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FAST TRACK

Proprotein Convertases Regulate Activity of Prostate Epithelial Cell Differentiation Markers and Are Modulated in Human Prostate Cancer Cells

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Abstract Prostate derived factor (PDF) is a member of transforming growth factor-β (TGF-β) superfamily proteins involved in differentiation of the prostate epithelium. Proprotein convertases (PCs) such as furin are thought to mediate the processing of TGF-β superfamily. In the present study, we demonstrated for the first time that human prostate cancer cell lines differentially synthesize and secret prostate derived factor (PDF), and that PDF secreted by LNCaP is processed by PCs. Exposure of LNCaP cells to the decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK), a synthetic furin-like protease inhibitor, inhibited PDF processing and resulted in the loss of luminal cell phenotype and induction of basal cell phenotype in LNCaP cells as demonstrated by alternations in the expression of cytokeratins 8, 14, 18, and 19, markers of prostate epithelial cell differentiation. These results suggest that proprotein convertases may be involved in the regulation of prostate epithelial cell differentiation, and may be an important target of prostate cancer therapy. J. Cell. Biochem. 88: 394–399, 2003.

Key words: proprotein convertases; TGF-β superfamily; prostate derived factor; furin; prostate

The prostate is a male sex accessory gland, and an organ of frequent benign and malignant growths, which constitute a major health hazard in men. Prostate Cancer is currently the most common cancer and the second leading cause of cancer deaths in men in the United States [Parker et al., 1997]. Transforming growth factor-β (TGF-β) superfamily proteins, which include bone morphogenetic proteins (BMP), play important roles in maintaining prostatic differentiation and regulating prostate cancer cell growth [Barrack, 1997; Lee et al., 1999; Wikstrom et al., 2001].

TGF-βs consists of an expanding number of proteins similar in structural and functional properties. They are first synthesized as larger biologically inactive precursors which are proteolytically processed at a dibasic site (R-X-X-R) to release mature TGF-βs. This processing site is a consensus cleavage motif for proprotein convertases including furin. In fact, it has been reported that furin, PACE-4, and PC5B have a potential to process proTGF-β and that furin [Dubois et al., 1995; 2001], PACE-4, PC6B, and PC7 also process proBMP-4 [Cui et al., 1998].

Previous studies have demonstrated that normal and malignant prostate cells produce TGF-βs [Harris et al., 1994; Perry et al., 1997; Yonou et al., 2001]. To induce biologic activity, secreted TGF-βs must be activated. But, in general, very little is known about secretion and processing of TGF-βs in prostate cells. Here we test the hypothesis that the lack of the capacity to process TGF-βs is closely linked to tumorigenesis in the prostate. To investigate the behavior of TGF-βs in prostate cancer cells, we studied a novel TGF-β superfamily protein, PDF [Paralkar et al., 1998]. PDF is a divergent member of TGF-β superfamily protein that is expressed highly in placenta and prostate. PDF is also known as placental bone morphogenetic

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protein (PLAB) [Hromas et al., 1997], placental transforming growth factor-β (PTGF-β) [Yokoyama-Kobayashi et al., 1997], macrophage inhibitory cytokine-1 (MIC-1) [Bootcov et al., 1997] and growth and differentiation factor-15 (GDF-15) [Bottner et al., 1999].

In the present study, we investigated the secretion of PDF and the effects of the synthetic furin inhibitor, CMK in different human cancer cell lines.

MATERIALS AND METHODS

Materials

CMK was obtained from Calbiochem (San Diego, CA) and dihydrotestostelrone (DHT) from Sigma (St. Louis, MO). Anti-proPDF antibody, anti-Cytokeratin 8, 18, 19 antibodies, and anti-prostate specific antigen antibody (PSA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cytokeratin 14 and 15 from Chemicon (Temecula, CA). *N*-t-butoxycarbonyl-Arg-Val-Arg-Arg-7-amino-4-methylcoumarine (Boc-RVRR-AMC) was obtained from Bachem, Bioscience (King of Prussia, PA). An antisera against mature PDF was raised by injecting a 20-aa peptide sequence (CQKTDTGVSLQTYDDLLAKD) located in the C-terminus of PDF to rabbits as described previously [Tan et al., 2000]. LNCaP, DU145, and PC3 cell lines were obtained from the American Type Culture Collection (Rockville, MD). RPMI-1640 was purchased from Cellgro Mediatech (Herndon, VA) and fetal bovine serum from Atlas biologicals (Fort Collins, CO).

