Differential expression of neutral endopeptidase-24.11 (neprilysin) and endothelin-converting enzyme in human prostate cancer cell lines

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ABSTRACT

Neutral endopeptidase-24.11 (neprilysin; NEP/CD10) is a cell surface metallopeptidase expressed by prostatic epithelial cells that degrades various bioactive peptides including endothelin. Endothelin-converting enzyme (ECE), the key enzyme of endothelin biosynthesis, catalyses the final processing step in the pathway. Neuropeptide substrates of NEP, including endothelin, have been implicated in the growth of androgen-independent prostate cancer. We have surveyed the expression of NEP and ECE in a range of prostate cancer cell lines. Western analysis reveals that ECE-1 is expressed abundantly in all the malignant cell lines tested, except for LNCaP. In contrast, LNCaP cells express high levels of NEP, while NEP was not detected in PC-3, DU145 and other metastatic cell lines that were tested. Of the normal immortalized prostate epithelial cell lines, PNT1a shows equivalent amounts of NEP and ECE. PNT2-C2 shows poor NEP expression but an abundance of ECE. P4E6, by comparison, has low levels of both ECE and NEP. These differences in expression may render these cell lines useful in experimental models for future study. Benign prostatic hyperplasia primary epithelial cells express much higher levels of NEP than malignant primary epithelial cells, but neither show ECE expression. On the other hand, surrounding stromal cell populations have detectable ECE levels. An absence of ECE in malignant and benign prostatic hyperplasia cells of primary epithelial origin suggests an important role for stromal interaction and paracrine production of ECE within the host. The upregulation of ECE expression in metastatic cells in culture may be indicative of its role in metastatic progression. A differential profile of ECE and NEP could contribute to an abundance of mitogenic peptides aiding the progression of androgen-independent prostate cancer.

INTRODUCTION

Death from prostate cancer is the second leading cause of death from cancer in Western males [1]. Prostate surgery or targeted irradiation can cure only cancer that is organ confined, yet up to 60% of patients present with metastases, particularly to the bone [2]. Treatment for cancer that has spread beyond the prostate relies on the androgen dependence of prostate epithelial cells for growth and survival. Chemical or surgical androgen ablation leads to significant periods of remission, but is ineffective once the tumours develop androgen independence. Such tumours are also refractory to conventional chemotherapy. The ability of some prostate
cancer cells to survive androgen withdrawal has led to a search for alternative growth pathways. Mitogenic peptides such as those of the bombesin family have been implicated in androgen-sensitive survival. Such peptides are normally metabolized at the cell surface by metalloenzymes like neutral endopeptidase-24.11 (neprilysin; NEP) and downregulation of NEP expression in prostate cancer has already been shown to be an important contributory factor [3].

Recently, clinical and pre-clinical results have indicated a mitogenic role for neuropeptides in prostatic disease [4]. Endothelin-1 (ET-1) peptide is not only an important vasoconstrictor, but also serves as an important growth stimulator in cancers, e.g. breast, cervical, pancreatic and prostate, by stimulating cell motility, angiogenesis and metastatic implantation by a two-way co-operation between the tumour and the stroma in which it is implanted [5]. ET-1 is produced by prostatic epithelium and prostate cancer cell lines, and plasma ET concentrations are significantly elevated in men with metastatic prostate cancer [6]. Therefore, a prospective therapeutic approach might be to block ET-1 activity in prostate cancer through the modulation of endothelin-converting enzyme (ECE), a metalloproteinase that converts the inactive precursor (big ET) into the potent ET peptide [7]. Little is known of the cellular localization or properties of prostatic ECE, although clearly such an activity exists. It has been shown recently that human ECE exists in four distinct isoforms (termed a, b, c and d) differing only in parts of their N-terminal cytoplasmic tails, which may influence their targeting and turnover [8].

Since the loss of NEP has been shown to contribute to the androgen-independent progression of human prostate cancer [3], the present study compares ECE and NEP expression in prostate cancer cell lines, and examines whether changes in ECE expression may correlate with phenotype in androgen-sensitive and androgen-independent cell lines.

**RESULTS AND DISCUSSION**

Established prostate epithelial cell lines from transformed and tumour origins were utilized in this study, as well as available tumour biopsy and radical prostatectomy material. Western analysis revealed that ECE-1 is expressed abundantly in all metastatic cell lines, but is expressed weakly in LNCaP cells (Figure 1). In contrast, LNCaP and were then washed and incubated for 1 h in secondary antibody, both at room temperature. After washing, enhanced chemiluminescence detection reagents were applied as recommended by the manufacturer (Amerham) and exposed to light-sensitive film.

**Antibodies**

NCL-CD10-270 monoclonal anti-NEP antibody was purchased from NovoCastra Labs (Newcastle upon Tyne, U.K.). The anti-ECE-1 monoclonal antibody was provided by Dr K. Shimada (Sankyo Research Institute, Tokyo, Japan). Anti-ECE-1a and anti-ECE-1c polyclonal antibodies are targeted to specific N-terminal sequences [10].

