Expression and Localization of Endothelin-Converting Enzyme-1 in Human Prostate Cancer

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Endothelin (ET)-1 can influence cancer invasion and metastasis by exerting an autocrine (epithelial) or paracrine (stromal) influence on growth. ET-1 is generated from big ET-1 by endothelin-converting enzyme (ECE)-1, which has four recognized isoforms, ECE-1a, ECE-1b, ECE-1c, and ECE-1d, differing only in their amino-terminal regions. This study investigated the expression and localization of the ECE-1 isoforms in prostate cancer (PC). The epithelial cell lines used were androgen-sensitive LNCaP, androgen-independent PC-3 and Du145, and nonmalignant transformed PNT1-a, PNT2-C2, and P4E6 prostate cells. Primary cells derived from malignant and benign tissue from radical prostatectomies were also exploited. Previously, we reported increased ECE-1 expression in androgen-independent PC cell lines, as compared with androgen-sensitive cells. Our present data show that transcripts for all ECE-1 isoforms were present in all epithelial cell lines analyzed. However, only the ECE-1c protein was detectable in PC-3, Du145, PNT2-C2, and PNT1-a cells. ECE-1c localized to both the cell surface and intracellular compartments in individual cell lines. In primary stromal cells, all individual ECE-1 isoforms were expressed at the mRNA level, with the exception of ECE-1a. ECE-1b and ECE-1c protein levels were higher in malignant stromal cells, as compared with benign cells. In stroma, ECE-1c protein was localized to the cell surface, with filamentous immunoreactivity throughout the cell, whereas ECE-1b immunoreactivity was punctate throughout the cytoplasm. The upregulation of the ECE-1c isoform in PC cell lines is being investigated further. Exp Biol Med 231:1106–1110, 2006

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Introduction

Prostate cancer (PC) is the most common cancer in the United Kingdom and the second most common cause of cancer death. In 2000, in the United Kingdom, 27,149 new cases were diagnosed and just under 10,000 deaths reported (United Kingdom Office for National Statistics, 2003). Conventional treatment for PC is almost ineffective in patients with metastatic disease, and approximately 70% of patients die within 5 years (1).

A mitogenic role for small regulatory peptides, such as endothelin (ET)-1, bradykinin, bombesin-like peptides, and neurotensin, has been indicated in various stages of PC (2, 3). ET-1, in particular, has been demonstrated in prostatic tissue *in vivo* and in human PC cell lines *in vitro*, and plasma ET-1 concentrations are significantly elevated in men with metastatic disease (4). Active ET-1 is generated from big ET-1 by endothelin-converting enzyme (ECE). Two ECE genes are recognized, ECE-1 and ECE-2 (5), both of which are membrane-bound, zinc-dependent metallopeptidases of the M13 family. ECE-1 exists as four distinct isoforms, termed ECE-1a, ECE-1b, ECE-1c (6), and ECE-1d (7). These isoforms differ only in their amino-terminal regions and are derived from a single gene *via* alternative promoters. They display similar enzyme kinetics, but have distinct subcellular locations: ECE-1a and ECE-1c are found at the cell surface, whereas ECE-1b and ECE-1d are located in intracellular membrane compartments (8). ECE-1c is the most abundant isoform and is widely distributed. We, and others, have shown that ECE-1 expression is significantly elevated in tumors (9–12). We previously demonstrated that ECE-1 is present in PC cell lines and primary tissue and is expressed both at the cell surface and intracellularly (9). Significantly elevated levels of ECE-1 were detected in primary malignant stromal cells, as compared with benign stroma (9).

This study addressed the expression and localization of the ECE-1 isoforms in PC stromal and epithelial components.

