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BRIEF REPORT

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The role of endocrine gland derived vascular growth factor/ Prokineticin-1 in human prostate cells

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Abstract: In steroidogenic tissues, a novel class of angiogenic molecules known as endocrine gland-derived vascular endothelial growth factors (EG-VEGF)/prokineticins are primarily produced. Here, we investigated how EG-VEGF/PROK1, a member of PROKs family, and its receptor are able to affect cellular motility in both non-neoplastic and cancerous human prostate cells. Using Western blot and motility test studies, EPN cells, a non-transformed cell line and Cancer Epithelial Prostatic Cells (CEPC) were employed as cellular models in the current investigation. Western blot examination of EPN normal prostate cells treated with EG-VEGF/PROK1 revealed that ERK1/2 was rapidly phosphorylated within 5, 10, and 20 minutes, while CEPC had high and sustained ERK1/2 activity at the same periods. Then, compared to normal EPN prostate cells, CEPC treated with EG-VEGF/PROK1 for up to 72 hours demonstrated enhanced cell motility. Based on our findings, EG-VEGF/PROK1 may play a role in prostate cancer progression by controlling angiogenesis and the motility of metastatic cells in CEPC cells, likely as a consequence of ERK1/2 activation, as contrasted to EPN normal prostate cells.

***Keywords*:** prostate cancer, EG-VEGF, EPN, CEPC, ERK1/2

Introduction

Angiogenesis plays a crucial role in the development, invasion, and metastasis of various cancers, including prostate cancer (*1,2*). Creating new microvascular endothelium is crucial for optimal prostate development, in fact (*3,4*). A link between the hormonal regulation of prostate tissue and angiogenesis caused by the paracrine actions of endothelial cells has been proposed. Prostate cells generate a number of angiogenic substances, including vascular endothelial growth factor (VEGF). The majority of prostate cancers have been shown to have higher VEGF levels and microvessel density, both of which are associated with a worse prognosis (*4-8*). Two novel endocrine gland-derived vascular angiogenic factors (EG-VEGF)/prokineticin-1 (PROK1) and prokineticin-2 (PROK2) that specifically affect endothelial cells (EC), have recently been identified (*9*). PROK1 is similar to Prok1, a previously cloned mammalian ortholog of mamba intestinal toxin-1(MIT-1) (*10*). However, other EC types, such as those derived from the aorta, umbilical vein, and dermis, are not affected by PROK1 and PROK2, which induce proliferation and migration of EC derived from adrenal capillaries (*11-13*). These peptides, which are structurally unrelated to VEGF and have 10 cysteine residues in the same places in all family members, control a variety of biological processes in steroidogenic and non-steroidogenic tissues, including smooth muscle contraction and, in particular, angiogenesis.

G-protein-coupled receptors recently discovered and given the names PROK-R1 and PROK-R2 (*11-13*) carry out the physiological actions of PROK1 and PROK2. Recombinant PROKs in nanomolar concentrations bind to and activate these receptors. In accordance with the effects of PROKs on smooth muscle contraction and angiogenesis (*11-13*), activation of PROK-R causes calcium mobilization, stimulation of phosphoinositide turnover, and activation of ERK1/2 pathways. The overexpression of PROK1 in a colorectal cancer cell line has recently been shown to cause angiogenesis and tumor growth after injection in a nude mouse model (*14*). Contrarily, PROK1 expression was diminished in advanced ovarian cancer but was still seen in the early stages of this illness (*15*), but its expression was typically undetectable in endometrial carcinoma (*16,17*). In order to assess the possible contribution of this factor to human prostate cancer, we examined the effects of PROK1 on ERK1/2 activation and cellular motility in normal and malignant human prostate cells.

