Tumor-Suppressive Effects of Neutral Endopeptidase in Androgen-independent Prostate Cancer Cells

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ABSTRACT

Expression of neutral endopeptidase (NEP) 24.11 is diminished in metastatic, androgen-independent prostate cancers (PCs; C. N. Papandreou et al., Nat. Med., 4: 50–57, 1998). To determine the effects on androgen-independent PC cells of overexpressing cell-surface NEP, an inducible tetracycline-regulatory gene expression system was used to stably introduce and express the NEP gene in androgen-independent TSU-Pr1 cells generating WT-5 cells, which expressed high levels of enzymatically active NEP protein when cultured in the absence of tetracycline. TN12 cells, which contain the identical vectors without the NEP gene and do not express NEP, were used as control. Expression of NEP in WT-5 cells after removal of tetracycline from the media resulted in a >80% inhibition in cell proliferation over a 1-week period (P < 0.005) compared with control cells. Tumor formation occurred in the prostate glands of orthotopically injected athymic mice killed at 30 days in 4 of 5 mice that were given injections of 2 × 10⁶ WT-5 cells and were fed doxycycline (NEP suppressed), and in all mice that were given injections of TN12 cells and were fed with or without doxycycline. In contrast, only 1 of 5 mouse prostates developed a tumor in mice that were given injections of WT-5 cells and that did not receive doxycycline. Analysis of the mechanisms of NEP-induced growth suppression revealed that NEP expression in WT-5 cells induced a 4-fold increase in the number of PC cells undergoing apoptosis, and increased the expression of p21 tumor suppressor gene protein and the level of unphosphorylated retinoblastoma protein as determined by Western blot. Flow cytometric analysis showed that induced NEP expression in WT-5 cells resulted in a G1 cell cycle arrest. These data show that NEP can inhibit PC cell growth and tumorigenicity and suggest that NEP has potential as therapy for androgen-independent PC.

INTRODUCTION

NEP 24.11 (neprilysin, enkephalinase, CD10, EC 3.4.24.11) is a Mr 90,000–110,000 cell-surface metallopeptidase that is normally expressed by numerous tissues, including prostate, kidney, intestine, endometrium, adrenal glands, and lung. This enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active peptides, including atrial natriuretic factor, substance P, bradykinin, oxytocin, Leu- and Met-enkephalins, neurotensin, bombesin, endothelin-1, and bombesin-like peptides (1–3). NEP reduces the local concentration of peptide available for receptor binding and signal transduction. The biological function of NEP appears to be organ-specific. In the central nervous system, NEP regulates enkephalin-mediated analgesia (4); in the kidney and vascular epithelium, the enzyme is involved in regulating levels of circulating atrial natriuretic factor (5); in the lung, NEP modulates tachykinins, such as substance P, that mediate inflammation (6); in the endometrium, NEP regulates endothelin-1, which causes vasoconstriction of endometrial arterioles during specific phases of the ovulatory cycle (7, 8). NEP has also been implicated in controlling cellular proliferation by hydrolyzing bombesin-like peptides, which are potent mitogens for fibroblasts and bronchial epithelial cells (9).

Loss or decreases in NEP expression have been reported in a variety of malignancies, including renal cancer (10), invasive bladder cancer (11), poorly differentiated stomach cancer (12), small cell and non-small cell lung cancer (13), endometrial cancer (14), and PC (15). Reduced NEP may promote peptide-mediated proliferation by allowing an accumulation of higher peptide concentrations at the cell-surface and may facilitate the development or progression of neoplasia (16, 17).

In PCs, NEP protein is expressed in androgen-sensitive LNCaP cells but not in androgen-independent PC cell lines (15). Furthermore, expression of NEP is transcriptionally activated by androgen in LNCaP cells and decreases with androgen-with-
drawal (15, 18). Consequently, we proposed that PC cells that survive androgen-withdrawal would emerge with reduced levels of NEP. This decrease in NEP expression may result in increased growth by allowing PC cells to use neuropeptides as an alternate source to androgen to stimulate cell proliferation. To delineate the role of NEP in the development and progression of androgen-independent PC, we used a tetracycline-repressible system to introduce a full-length NEP cDNA into TSU-Pr1 cells. We report that overexpression of cell-surface NEP inhibits PC cell growth by inducing G1 cell cycle arrest and inhibits PC cell tumorigenicity in an orthotopic model of PC.