Materials and Methods

Materials. Antibodies to the ECE-1 isoforms were produced as described, and characterized previously (13). Primary prostate stromal cells (14) and P4E6, PNT1a, and PNT2C2 cell lines (15) were provided by YCR Cancer Research Unit (York, United Kingdom). The following isoform specific primers were synthesized by MWG Biotech (Eversberg, Germany): ECE-1a forward: 5′-TGCAGCGGAACCCCTTCCTCCA-3′, reverse: 5′-GAAGAAGTCATGGCAGGGGTC-3′; ECE-1b, forward: 5′-ATGTCGACGTACAAGCGGGCCA-3′, reverse: 5′-GAAGAAGTCATGGCAGGGGTC-3′; ECE-1c forward: 5′-CGGAGCACGCGAGCTATGATG-3′, reverse: 5′-GAAGAAGTCATGGCAGGGGTC-3′; and ECE-1d forward: 5′-TGCATTTGGCCTTGCAGATGTC-3′, reverse: 5′-GAAGAAGTCATGGCAGGGGTC-3′.

Methods. *Cell Culture.* Cell lines were routinely cultured in RPMI-1640 containing 2 m*M* L-glutamine and 10% (v/v) fetal bovine serum (FBS) with the following exceptions: P4E6 cells were cultured in keratinocyte serum-free medium (SFM) supplemented with bovine pituitary extract (BPE; 25 μg/ml), recombinant epidermal growth factor (rEGF; 0.2 ng/ml), 2 m*M* L-glutamine, and 2% FBS. All cells were routinely grown in antibiotic-free media at 37°C and 5% CO_2. The cell culture reagents were purchased from BioWhittaker (Wokingham, United Kingdom), with the exception of the keratinocyte-SFM, BPE, and rEGF, which were obtained from GIBCO BRL (Paisley, UK). Primary stromal cells were maintained in RPMI-1640 supplemented with 2 m*M* L-glutamine, 10% (v/v) FBS, 1% (v/v) penicillin, and 50 U/ml streptomycin.

Immunofluorescence. Cells were grown to 60% confluency, and fixed and permeabilized for 10 mins in methanol/acetone (1:1 ratio), at room temperature. Nonspecific binding sites were blocked for 30 mins in blocking buffer (Tris-buffered saline, 1% [v/v] normal goat serum, and 0.2% [w/v] gelatin). Primary antibodies were used at the following concentrations: 1:50 ECE-1b and 1:100 ECE-1c. For negative controls, the primary antibody was replaced with pre-immune serum. Cells were incubated for 30 mins at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:1000, Jackson ImmunoResearch Laboratory, West Grove, PA). Cells were examined using an Olympus IX70 inverted wide-field fluorescence microscope. Images were captured using Delta Vision software (Applied Precision, Issaquah, WA).

Immunoblotting. Protein was isolated from cell membrane fractions after disruption using pressurized N_2 gas (750 psi) in a Parr cell disruption bomb. Proteins were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Invitrogen, Paisley, UK), and blocked in 0.1% Tween-20 in 10 m*M* Tris-HCl, pH 7.4 with 5% (w/v) milk powder and 2% (w/v) bovine serum albumin. Membrane proteins were incubated with the following antibodies: 1:100 ECE-1a, 1:100 ECE-1b, and 1:500 ECE-1c. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Ltd., Amersham, UK).

Reverse Transcriptase Polymerase Chain Reaction. Total RNA was isolated using the RNeasy kit (Qiagen, Sussex, UK) according to the manufacturer's instructions. Each RNA (1 μg) was subjected to reverse transcriptase (RT) polymerase chain reaction (PCR) using the GeneScript one-step kit (GeneSys Ltd., Sussex, UK) according to manufacturer's guidelines. The reverse transcription was performed for 30 mins at 50°C, followed by a denaturation step of 94°C for 2 mins for first-strand synthesis. The following program was used for second-strand synthesis and amplification: 94°C for 20 secs denaturation, 55°C for 30 secs annealing, and 68°C for 1 min extension, for 10 cycles. The same program was repeated 25 times with an extra cycle extension of 5 secs per cycle followed by a final extension step at 68°C for 7 mins. The primer sequences used for RT-PCR are given in the Materials section.

