone exhibited the additive effect on parental LNCaP cells (Fig. 4C). These data suggest that the PSA-inducing pathway mediated by PSAF may be different from that mediated by androgen receptor. To further examine the biochemical nature of the PSAF, we tested various known factors within the optimal concentration of growth stimulation for LNCaP and other cell types (27, 33–36). Results of these studies showed that no apparent increases in the steady-state levels of PSA mRNA can be detected in the parental LNCaP cells in the presence of GFs such as TGF-α, TGF-β, bFGF, NGF, HGF, and KGF (data not shown); neurotransmitters and that the induction may not be elicited through signal transduction pathways mediated by androgen receptor, protein kinase C, or protein kinase A.

**Partial Characterization of the PSAF from CM of the C4 Subline.** To characterize the action of PSAF on up-regulating PSA mRNA, parental LNCaP cells were incubated with 2-fold concentrated CM from the C4 subline for various periods. The CM was removed at the end of the incubation period, and the cells were changed to serum-free medium and assayed at 48 h posttreatment. Northern blot analysis of PSA mRNA revealed that a short-term exposure (1 h) of C4 CM to parental LNCaP cells is sufficient to elicit the signal(s) to increase the steady-state levels of PSA mRNA when determined at 48 h (Fig. 6A). The active PSA-inducing factor was tested by standard biochemical methods as shown in Fig. 6B. PSAF is sensitive to heat and acid treatment but resistant to trypsin digestion. Furthermore, the majority of PSAF activity is still retained in 60–80% of the ammonium sulfate fraction (Fig. 6C). This result suggests that PSAF must be a protein factor that resists protease digestion because of its unique amino acid composition.

**DISCUSSION**

Serum PSA is regarded as a useful marker for the diagnosis and prognosis of men with prostate cancer (37). Elevation of serum PSA was found in prostate cancer and benign prostate hyperplasia patients but not in prostate cancer patients who are undergoing prolonged remission and not in healthy men or women or patients with nonprostatic malignancies (37). Until recently, clinical data have indicated that serum PSA levels roughly correlate with tumor volume and stage of the prostate cancer (11–13, 37–39), suggesting that PSA may be the most useful prostate-specific marker available for the monitoring of prostate cancer progression and its therapeutic responsiveness. Although the clinical importance of PSA in monitoring prostate cancer progression is undisputed, wide variations exist in many patients with either localized or metastatic disease (12, 13, 37, 39). Evidence suggesting that factors other than androgen may be involved in the regulation of PSA includes the following: (a) Serum PSA levels in Stage D prostate cancer patients with bony metastasis are estimated to be higher than those found in other stages (37), which may be due to the higher tumor volume. In contrast, patients have elevated PSA levels with small tumor volume, which implies that factor(s) in the bone marrow may induce PSA expression by human prostate cancer cells. These results imply that factor(s) in the bone marrow may induce PSA expression by human prostate cancer cells. (b) Serum PSA levels rebound in castrated men with prostate cancer (11–13, 37) and in the mouse model of human prostate cancer (24). These observations are consistent with the suggestion that nontesticular factor(s) may play a role in regulating PSA expression by human prostatic cancer cells. These results suggest that nonandrogenic tissue factor(s) may regulate PSA expression by developing human prostatic epithelial cells. (c) In both cultured primary human prostatic epithelial cells (40) and LNCaP cells (19–24), androgen, GFs, and extracellular ma...
pomethylation of PSA gene in these cells. We exclude the possibility derived several LNCaP sublines from those AI tumors. Among these dent human prostatic carcinoma cells.

The same protein content (900 ng) from each fraction and crude were incubated with at the indicated concentrations, and each fraction was dialyzed with a 3500 molecular

CM from C4 subline was treated with 1 N HCl (4°C, overnight) and then neutralized with trypsin (10 mg/ml, 37°C, 2 h), then neutralized with trypsin inhibitor before adding to parental LNCaP cells. CM from C4 subline was precipitated with ammonium sulfate at the indicated concentrations, and each fraction was dialyzed with a 3500 molecular weight cut-off membrane against distilled water for 24 h and concentrated by lyophilizer. The same protein content (900 ng) from each fraction and crude were incubated with parental LNCaP cells for 48 h. The steady-state levels of PSA and ODC mRNA were determined 48 h after treatment.

trix were found to affect PSA production by these cultured human prostatic epithelial cells in vitro. Because of the potential significance of understanding of the nonandrogenic factor(s) in PSA regulation by human prostatic epithelial cells, we reported here that the identification and partial characterization of PSAF from C4 subline which may be responsible for regulating PSA expression by androgen-independent human prostatic carcinoma cells. A LNCaP human prostate cancer model that extends from AD to AI progression was established in our laboratory (41). We observed that the serum PSA levels produced by LNCaP tumors decreased dramatically shortly after castration. However, serum PSA rebounded at 3 weeks postcastration, indicating that the AD progression to AI can be studied in this experimental animal model. Subsequently, we have derived several LNCaP sublines from those AI tumors. Among these sublines, we found that C4 subline became tumorigenic in both intact and castrated hosts and secreted elevated levels of PSA both in vivo and in vitro in the absence of androgen steroids. In order to understand the underlying mechanism(s) for regulating the elevated levels of PSA mRNA C4 cells, first we examine both DNA amplification and hy-

pomethylation of PSA gene in these cells. We exclude the possibility that either the amplification or the hypomethylation of the PSA gene can contribute to the overexpression of the PSA gene in Southern blot analyses (Fig. 2). However, CM produced from the C4 subline is capable of increasing the steady-state levels of PSA mRNA in the parental LNCaP cell line. The induction of PSA mRNA and protein by PSAF in LNCaP cells appears to be concentration dependent and exhibits organ specificity (Fig. 3B); this effect is specific, as illus-


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