Cell Culture

Human prostate cancer cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and cultures maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

RT-PCR Analysis

Total RNA was prepared from various cell lines using RNeasy (Qiagen, Valencia, CA) according to the manufacture's instruction. cDNA was prepared using Superscript (Invitrogen, Carlsbad, CA). The efficiency of each cDNA reaction was assayed by amplification of β-actin transcripts with primers for β-actin (Promega, Madison, WI). Primers used to amplify PDF gene were: sense, 5′-CATTCAAAAGACCGACACC; antisense, AGGTGCACAGTGGAAGGA-3′. The PCR condition of PDF was as follows: 1cycle at 94°C for 3 min, 30 cycles at 95°C for 15 s, 54°C for 30 s, 72°C for 1 min. The PCR reaction used a volume of 50 μl with 2.0 mM $MgCl_2$ and 2.5 U of PLATINUM Taq DNA Polymerase (Invitrogen, Carlsbad, CA). To check the DNA contamination, control experiments in which no reverse transcriptase was added prior to the PCR were performed. Amplification products (10 μl) were separated by 8% polyacrylamide gel electrophoresis.

Western Blot Analysis

Cells (approximately 1×10^6 cells) were seeded in 100 mm cell culture dishes and cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 5% CO_2. Subconfluent cells were washed with phosphate buffered saline (PBS), lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) and used for cell lysate. For the detection of PDF in conditioned media, subconfluent cells was serum-starved for 24 h. The serum free media were collected and precipitated with 10% trichloroacetic acid. After centrifugation, pellets were dissolved in the buffer which consisted of 8 M urea, 50 mM Tris (pH 8.0), and 0.1% NP-40. Samples were mixed in 5 × Laemmli sample buffer with 2% betamercaptoethanol, subjected to electrophoresis on a 15% SDS–polyacrylamide gel, and transferred to PVDF membranes. Membranes were blocked with PBS containing 0.1% Tween 20 and 5% non-fat dry milk for 1 h at room temperature and incubated with the appropriate primary antibodies and antisera (1:500–2,000) overnight at 4°C. After the membranes were washed with PBS containing 0.1% Tween 20 three times, the membranes were incubated with appropriate horse-radish peroxidase-conjugate secondary antibodies (1:2,000) for 1 h at 4°C. The protein bands were visualized by chemiluminescence using SuperSignal West Pico ECL kit (PIERCE, Rockford, IL). This protocol was used for the detection of PDF, cytokeratin, and PSA.

Proprotein Convertase Activity Assay

For determination of cellular PCs activity, 30,000 cells were seeded in 48-well plates for 24 h. Next day, growth medium and CMK were added (final concentration; 100 μM). After 24 h incubation, media were replaced and cells were washed with PBS. Additional 48-well plates were prepared same way for the determination of cell number, which was used for

normalization. 150 μl of assay medium which consists of RPMI-1640 with 0.25% Triton X-100 for permeabilization and boc-RVRR-amc (100 μM) as a fluorogenic substrate were added to each well. Fluorescence was measured at 360 nm excitation and 460 nm emission wavelengths after 4 h of substrate addition. The data were normalized to cell number. Human recombinant furin (Sigma) was used for positive control.

Statistical Analysis

Data are presented as the mean ± SD of three culture wells in each of two to six independent trials. Statistical analysis was performed using Student's t test. $P < 0.05$ was considered significant.

RESULTS

PDF Synthesis and Secretion in Different Human Prostate Cancer Cell lines

Previous studies have demonstrated expression of PDF in normal and malignant prostate cells [Paralkar et al., 1998; Thomas et al., 2001]. PDF gene expression was reduced in primary prostate cancer tissues as compared to non-neoplastic prostate tissues [Thomas et al., 2001]. To test our hypothesis that alteration in PDF processing or expression may be linked to tumorigenesis, we examined cellular and secreted PDF in different androgen-dependent (LNCaP) and androgen-independent (PC3 and DU145) cell lines. Total cellular RNA isolated from different prostate cancer cell cultures was subjected to RT-PCR. PDF mRNA expression was detected in LNCaP cells and PC3 cells, but not in DU145 cells (Fig. 1A). The presence of PDF protein was further confirmed by immunoblot analysis. ProPDF was detected in LNCaP and PC3 cell lines by an anti-proPDF antibody, which recognized proPDF but not mature PDF. However, an anti-mature PDF antisera failed to detect mature PDF in cell lysates from all cell lines. Mature PDF, and not proPDF, was detected in LNCaP conditioned media, but only proPDF was detected in PC3 conditioned media (Fig. 1B). These results demonstrated that mature PDF was secreted and processed only by LNCaP cells, and not by DU145 and PC3 cells. That proPDF was secreted by PC3 cells without processing suggest that these cells may be deficient in PCs. Furthermore, DU145 cells did not express PDF.