MATERIALS AND METHODS

Cell Culture. PC cell lines were maintained in RPMI 1640 supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 units/ml streptomycin and penicillin, and 10% FBS. TSUGK27 is a TSU-Pr1 cell line stably transfected with pGK hygro and PUHD 15–1 (4.4 kb) containing the coding sequence for the COOH-terminal domain of VP16 (named the tetracycline-responsible transactivator or tTA), downstream of the hCMV promoter (19). This cell line was cultured in media containing 150 μg/ml of hygromycin and 1 μg/ml of tetracycline.

Plasmid Construction and Gene Transfer. To construct the tetracycline-repressible NEP-expression vector, 1.7 kb SacII–XhoI and 1.6 kb XhoI–XbaI DNA fragments containing the entire wild-type NEP coding sequence, were isolated from the pClShENK vector (provided by Arris Pharmaceutical Corp., San Francisco, CA) and ligated into the pTRE vector (Clontech Laboratories, Inc., Palo Alto, CA) at the SacII and XbaI sites to generate pwtNEP. The pTRE empty vector was used as control. TSUGK27 cells were cotransfected with either pwtNEP or pTRE vectors together with pSV2, which contains a neomycin-resistant gene under control of the SV40 promoter using LipofectAMINE following the manufacturer’s instructions with minor modifications (Life Technologies, Inc., Gaithersburg, MD). Briefly, 2 × 10⁶ cells were seeded in 6-well plates 16 h prior to transfection. One μg of target plasmid DNA together with 0.1 μg of pSV2 plasmid (ratio: 10:1) in serum-free MEM was mixed with 5 μl of LipofectAMINE at room temperature for 30 min. Cells were then washed with serum-free MEM, incubated in the DNA-liposome mixture at 37°C for 5 h followed by MEM containing 5% FBS for 18–24 h. Cells were refed with medium containing 500 μg/ml G418, 150 μg/ml hygromycin, and 1 μg/ml tetracycline for 12–15 days (Life Technologies, Inc.) and surviving clones were expanded to cell lines for further analysis.

Proliferation Assays. Proliferation assays were performed as described previously (20). Briefly, 10,000 cells/well were plated in duplicate 12-well tissue-culture plates (Falcon Division, Becton Dickinson, Cockeysville, MD) in RPMI medium containing 10% FBS and counted over 3–7 days using a Coulter counter ZM (Coulter Electronics, Hialeah, FL). All of the experiments were performed in triplicate on at least two separate occasions. Statistical analyses were performed using a nonpaired Student’s t test.

Enzyme Assays. Enzyme assays were performed to determine NEP-specific enzyme activity as described previously (20) using Suc-Ala-Ala-Phe-pNA (Bachem Bioscience, Inc., Philadelphia, PA) as substrate. Briefly, cells were rinsed in cold lysis buffer (50 mM Tris/150 mM NaCl) and lysed in lysis buffer containing 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), which did not affect NEP enzyme specific activity. Protein concentrations were measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Thirty μl of cell membrane suspension were added to a mixture of 200 μl of 100 mM Tris-HCl (pH 7.6), 10 μl of 20 mM substrate (dissolved in DMSO), and 10 μl of aminopeptidase N enzyme solution (EC 3.4.11.2; Boehringer Mannheim, Indianapolis, IN) and were incubated at 37°C for 10 min. The reaction was stopped by adding 10% trichloroacetic acid, and the mixture was centrifuged at 2500 rpm for 5 min; 250 μl of supernatant was then removed for colorimetric analysis. The absorbance of the chromogen was immediately read at 540 nm against a reaction mixture without cell membrane as blank. Specific activities were expressed as pmol/μg protein/min and represent an average of two separate measurements performed in duplicate. The SE of measurement of independent duplicate experiments was ~10–20% of the mean value.