Results

ECE-1 Isoform Expression in PC Cell Lines. Individual ECE-1 isoform mRNA expression was determined in established malignant PC cell lines and transformed epithelial cell lines using RT-PCR. After the first round of PCR, faint bands of the expected size were detected in all cell lines analyzed; these were significantly enhanced after a second round of amplification (Fig. 1A). This was true for all ECE-1 isoforms, with the exception of ECE-1c, which did not require reamplification.

ECE-1 isoform protein expression was determined in the established malignant PC cell lines, LNCaP (androgen-sensitive), PC-3, Du145 (androgen-independent), and the transformed prostate epithelial cell lines, PNT-1a, PNT2-C2, and P4E6, by Western blot analysis (Fig. 1B). All cell lines lacked detectable ECE-1a and ECE-1b protein expression (data not shown). LNCaP cells had low ECE-1c protein expression, whereas PC-3 and Du145 had comparatively high levels of ECE-1c protein expression (Fig. 1B). The transformed cell lines, PNT2-C2 and PNT1-a, exhibited relatively high immunoreactivity for ECE-1c, whereas P4E6 levels were undetectable (Fig. 1B). We do not presently have an ECE-1d antibody; therefore, we were unable to perform protein analysis for ECE-1d.

Subcellular Localization of ECE-1c in PC Cell Lines. Immunofluorescence analysis was used to investigate the subcellular distribution of ECE-1c protein in prostate epithelial cell lines. Methanol/acetone–fixed PC-3 cells revealed predominantly cell surface immunoreactivity