Materials and Methods

Prostate cell lines and primary prostate cell cultures

Different centrifugations of minced and prostatic tissues treated with collagenase (Collagenase IV, Gibco-BRL, Milan, Italy, 10 mg/mL) separated cancer epithelial prostatic cells (CEPC). The CEPC were plated on keratinocyte-SFM (KFSM) medium from Gibco-BRL in Milan, Italy, which also included 5% fetal bovine serum (FBS), bovine pituitary extract (10 mg/mL), epidermal growth factor (10 ng/mL), cholera toxin (10 ng/mL), and antibiotics (fungizone and penicillin-streptomycin). After EDTA-trypsin treatment, cultures were separated and utilized at first passage at confluence. According to previously documented techniques (*18,19*), the epithelial type was determined if cytokeratin immunostaining was positive in almost all of the cells. The high expression of the proliferative antigen Ki67 and, more specifically, the high expression of mutated p53 protein, as shown by immunoreactivity with the monoclonal antibodies clone Ki-67 (DAKO, Milan, Italy) and Pab 240 (Serotec, Delta Biological, Italy), respectively, confirmed the malignant nature of cells derived from prostate carcinomas. The experimental methods were performed at least three times with four cell strains taken from CEPC. Our group (*19*) identified and characterized a non-transformed cell line known as EPN cells, which were grown in HAM-F12 enriched with 3% FBS and antibiotics (fungizone and penicillin-streptomycin). Every culture was kept alive at 37°C in a humidified 5% CO_2 environment.

Western blot analysis and protein extraction

Samples of prostate cells were homogenized directly into the lysis buffer, which contains the following ingredients: 50 mM HEPES, 150 mM NaCL, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mg aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate. Centrifugation was used to clarify the lysates at a rate of 14,000 × 10 min. The amount of protein was determined using a Bio-Rad test (Bio-Rad, München, Germany), and it was then boiled for five minutes in Laemmli buffer (Tris-HCl pH 6.8, 0.125 M, SDS 4%, glycerol 20%, 2-mercaptoethanol 10%, and bromophenol blue 0.002%), as previously reported (*19-25*). Proteins were exposed to reducing conditions SDS-PAGE (15% polyacrylamide). Using pre-stained protein standards, the full transfer of proteins to nitrocellulose membranes (Immobilon Millipore Corporation) during electrophoresis was determined (Bio-Rad, Hercules, CA). The membrane was incubated with the primary antibody against ERK1 (1:1,000; #sc-94-G, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and P-ERK1 for 1 h after blocking with TBS-BSA (25 mM Tris, pH 7.4, 200 mM NaCL, 5% bovine serum albumin), at room temperature. After 45 minutes at room temperature, membranes were treated with the horseradish peroxidase-conjugated secondary antibody (1:4,000), and an ECL system was used to monitor the response (Amersham Life Science, UK).

Wound recovery

Confluent cells were cultivated in KSFM containing 3% FCS before being maintained for 48 hours in serum-free media. In order to lower the content of endogenous steroids, the monolayers were scraped with a sterile, disposable 200 µL plastic pipette tip and then treated with KSFM, KSFM added with 5% charcoal-treated FCS (5% DCC), or 3% FCS. Then, monolayers were captured at various time frames *via* photography (24 h). The shown figure is representative of three independent assays.

PCR

As previously disclosed (*26*), reverse transcriptase-polymerase chain reaction (RT-PCR) RNAs were reverse transcribed using 5 µg of total RNA (*25*). We performed an RNA transcription without the use of reverse transcriptase to generate a negative control for the amplification reactions. A total of 50 µL of cDNA (400 ng) amplified using RT of RNAs was mixed with 10 mmol Tris-HCl, 1.5 mmol MgCl2, 50 mmol KCl (pH 8.3), and 100 ng 5'-3' end primers. 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec each made up the PCR conditions. In a semiquantitative PCR, these genes were amplified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (22 cycles) as previously reported to assess the variability in the expression of PROK1 and PROK-R1 (*25*). For GAPDH, we employed oligonucleotide sequences (*25*). Additionally, we utilized the following primers: PROK-R1 sense (5'-GCG GCA TTG GAA ACT TCA-3') and antisense (5'-GGC CCA CGA ATT CTA TGC C-3') for PROK-R1and PROK1 sense (5'-CGC GAG TCT CAA TCA TGC TCC T-3') and PROK1 antisense (5'-GGC AAG GCG CTA AAA ATT GAT G-3'). After that, PCR products were separated using a 100-bp DNA ladder from Life Technologies as a size marker on a 1.2% agarose gel that also included ethidium bromide.

Statistic evaluation

The results, which came from at least three different experiments with each point carried out in triplicate, were presented as mean + SE (standard error of the mean). The analysis of variance was used to compare the means.