Cell Cycle Analysis. Flow cytometry analysis was performed as described previously (21). Briefly, cells were rinsed twice in cold PBS, trypsinized, washed twice with PBS, and fixed in cold ethanol overnight. The following day, cells were resuspended in 500 μl of PBS, digested with 20 μg/ml RNase at 37°C for 1 h, chilled on ice for 10 min, and stained with propidium iodide (50 μg/ml) by incubation for 1 h at room temperature in the dark. Cell cycle distribution was analyzed by flow cytometry using a Becton Dickinson FACS system.

Apoptosis Assay. Apoptotic cells stained with acridine orange and ethidium bromide were assessed by fluorescence microscopy. Briefly, 2 μl of stock solution containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide were added to 25 μl of cell suspension. Total number of cells, as well as apoptotic cells that showed shrinkage, blebbing, and/or apoptotic bodies, were counted. DNA fragmentation analysis was performed as described previously (22).

Protein Extraction, Immunoprecipitation, and Western Blot Analysis. Protein was extracted from exponentially growing cells and was analyzed by Western blotting as described previously (23) using primary antibodies 5B5 (anti-NEP; Arris Pharmaceutical Corp.), p21, retinoblastoma, Bcl-2, p53, and cyclins A and D (Oncogene Research Products, Cambridge, MA). Blots were incubated with enhanced chemiluminescent (ECL) detection reagents (Amersham Pharmacia Bio- tech, Arlington Heights, IL), and proteins were detected by autoradiography by exposure of blots to Kodak XAR film for 2–15 min. Membranes were stained with 0.2% Ponceau red to assure equal protein loading and transfer. Immunoprecipitation using mAb J5, which recognizes NEP (CD10; Beckman-Coulter Pharmaceutical, Inc., Hialeah, FL), was performed as described previously (24).

Orthotopic Injection. Male nude mice (25–30 g) were anesthetized with 300 μl of 5 mg/ml pentobarbital (10 μl/g body weight) administered i.p. The abdomen was sterilly prepped with Betadine, and a 1.5-cm vertical incision was made in the middle line of the abdomen through the skin and peritoneum to expose the bladder and seminal vesicles. A 26-gauge...
needle was inserted into the parenchyma of the dorsal lobe of the prostate and advanced just below the capsule, into which $2 \times 10^6$ tumor cells diluted in 0.1 ml of sterile PRMI1640–10% FBS were injected. The lack of significant extraprostatic leakage and formation of a visible bulla between prostate parenchyma and capsule were criteria for a successful injection. The incision was closed using metal clips. Dox-Diet was obtained from Bio Serve (Frenchtown, NJ).

NMR Imaging Assay. Mice were imaged on a 4.7T 33-cm bore Bruker Omega NMR system. Images were obtained using a homebuilt 4-turn foil solenoid radiofrequency coil tuned to 200 MHz. Image acquisition parameters included eight slices, field of view of 25 mm, slice thickness of 2 mm, 128 $\times$ 128, matrix (200 units in plane resolution), repetition interval ($TR$) of 500 ms, and echo time ($TE$) of 16 ms.