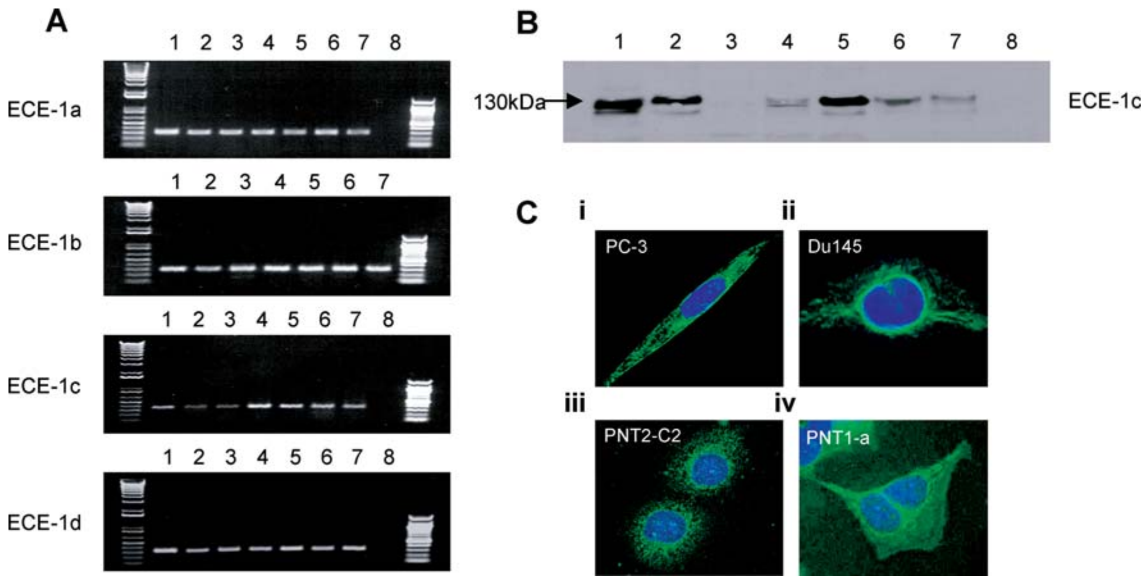

Figure 1. ECE-1 isoform expression and localization in prostate cell lines. (A) Total RNA was isolated from the following: Lane 1, human umbilical vein endothelial cell (HUVEC) line, positive control. Lane 2, androgen-sensitive LNCaP. Lanes 3 and 4, androgen-independent PC-3 and Du145, respectively. Lanes 5, 6, and 7, the virally transformed nonmalignant PNT2-C2, PNT1-a, and P4E6 cell lines. Each 1-μg RNA sample, alongside a negative control (minus template, Lane 8), were subjected to RT-PCR using primers specific for each ECE-1 isoform. To detect ECE-1a, ECE-1b, and ECE-1d, amplicons were purified and reamplified by PCR using nested primers to the ECE-1 isoforms. (B) ECE-1c protein expression in PC cell lines. Each lane contains 20 μg of membrane protein. Lane 1, positive control (500 ng of transfected Chinese hamster ovary [CHO] cells). Lane 2, HUVECs (positive-control cell line). Lane 3, LNCaP. Lanes 4 and 5, PC-3 and Du145, respectively. Lanes 6, 7, and 8, PNT2-C2, PNT-1a, and P4E6, respectively. (C) ECE-1c protein expression and localization in prostate cell lines. (i) PC-3; (ii) Du145; (iii) PNT2-C2; and (iv) PNT1-a cells that were fixed and permeabilized using methanol/acetone (1:1 ratio), at room temperature. Nonspecific binding sites were blocked with 1% normal goat serum, and the cells were incubated with a 1:100 dilution of an antibody to ECE-1c. For negative controls, the primary antibody was replaced with pre-immune serum. Cells were then incubated with FITC-conjugated anti-rabbit IgG and 4′,6-diamidino-2-phenylindole (DAPI), and examined using an inverted wide-field microscope. Color figure is available in the on-line version of the journal.

(Fig. 1Ci). ECE-1c localization in Du145 cells seemed to be intracellular, with bright staining in the perinuclear region (Fig. 1Cii). In PNT2-C2 cells, ECE-1 protein immunoreactivity was observed to be diffuse and punctate (Fig. 1Ciii), whereas PNT1-a cells had immunoreactivity throughout the cytoplasm (Fig. 1Civ).

ECE-1 Isoform Expression in Stromal Cells Derived from Benign and Malignant Prostate Tissue. RT-PCR was used to ascertain ECE-1 isoform mRNA expression in both benign and malignant prostate stromal cells. PCR products of the size expected for ECE-1b, ECE-1c, and ECE-1d were clearly visible in both benign and malignant cells (Fig. 2A). ECE-1a, however, was not clearly detectable in either benign or malignant stromal cells (Fig. 2A).

ECE-1 isoform protein levels in benign and malignant stromal cells were determined by Western blot analysis (Fig. 2B). Both benign and malignant stromal cells lacked ECE-1a protein expression (data not shown). ECE-1b protein was expressed in malignant primary stromal cells only (Fig. 2B). Higher levels of ECE-1c protein were found in malignant primary stromal cells relative to benign primary stromal cells (Fig. 2B).

Subcellular Localization of ECE-1b and ECE-1c in Primary Stromal Cells Derived from Benign and Malignant Tissue. Immunofluorescence analysis was used to investigate the subcellular distribution of ECE-1b and ECE-1c in primary stromal cells derived from both benign and malignant prostate tissue. ECE-1b immunoreactivity of benign primary stromal cells was barely visible (Fig. 2C). In malignant primary stromal cells, ECE-1b immunoreactivity was observed as punctate cytoplasmic fluorescence (Fig. 2C), whereas ECE-1c was observed as bright filamentous immunoreactivity throughout the cell and on the cell surface (Fig. 2D). Benign and malignant primary stromal cell immunoreactivity for ECE-1c differed in intensity only, with brighter immunoreactivity in malignant primary stromal cells (Fig. 2D). This data correlates with the immunoblotting data.

Discussion

Total ECE-1 expression is reported to be significantly elevated in malignant tumors (9, 11, 12, 16). We previously demonstrated that ECE-1 is present in androgen-independent PC cell lines and in primary prostatic stroma, with higher levels of ECE-1 in malignant compared with benign stromal cells (9). The increased stromal ECE-1 expression is attributed to PC cell invasion *in vitro* (9). The present study investigated the expression and localization of the four known ECE-1 isoforms in PC.