Results and Discussion

Both non-neoplastic and cancerous epithelial prostate cells express PROK1 and PROK-R1. In order to determine how these genes are expressed in CEPC primary cultures and the EPN cell line, we used semiquantitative RT-PCR. EPN and CEPC both contained the mRNAs for PROK1 and PROK-R1 (Figure 1A).

Cultures of EPN and malignant (CEPC) human prostate cells, fasted for 48 h and maintained in 0.5% FCS, were treated with 40 nM PROK1 to see whether it activated the ERK1/2 pathway. Then, in cell lysates made after 5, 10, and 20 min of treatment, Western blot analyses were performed to confirm that ERK1/2 was activated. Figure 1B demonstrates that within 5 min, PROK1 produced a fast phosphorylation of Erk1/2 in EPN cells, which persisted for up to 20 min. After 5 minutes, PROK1 also significantly enhanced ERK1/2 phosphorylation in CEPC cultures, however unlike in normal cells, the elevated phosphorylation levels were greater than in the normal EPN cell line (Figure 1C). Since there was no change in the ERK1/2 protein, staining with anti-Erk1/2 and anti-phospho-Thr/Tyr antibodies demonstrated that the action of PROK1 on ERK1/2 was caused by phosphorylation of the enzyme rather than an increase in ERK1/2 expression (Figure 1C).

We examined the role of PROK1 in the control of prostate epithelial cells' motility using a wound-healing experiment in EPN and CEPC cells. Before being scraped and thereafter being stimulated with FCS, confluent cell monolayers were kept in serum-free media for 48 hours. Cell motility in CEPC cells was higher than in EPN cells after 15 hours of serum administration. Contrarily, when cells were kept in serum-free media, neither EPN nor CEPC showed much evidence of cell motility (Figure 1, D and E). Contrary to what may be expected, the scrape wound persisted in the monolayer of EPN cells for 24 hours after PROK1 (40 nM) was added to the CEPC cells (Figure 1, D and E). Few CEPC cells began to migrate from the wound's edges in the absence of serum, while EPN cells remained stationary.

Earlier, we reported that whereas PROK1 expression is scarcely perceptible in the non-neoplastic human prostate, it is significantly increased in prostate cancer. We specifically demonstrated the expression of PROK1 by prostate epithelial cells and the association between expression levels and Gleason scores (*25*). Additionally, we discovered that the amount of the PROK1 protein increased significantly and steadily as the grade of the prostate cancer increased from low to medium to high, showing the significance and specificity of PROKs as predictive biomarkers for prostate cancer progression (*25*). Further evidence that PROKs may directly affect epithelial cells comes from the finding of EG-VEGF receptor (PROK-R1) transcripts in both normal (EPN)