**Immunocytochemistry**

Cells were grown on cover slips in RPMI 1640 medium supplemented with 10% foetal calf serum. Cells were fixed with ice-cold methanol at –20 °C for 5–10 min and incubated with 10% blocking serum. Primary antibody (1:20 anti-ECE and 1:80 anti-NEP) and biotinylated secondary antibody were each applied for 1 h at room temperature. Cells were incubated with a streptavidin–horseradish peroxidase (Amersham) conjugate for 15 min and were fixed again in ice-cold methanol for 5 min, before applying the diaminobenzidine substrate (Sigma). Negative controls (excluding primary antibody) showed no non-specific staining (results not shown).

**METHODS**

**Cell lines**

PNT1a, PNT2-C2 and P4E6 (normal, immortalized prostate epithelial cell lines), LNCaP (androgen-sensitive) and PC-3 (androgen-independent) were obtained as described previously [9]. Androgen-independent DU-145, Tsu-Prl and PPC-1 cells lines were kindly donated by Professor D. Nanus, Cornell Medical Center, New York, NY, U.S.A.

**Western blot analysis**

Protein was extracted either from total cell lysates or from cell membranes and subjected to Western analysis. Filters were incubated for 1 h in primary antibody, and were then washed and incubated for 1 h in secondary antibody, both at room temperature. After washing, enhanced chemiluminescence detection reagents were applied as recommended by the manufacturer (Amerham) and exposed to light-sensitive film.

**Figure 1** ECE-1 expression in cell membrane preparations

Each lane contains 20 μg of membrane protein. Both androgen-independent cells and normal immortalized cells show ECE-1 expression, whereas LNCaP cells almost completely lack ECE. PNT1a and P4E6 also express ECE-1.
cells were seen to express high levels of NEP, while PC-3, DU-145 and other metastatic cell lines tested lacked detectable NEP activity (Figure 2). This striking differential pattern of ECE and NEP expression is further confirmed by immunocytochemical analysis (Figure 3). ECE-1 expression was absent in malignant primary epithelial cells from radical prostatectomies (Figure 4). In contrast, primary stromal cell cultures from the same malignant tissue reveal the presence of ECE-1 (Figure 5). These results are from low-grade tumours. Therefore, the absence of epithelial ECE suggests a role for stromal interaction and paracrine production of ECE, and hence ET, within the host. The observed autocrine upregulation of ECE in metastatic epithelial cell lines in culture (Figure 1) is not unusual, since an ET-1-mediated autocrine loop has been implicated in the growth of ovarian tumour cells [11].

**Figure 3** Immunostaining for ECE and NEP on cells grown in culture

Monoclonal antibodies directed against ECE and NEP were used at dilutions of 1:20 and 1:80 respectively. ECE-1 expression is apparent in androgen-independent cells, while NEP is only detectable in androgen-sensitive LNCaP cells. PC-3 NEP expression is representative of all androgen-independent cells tested.
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Figure 5 Immunodetection of NEP in primary cell cultures
Radical prostatectomy material from patients with BPH (benign prostatic hyperplasia) or malignant prostates was established either as epithelial or stromal cultures. Each lane contains 5 μg of membrane protein. NEP is present in two out of three BPH samples and is barely detectable in malignant epithelia.

Recent immunohistochemical analysis of high grade prostate tumour biopsies (B. A. Usmani, unpublished work) has revealed a dramatic upregulation of ECE expression within the stroma (results not shown). These results imply an increased role for stromal involvement during prostate cancer progression. Based on these observations, and other evidence from the literature, the model we would like to propose is that within a growing tumour environment, NEP, which is normally expressed, is downregulated, while in the surrounding stroma the upregulation of ECE expression provides a local source of the powerful mitogenic peptide ET. It is the relative expression of these two peptidases acting at the cell surface, which will control the ‘aggressiveness’ of the malignant neoplasm. Tumour–stromal interaction has already been reported by Egidy et al. [12] in human colon cancer with respect to the ET system. Further evidence for this model may be drawn from the findings reported by Suzuki et al. [13]. These authors have shown an imbalance between NEP and ET-1 expression in human endometrial carcinoma, whereby NEP expression is downregulated and ET-1 expression is upregulated in the stroma. We have also reported the loss of NEP during progression of metastatic prostate cancer [3]. Clearly, a loss of NEP has been shown to contribute to tumour progression in human prostate and other cancers [3]. Work is under way at present to confirm a reciprocal role for ECE and, perhaps more importantly, the expression of particular isoforms of ECE which might contribute to the androgen-independent progression of human prostate cancer and could, therefore, be individual targets in future therapy.

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REFERENCES

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