RESULTS

Expression of NEP in TSU-Pr1 Cells. TSUGK27 cells, a derivative of TSU-Pr1 cells that contain the tetracycline regulator and a hygromycin resistance plasmid (19), do not express NEP protein nor exhibit NEP enzyme activity. TSUGK27 cells were transfected with pwtNEP (or pTRE control vector) together with a neomycin resistance vector and antibiotic-resistant stable clones, screened by measuring NEP catalytic activity in cells cultured in the presence or absence of 1 $\mu$g/ml tetracycline (Fig. 1A). Removal of tetracycline from the medium resulted in a significant increase in NEP enzyme activity in at least four clones (WT5, WT6, WT12, and WT24). TSUGK27 cells, which contained the pTRE empty vector without the NEP gene (TN cells), did not exhibit enzyme activity. Integration of pTRE into genomic DNA of TN cell lines was verified by Southern blotting with a probe specific for pTRE vector sequence (not shown). Western blot of NEP protein immunoprecipitated with mAb J5, which recognizes NEP, showed the presence of NEP protein in WT5 and WT24 cells after the removal of tetracycline from the medium, similar to what was observed in LNCaP cells, which constitutively express NEP, but which was not observed in control TN12 or TN15 cells (Fig. 1B). Flow cytometry confirmed the expression of cell-surface NEP in WT cell lines (data not shown). WT5 cells were further characterized for NEP enzyme inducibility. NEP enzyme activity was suppressed when WT-5 cells were cultured in 1 $\mu$g/ml tetracycline, and enzyme activity increased with decreasing concentrations of tetracycline ranging from 1000 to 0 ng/ml (Fig. 1C). Enzyme activity reached maximum levels at 72–96 h after the removal of tetracycline from the medium (Fig. 1D). Thus, induction of NEP
enzyme activity was both time course-dependent and dose-dependent.

**NEP Expression Induces Growth Inhibition in Vitro and Inhibits Tumorigenicity in Athymic Mice.** WT5 and control TN12 cells were cultured for 7 days with and without tetracycline (Tet), and the cell number was determined. Data are representative of one experiment performed in triplicate. Error bars, SE. B, NMR imaging of mouse prostates into which PC cells were injected. High spatial resolution 1H NMR images of the pelvis were obtained of mice in which $2 \times 10^6$ cells of TN12 or WT5 cells had been injected orthotopically into the prostate. Mice were fed with regular feed or Dox-Diet (feed containing doxycycline), beginning 3 days prior to orthotopic injection. All of the mice were killed 30 days after implantation. Imaging was performed prior to sacrifice in randomly selected animals. Top two panels, images of prostate glands into which TN12 control cells were injected in mice that were fed with Dox-Diet (top left) or regular feed without doxycycline (top right). Note tumor formation in both animals. Bottom two panels, images of prostate glands into which WT-5 cells were injected in mice that were fed with Dox-Diet (bottom left) or regular feed without doxycycline (bottom right). Tumor formation is absent in mice not receiving doxycycline (NEP expressed). T, tumor; B, bladder; P, prostate.

**Fig. 2** A, NEP expression induces growth inhibition in *vitro*. WT5 and TN12 were cultured for 7 days with and without tetracycline (Tet), and the cell number was determined. Data are representative of one experiment performed in triplicate. Error bars, SE. B, NMR imaging of mouse prostates into which PC cells were injected. High spatial resolution 1H NMR images of the pelvis were obtained of mice in which $2 \times 10^6$ cells of TN12 or WT5 cells had been injected orthotopically into the prostate. Mice were fed with regular feed or Dox-Diet (feed containing doxycycline), beginning 3 days prior to orthotopic injection. All of the mice were killed 30 days after implantation. Imaging was performed prior to sacrifice in randomly selected animals. Top two panels, images of prostate glands into which TN12 control cells were injected in mice that were fed with Dox-Diet (top left) or regular feed without doxycycline (top right). Note tumor formation in both animals. Bottom two panels, images of prostate glands into which WT-5 cells were injected in mice that were fed with Dox-Diet (bottom left) or regular feed without doxycycline (bottom right). Tumor formation is absent in mice not receiving doxycycline (NEP expressed). T, tumor; B, bladder; P, prostate.
ever, no tumor was detected in the animal that was given injections of WT5 cells and did not receive tetracycline (NEP expressed). Autopsies of all of the animals revealed 100% tumor formation in animals receiving TN12 cells, and in four (80%) of five animals given injections of WT5 cells and fed with tetracycline. Only one of five animals that were given injections of WT5 cells and did not receive tetracycline developed a tumor, which was appreciably smaller than other tumors formed (Table 1).