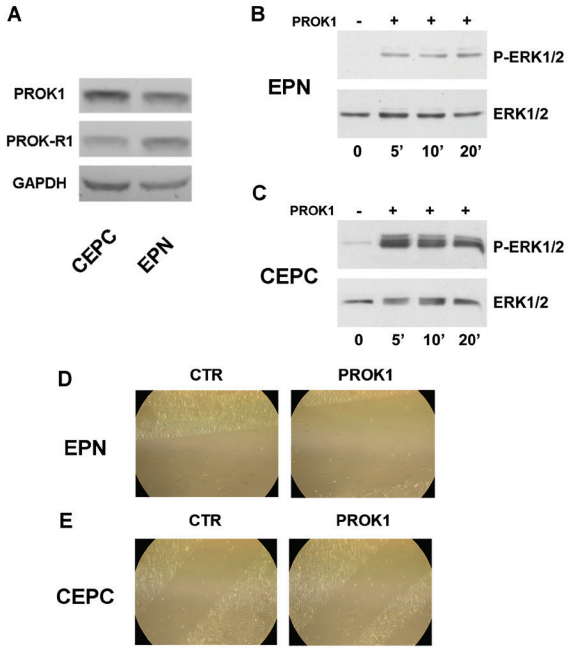

Figure 1. PROK1 activity in prostate-derived cell models. (A) GAPDH, PROK1, and PROK-R1 primers were used to reverse transcribe and amplify total RNA, respectively. PCR results were run through a 1.2% agarose gel electrophoresis, stained with ethidium bromide, and then captured on camera. Using GAPDH as an internal reference, semiquantitative RT-PCR was used to ascertain the mRNA levels of PROK1, PROK-R1. PCR results were run through a 1.2% agarose gel electrophoresis, stained with ethidium bromide, and then captured on camera. 40 nM PROK1 was applied to EPN **(B)** and CEPC **(C)** for the specified durations. Lower panels of (B) and **(C)** show blots with antibodies against Erk1/2 protein, whereas the top panels of **(B)** and **(C)** display blots treated with antibodies raised against phospho-Erk1/2. There are two distinct bands at 44 and 42 kDa. Three different experiments are shown by the blots. EPN **(D)** and CEPC **(E)** were damaged while serum-starved, cultured in KSFM (SF), and KSFM was subsequently supplemented with 40 nM of PROK1. After 24 hours of therapy, the morphology of cell motility is shown.

and cancerous (CEPC) prostate cells (*25*). The biological foundation of tumor angiogenesis has long piqued researchers' curiosity in cancer. Blood vessel changes are associated with abnormal pathways, apoptosis, androgen receptor signalling, signal transduction, cytokines, and cell adhesion molecules. The VEGF pathway is one of the main regulators of this process. This element, which is widely expressed in both non-neoplastic and cancerous tissues, is primarily accountable for the formation and upkeep of the aberrant tumor vascular network. Current preclinical and clinical investigations in cancer on the suppression of the VEGF pathway were inspired by these findings. Anti-VEGF medication has extra systemic consequences, such as causing endothelial cell death and altering the functioning of the vascular bed, despite the fact that it may have antiangiogenic qualities and seem promising. LeCouter *et al*. discovery's of the EG-VEGF gene, a novel angiogenic agent mostly produced in endocrine cells that specifically works on the endothelium of endocrine gland cells, has opened up

new perspectives on the pathogenesis and treatment of endocrine-related tumors (*9*). In fact, the discovery of angiogenic tissue-selective factors may open the door to tissue-specific angiogenic treatments with minimal systemic side effects. The fundamental reason for the divergence between EG-VEGF and VEGF is the selectivity of expression region. In actuality, PROK1 is only present in cells that produce hormones, such as those in the ovaries, testes, and adrenal gland (*9,27-29*). About how PROKs are expressed in cancerous tissues, not much is known. Recent research showed that colorectal cancer cells express PROK1. Antisense PROK1 injections into mice also resulted in angiogenesis and tumor development suppression. Prostate cancer and PROKs have not been linked, and nothing is known about the expression of PROK-R in non-neoplastic and cancerous human prostate (*14,15*).

In the current work, we demonstrate that the malignant epithelial prostate cells (CEPC) and EPN, a human prostate epithelial cell that has not undergone transformation, have distinct levels of MAP kinase (ERK1/2) activity. This may be partially explained by our recent discovery that normal and cancerous human epithelial prostate cells exhibit varying degrees of fast, non-genomic effects of PROK1 on Erk1/2 (*30*). We demonstrate that a 5-minute PROK1 therapy causes the Erk1/2 pathway to be activated in both CEPC and EPN. Erk1/2 phosphorylation levels being altered suggests that regulatory kinases and/or phosphatases upstream and/or downstream of Erk1/2 are altered in tumor cells because of the pathway's rapid stimulation, which is comparable to many extracellular signals that start cytoplasmic signal transduction pathways. Actually, multiple cancer processes have been altered, resulting in altered cell phenotypes that affect the regulation of cell proliferation. Migration of tumor cells to distant organs is not a routine process. Breast, prostate, and lung cancers – the most prevalent solid tumors – metastasize particularly to the bones (*30*). The preferred spreading of certain tumors to the bones is determined by the biological characteristics of the cancer cells and the environment of the metastatic target site (*30*).

Cell motility is a crucial element in the spread of prostate cancer. Our findings suggest that PROK1 may be a novel potential component involved in the stimulation of cell migration and motility. Finding the genes and related molecular mechanisms that cause epithelial prostate cells to transdifferentiate into a transformed phenotype is essential for developing novel prostate cancer treatment methods. Therefore, the presence or absence of molecules associated to motility may be exploited to help with the diagnosis and prognosis of human prostate cancer. Modified expression of these genes may govern the metastatic potential of any specific prostate tumor. Our study demonstrates that PROK1 has stimulating effects on prostate epithelial tumor cell growth and migration *in vitro*, suggesting a role in the neoplastic progression.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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