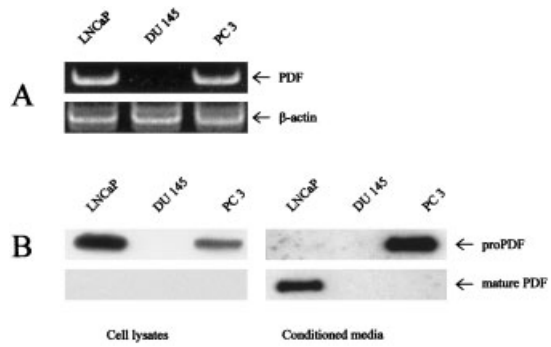

Fig. 1. **A**: PDF mRNA expression in different human prostate cancer cell lines. PDF mRNA expression was analyzed by RT-PCR as described in Materials and Methods. PDF-specific primers yield a 100 bp fragment. **B**: Differential PDF synthesis and secretion by different human prostate cancer cells. Immunoblots were performed with anti-proPDF antibody (~40 kD proPDF form) and anti-PDF antisera (~17 kD mature PDF form).

Inhibition of PDF Processing by Furin-Like Protease Inhibitor, CMK

Since BMP-4 and TGF-β1 were processed by PCs [Dubois et al., 1995; Cui et al., 1998], we hypothesized that the PDF processing in LNCaP cells was due to PCs. To test this hypothesis, we examined PC activity in prostate cancer cells and the inhibition of PCs activity by CMK. Cells were treated with CMK in the presence of fluorogenic substrate, boc-RVRR-amc, and PC activity was assayed. Our results demonstrated significantly higher activity in LNCaP cells than in PC3 and DU145 cells and the inhibition of PCs by CMK for 24 h (Fig. 2). We also examined PC dependent PDF processing in LNCaP cells in the presence of CMK by immunoblotting for PDF. The treatment with CMK resulted in the reduction of PDF processing in LNCaP conditioned media in a dose-dependent manner, and concomitant increase in proPDF (Fig. 3). CMK did not affect cellular PDF.

Effect of CMK on Prostate Luminal and Basal Cell Phenotype Markers in LNCaP Cells

In the prostate, luminal epithelial cells differentiate from basal epithelial cells. The cell types are distinguished by the expression of cytokeratin 8 and 18 in the luminal and cytokeratin 5, 15, and 14 in the basal epithelial cells [Xue et al., 1998]. Cytokeratin 19 is suggested to be a marker of intermediate stage in the differentiation process of prostate cells [Hudson

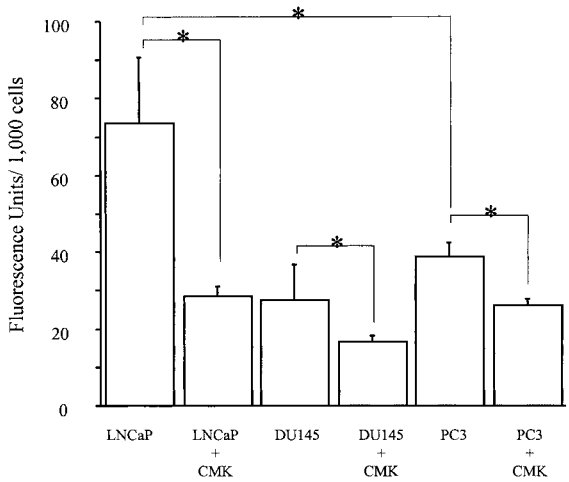

Fig. 2. Determination of PCs activity by measuring the cleavage of fluorogenic substrate, boc-RVRR-amc, in human prostate cancer cell lines and the effect of 100 μM CMK. Cells were incubated in the presence or absence of 100 μM CMK for 24 h. Thereafter fluorogenic substrate (100 μM) was added and cells were incubated for additional 4 h. $*P < 0.001$.