NEP Expression Induces Apoptosis, G1 Cell Cycle Arrest, and Expression of p21 Protein. An analysis of WT5 cells after the removal of tetracycline from the media for 7 days revealed nuclear fragmentation and chromatid condensation, consistent with cells undergoing apoptosis (Fig. 3A), which was confirmed by gel electrophoresis showing nucleosomal fragments in the DNA derived from WT5 cells cultured without tetracycline (Fig. 3B). As shown by flow cytometric analysis, the removal of tetracycline for 7 days induced apoptosis in 14.8% of WT-5 cells. This apoptosis was not observed in TN12 cells nor in WT5 cells cultured in tetracycline, which suppresses NEP expression. Cell cycle analysis of TN12 cells before and after the removal of tetracycline from the media revealed no differences in the distribution of cells within phases of the cell cycle (Fig. 3C). Similar to TN12 cells, 54.7% of WT-5 cells cultured in medium containing tetracycline (which suppresses NEP expression) were in the G1 phase of the cell cycle. However, induction of NEP expression after the removal of tetracycline from media resulted in G1 arrest, with 73.9% of WT-5 cells in G1 phase. The analysis of cell cycle proteins that may contribute to an arrest in G1 revealed a 3- to 4-fold increase in p21 protein levels and a reduction of phosphorylated retinoblastoma protein (ppRb) to the unphosphorylated form in WT5 cells cultured without tetracycline (Fig. 3D). In contrast, no alterations in the expression pattern of cyclin A, cyclin D, or p27 were observed.

DISCUSSION

NEP inactivates neurotensin, bombesin, and endothelin-1, all of which have been implicated in progression to androgen-independent PC (25-27). We previously reported that NEP expression is decreased in androgen-independent PC cell lines

in vitro, and in tumor cells of metastatic biopsy specimens in vivo from patients with androgen-independent PC (15). Expression of NEP is transcriptionally activated by androgen in androgen-dependent PC cells and decreases with androgen withdrawal (15). Consequently, PC cells that survive androgen withdrawal can emerge with reduced levels of NEP. This decrease in NEP expression can contribute to the development of androgen-independent PC by allowing PC cells to use neuropeptides as an alternative source to androgen to stimulate cell proliferation.

The current study was aimed at further delineating the antitumor effects of NEP and its potential use for inhibiting androgen-independent PC cell growth. In this regard, we had previously shown that androgen-induced growth repression of androgen-independent PC3 cells expressing androgen receptor (PC3/AR) and of an androgen-independent subline of LNCaP cells results in part from androgen-induced expression of NEP in these cells (20). However, the growth inhibition observed was modest (~20–30%) and the level of NEP specific enzyme activity achieved after androgen-stimulation was relatively low. Recombinant NEP also inhibits androgen-independent PC cell growth, but it is difficult to obtain sustained serum levels of NEP in mice using recombinant NEP (13), and we did not observe significant inhibition of tumorigenicity of PC xenografts in athymic mice receiving i.p. recombinant NEP daily for 30 days. Therefore, we constructed an inducible system of NEP expression to more fully examine the effects of expressing cell-surface NEP in androgen-independent PC cells.

Our data show that NEP can regulate androgen-independent PC cell proliferation. Moreover, expression of NEP inhibits xenograft tumor formation in the prostate gland of athymic mice, which suggests that NEP can function as a tumor suppressor of PC. The mechanism of NEP-induced growth inhibition presumably involves the inactivation of its neuropeptide substrates, such as bombesin, endothelin-1, and neurotensin, each of which has been implicated in androgen-independent PC growth (28). We observed that overexpression of NEP resulted in an increase in cells undergoing apoptosis. Recent studies suggest that bombesin and endothelin-1 can act as survival factors and inhibit apoptosis (29–31). Thus, expression of NEP, which leads to inactivation of these neuropeptides, would allow for greater numbers of cells to undergo cell death. In addition, overexpression of cell-surface NEP led to G1 cell cycle arrest, presumably mediated in part by the induction of p21 protein expression and dephosphorylation of Rb. We recently have shown that overexpression of NEP in WT-5 cells inhibits neuropeptide-mediated phosphorylation of FAK (p125FAK).4 Zhao et al. (32) reported that overexpression of a dominant-negative FAK mutant inhibited cell cycle progression at G1 and induced p21 expression, which leads to the possibility that the induction of growth arrest by NEP results in part from the inhibition of FAK phosphorylation.