Analysis of ECE-1 isoform expression and localization revealed ECE-1c to be the main isoform expressed in

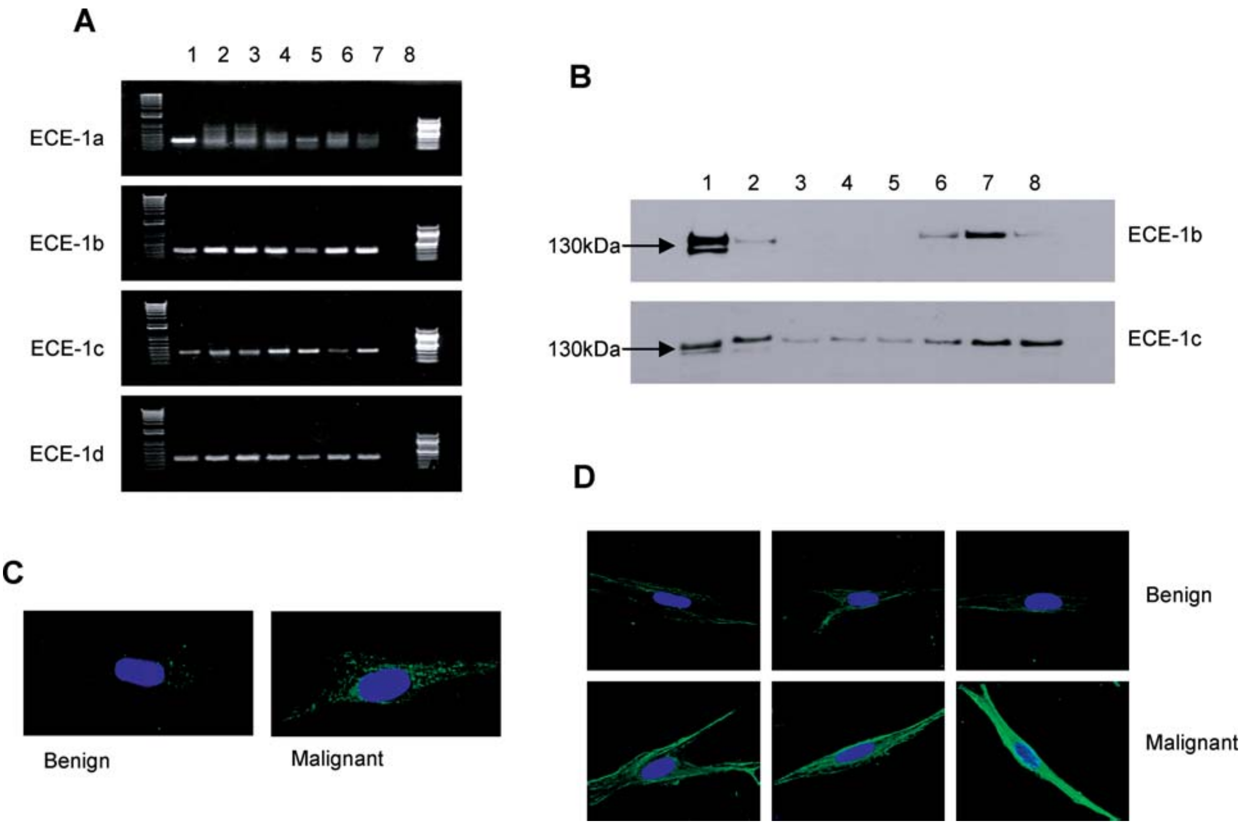

Figure 2. ECE-1 isoform expression and localization in primary stromal cells derived from benign and malignant tissue. (A) Total RNA was isolated from the following: Lane 1, HUVEC line, positive control. Lanes 2, 3, and 4, primary stromal cells derived from benign prostate tissue (BPH). Lanes 5, 6, and 7, primary stromal cells derived from Gleason score 7 malignant prostate tissue. Samples are from three separate patients. Each 1-μg RNA sample, alongside a negative control (minus template; Lane 8), was subjected to RT-PCR using primers specific for each ECE-1 isoform. (B) ECE-1b and ECE-1c protein expression in PC cell lines. Each lane contains 50 μg of membrane protein (ECE-1b) or 20 μg of membrane protein (ECE-1c). Lane 1, positive control (500 ng of transfected CHO cells). Lane 2, positive-control cell lines, EaHY926 (ECE-1b) or HUVEC (ECE-1c). Lane 3, 4, and 5, primary stromal cells derived from benign prostate tissue (BPH). Lanes 6, 7, and 8, primary stromal cells derived from Gleason score 7 malignant prostate tissue. Samples are from separate patients. Cells were fixed and permeabilized using methanol/acetone (1:1 ratio), at room temperature. Nonspecific binding sites were blocked with 1% normal goat serum and the cells were incubated in (C) 1:20 anti–ECE-1b or (D) 1:100 anti–ECE-1c. For negative controls, the primary antibody was replaced with pre-immune serum. Cells were then incubated with FITC-conjugated anti-rabbit IgG and DAPI, and examined using an inverted wide-field microscope. Color figure is available in the on-line version of the journal.

prostate epithelial cells, with increased expression in metastatic, androgen-independent cell lines. The relative expression of each of the isoforms has previously been reported to differ significantly between tissues, with ECE-1c being the predominant isoform in many human tissues tested, such as heart, kidney, testis, liver, and lung (6, 17). ECE-1c is also the most abundant isoform in lung cancer–derived cell lines (17). In addition, the ECE-1 isoforms can exhibit distinct subcellular localizations. For example, ECE-1a and ECE-1c are present at the cell surface, whereas ECE-1b and ECE-1d are located on intracellular membrane compartments (8). Recently, ECE-1a has been shown in the nuclear compartment of transiently transfected cells and native endothelial cells (18).