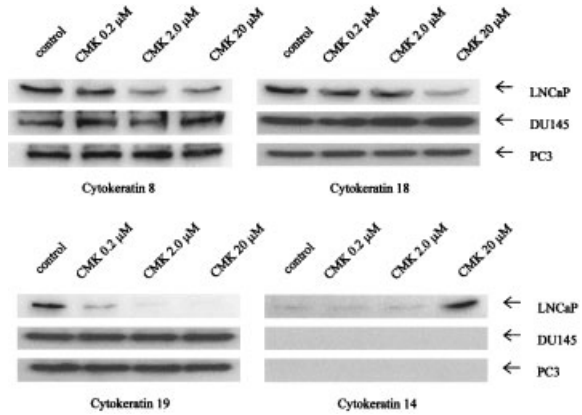

Fig. 4. The effects of CMK on prostate epithelial cell differentiation markers. Cells were treated by CMK in the presence of serum-free RPMI-1640. Culture medium and CMK were replaced every 24 h. Cells were treated for 72 h, lysed, and electrophoresed on 15% SDS–PAGE. Immunoblots were performed with anti-cytokeratin 8 (~52 kD), 14 (~52 kD), 18 (~45 kD), and 19 (~44 kD) antibodies. Cytokeratin 8 and 18 represent luminal epithelial cell phenotype. Cytokeratin 19 and 14 represent intermediate differentiation and basal epithelial cell phenotype, respectively.

et al., 2001]. Recent data have demonstrated that TGF-β induced the upregulation of luminal and the downregulation of basal cytokeratin gene expression in NRP-152 rat prostate basal epithelial cells [Danielpour, 1999]. We examined whether CMK regulate the differentiated phenotype of LNCaP cells as a result of inactivation of TGF-β superfamily processing, including PDF. Prostate cancer cell lines were treated with various concentrations of CMK for 72 h, and cell lysates were analyzed by Western blot. Results showed that CMK downregulated the expression of cytokeratin 8, 18, and 19 in LNCaP cells, but not in DU145 and PC3 cells. Cytokeratin 14 was upregulated in a dose dependent manner in LNCaP and was not detected in DU145 and PC3 cells (Fig. 4).

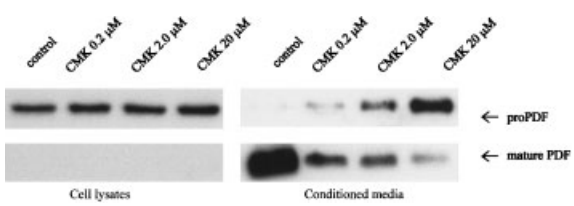

Fig. 3. Inhibition of PDF processing by CMK in LNCaP cells. Cells were cultured and serum-starved with and without various concentrations of CMK for 24 h. Cells and supernatants were electrophoresed on 15% SDS–PAGE. Immunoblots were performed with anti-proPDF antibody (~40 kD proPDF form) and anti-PDF antisera (~17 kD mature PDF form).

Proprotein Convertase Inhibition Decreases DHT-Induced PSA Secretion in LNCaP Cells

PSA is a widely used and important serological marker for prostate cancer in patients. PSA expression is normally regulated by androgens in well-differentiated prostate epithelial cells [Hayward and Cunha, 2000]. Increased plasma TGF-β levels in the prostate cancer patients are correlated with elevated PSA levels [Adler et al., 1999]. A role of smad3, an intracellular signaling mediator of TGF-β, in the regulation of PSA gene expression via cooperation with androgen receptor has been reported in LNCaP cells [Kang et al., 2001]. Therefore, it is important to investigate the effect of CMK on androgen induced PSA expression to understand the mechanism of action of TGF-β superfamily including PDF in prostate tumorigenesis. LNCaP cells were treated with various concentrations of CMK for 24 h in the presence or absence of DHT, and cell lysates were analyzed by Western blot. Our results demonstrated that androgen-induced PSA expression was inhibited by the addition of CMK, whereas the levels of endogenous PSA in the absence of DHT were not affected (Fig. 5). Since PSA is not expressed in

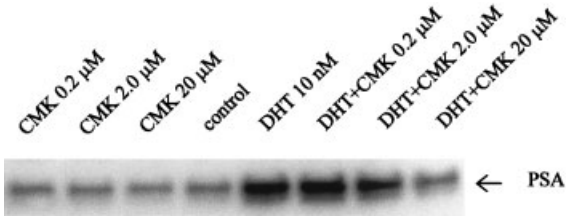

Fig. 5. The effect of CMK on the DHT-stimulated PSA expression in LNCaP cells. DHT and CMK were added and cells were incubated for 24 h. Cell lysates were electrophoresed on 15% SDS–PAGE. Immunoblots were performed with anti-PSA antibody (~37 kD).