Table 1  NEP inhibits xenograft tumor formation in the prostates of athymic mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dox</th>
<th>Tumor formation</th>
<th>Tumor sizea (mm³)</th>
<th>Mouse weightb (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-5 ±</td>
<td>4/5 (80%)</td>
<td>0.43 (0.11)</td>
<td>6.1 (2.4)</td>
<td></td>
</tr>
<tr>
<td>WT-5 −</td>
<td>4/5 (20%)</td>
<td>0.07 (0.07)</td>
<td>5.7 (2.8)</td>
<td></td>
</tr>
<tr>
<td>TN-12 ±</td>
<td>4/4 (100.0%)</td>
<td>0.91 (0.35)</td>
<td>5.5 (2.3)</td>
<td></td>
</tr>
<tr>
<td>TN-12 −</td>
<td>4/4 (100.0%)</td>
<td>1.04 (0.42)</td>
<td>6.2 (1.9)</td>
<td></td>
</tr>
</tbody>
</table>

a Tumor size measured in mm³ and calculated as length × width × depth/2. Tumor measurements were performed by two researchers independently; in parentheses, SE.
b Average change in animal weight over 30 days of experiment; in parentheses, SE.

LNCaP cells constitutively express NEP and exhibit some

5 C. N. Papandreou and D. M. Nanus, unpublished data.
of the characteristics observed in WT-5 cells expressing NEP, including a longer doubling time (33), diminished ability to migrate in extracellular matrix (34), and decreased tumorigenicity in athymic mice (34, 35), compared with androgen-independent PC cells that lack NEP expression. In addition, the inhibition of NEP enzyme activity in LNCaP cells results in an increase in FAK phosphorylation. However, the complete phenotype observed in WT-5 cells that are induced to express NEP is not present in LNCaP cells. This may result from the lower levels of NEP protein and enzyme activity in LNCaP cells.

Fig. 3 NEP expression induces apoptosis and cell cycle G₁ arrest. A, photomicrograph of WT5 cells stained with acridine orange and ethidium bromide cultured in the presence (left) or absence of tetracycline (right). Apoptotic cells were determined by fluorescent staining as described in “Material and Methods.” Arrow, apoptotic bodies in cells, in which NEP expression is induced by removing tetracycline. B, DNA fragmentation gel electrophoresis of WT5 cells cultured in the presence or absence of tetracycline (Tet) for 3, 5, and 7 days. DNA laddering occurred in cells cultured without tetracycline (NEP expressed) for 7 days. C, flow cytometric analysis of TN-12 and WT5 cells cultured in the presence or absence of tetracycline for 7 days. There is no significant difference in cell cycle histograms between TN-12 cultured with tetracycline (top left) or without tetracycline (top right), or with WT-5 cultured in tetracycline (bottom left). After the removal of tetracycline from WT5 for 7 days, an increase in cells in G₁, and in the percentage of apoptotic cells occurs (bottom right). D, cell lysates from WT5 cells cultured in the presence or absence of tetracycline for 3, 5, and 7 days were separated on SDS-PAGE, transferred to nitrocellulose, and probed with Abs to the proteins indicated. There were an increase in p21 protein and a decrease in the level of phosphorylated retinoblastoma (Rb) protein. Similar results were obtained on at least one other occasion using different cell lysates.
compared with WT-5, or from a lack of downstream mediators of NEP action that are present in TSU-Pr1 cells but not in LNCaP cells.

In summary, the current study emphasizes the consequences of decreased NEP expression in PC cells by studying the effects of reexpressing cell-surface NEP. These experiments suggest overexpression of NEP in PC cells results in multiple effects, including growth inhibition, induction of apoptosis, cell cycle arrest, and the inhibition of tumor formation. Augmentation of NEP expression by delivery of exogenous cell-surface NEP using gene constructs is a potential approach to the treatment of hormone-refractory PC.

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