In this study, immunofluorescence analysis revealed distinct expression patterns of ECE-1c in different PC cell lines. PC-3 cells showed predominantly cell-surface immunoreactivity, consistent with recent reports (6, 7, 19–21), whereas Du145, PNT2-C2, and PNT1-a cells displayed intracellular immunoreactivity. Western blot analysis revealed higher levels of ECE-1c protein in malignant primary stromal cells compared with benign primary stromal cells. Bright filamentous immunoreactivity was observed for ECE-1c throughout the cell and on the cell surface. The increase in ECE-1c in the stromal compartment of malignant cells may implicate a paracrine activation of ET-1 by ECE-1c. For example, big ET, the inactive precursor of ET, resides in the stroma and colocalizes with α-actin of smooth-muscle fibroblasts in rat prostate (22). Similarly, ECE-1 has been shown to colocalize with α-actin in human vascular smooth-muscle cells (23). This subcellular localization is similar to the immunoreactivity pattern observed for ECE-1c in our study. We suggest, therefore, that the close proximity of big ET to ECE-1 in the stroma of the prostate would allow ET-1 to be synthesized on demand from adjacent epithelial cells. In addition, the comparable levels of membrane-associated ECE-1c in PC-3, the most malignant of the cell lines, with those observed in malignant prostatic stroma, is indicative of the epithelial-mesenchymal transition undergone by these cells. As such, they would be self-sufficient and relatively stromal-independent in the supply of ET-1.

In addition to increased ECE-1c levels, higher levels of ECE-1b protein were also detected in malignant primary stromal cells compared with benign primary stromal cells, but with predominantly intracellular immunoreactivity. ECE-1b has been reported to be present in late endosomes (8) and shown to cycle between the plasma membrane and endosomes. Cycling ECE-1b might act as a vehicle for regulating the distribution of the other ECE isoforms by associating with them through heterodimer formation (8). For example, when the plasma membrane isoforms ECE-1a and ECE-1c were expressed alongside the intracellular isoform ECE-1b, both ECE-1a and ECE-1c were redirected to intracellular vesicles, resulting in reduced extracellular activity (8).

A better understanding of the relative roles of ECE-1 isoforms in the development, invasion, and progression of prostate tumors will help in the potential design of therapeutic agents to treat PC.

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