PC3 and DU145 cells, these experiments were not conducted in these cell types.

DISCUSSION

In this study, we demonstrated for the first time differential production, secretion, and processing of PDF in various prostate cancer cell lines, LNCaP, PC3, and DU145. We also demonstrated proprotein convertase dependent alterations of prostate epithelial differentiation assessed by cytokeratin expression patterns and androgen dependent PSA production.

Several groups have described the correlation between TGF-βs and development and tumorigenesis of the prostate [Barrack, 1997; Lee et al., 1999; Wikstrom et al., 2001]. Previous studies have also demonstrated the effects of TGF-βs on and the synthesis and localization of TGF-βs in normal and malignant prostate cells [Harris et al., 1994; Perry et al., 1997; Yonou et al., 2001].

The data presented here demonstrated PDF secretion and processing in prostate cancer cell lines. PDF was synthesized as a proPDF form in LNCaP and PC3 cells, and activated by PCs in LNCaP, but not in PC3 cells. The differences in cell phenotypes of LNCaP and PC3 cells may be in part due to impaired maturation of the TGF-β superfamily members in PC3 cells. Prostate cancer cells including LNCaP and PC3 have been shown to produce a variety of BMPs, which stimulate osteoblastic bone formation and cause osteoblastic metastasis [Yoneda, 1998]. Similarly the mRNA expression of various BMPs has been reported in LNCaP and PC3 cells. In vivo, LNCaP tumor stimulate osteoblastic responses, while PC3 tumor result in extensive bone destruction and osteolytic responses [Soos et al., 1997; Shevrin et al., 1988]. Differences in the activation of TGF-βs including PDF by PCs may contribute to these responses.

Our results have demonstrated different activity of PCs between human prostate cancer cell lines. The activity of PCs was significantly higher in LNCaP cells compared to DU145 and PC3 cells. The treatment of cells with CMK inhibited activity of PCs in all cell lines. To our knowledge, these observations are the first to show differences in PCs activity among different human prostate cancer cell lines. Our observations clearly demonstrate that PCs activity in LNCaP cells is responsible for PDF processing. Although PC3 cells had low activity of PCs, we were unable to detect processing of PDF. This suggests that a specific PC active on PDF is deficient in PC3 cells.

We also demonstrated the inhibition of PDF processing by CMK caused alterations in the regulation of luminal and basal prostate epithelial cell differentiation markers in prostate cancer. Our results showed the downregulation of cytokeratin 8, 18, and 19 and the upregulation of cytokeratin 14 by the addition of CMK in LNCaP cells, indicating the loss of differentiated cell characteristics. It had recently been demonstrated that TGF-β induced the upregulation of luminal and downregulation of basal cytokeratins gene expression in rat normal prostate cell line [Danielpour, 1999]. Therefore, our results suggest that the inhibition of PCs activity reduces the processing of TGF-βs including PDF and results in the loss of differentiated cell phenotype. Our results showed that CMK treatment did not affect the expression of cytokeratins in DU 145 and PC3 cells, which could be due to the lower activity of PCs or absence of specific PC.

In addition, we demonstrated that CMK inhibited DHT-induced PSA expression. PSA is expressed in well-differentiated prostate epithelial cells and regulated by androgens [Hayward and Cunha, 2000]. Cooperation between androgen receptor and smad3 has been shown to induce PSA gene expression [Kang et al., 2001]. Our results are consistent with these observations. Thus, it is possible that inhibition of processing of TGF-β superfamily proteins, including PDF may result in the reduction of the androgen-induced PSA expression.

In conclusion, our results provide the evidence that PCs play an important role in the

processing of PDF, a member of the TGF-β superfamily, in prostate cancer cells and regulate prostate epithelial cell differentiation.

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