Chemosensitization of Androgen-Independent Prostate Cancer with Neutral Endopeptidase

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ABSTRACT

Purpose: We investigated whether neutral endopeptidase (NEP) could augment chemosensitivity to anticancer drugs by promoting protein kinase C (PKC) activitymediated mitochondrial apoptosis in prostate cancer (PC) cells.

Experimental Design: Human PC cell lines LNCaP and PC-3, and a normal prostate epithelial cell line (PrEC) were used. The protein expression was detected by Western blot analysis, and the protein turnover was determined by pulse-chase assay. Apoptotic ratio was measured by annexin V staining.

Results: Western blot analyses and pulse-chase assays showed that the specific NEP inhibitor CGS24592 decreased PKCα protein expression by promoting PKCα protein degradation in NEP-expressing LNCaP cells. Conversely, recombinant NEP (rNEP) increased PKCα protein expression by delaying PKCα protein degradation in NEP-negative PC-3 cells. Apoptosis assays showed that rNEP promoted anticancer drug-induced apoptosis in PC-3 cells specifically through PKCα activity that mediated anticancer drug-induced mitochondrial change such as cytochrome-c release and caspase-9 activation. Of note, rNEP was able to increase PKCα protein expression predominantly in PC-3 cells rather than in PrEC cells. Treatment with rNEP before subtoxic concentrations of etoposide (0.1 μM) significantly promoted mitochondrial apoptosis compared with only etoposide in PC-3 cells (P < 0.01) but not in PrEC cells.

Conclusions: These results suggest that NEP enzyme activity contributes to anticancer drug-induced PC cell apoptosis dependent on PKCα-mediated mitochondrial events. More importantly, the combination of NEP with anticancer drugs may be a promising therapeutic modality because rNEP is able to augment chemosensitivity in androgen-independent PC with minimal toxicity in normal tissues.

INTRODUCTION

The development of androgen-independent prostate cancer (PC) is the cause of treatment failure, resulting in ~30,000 deaths from PC in the United States each year (1). Recent studies show that chemotherapy has a modest benefit in some patients with androgen-independent PC (2, 3). However, virtually all patients relapse and die of progressive disease. The mechanisms of sensitivity and resistance of PC cells to chemotherapeutic agents in not well defined. Various anticancer drugs, such as etoposide, that directly or indirectly induce DNA damage, cause proliferating cells to undergo cell cycle arrest followed by growth inhibition and/or apoptotic cell death. However, the relationship between cell cycle arrest and apoptosis is poorly understood. We and others have recently reported that etoposide-resistant cancer cells sense DNA damage followed by G2-M cell cycle arrest but fail to undergo apoptotic cell death (4, 5). PC cells, although numerous studies demonstrated that androgen-independent PC-3 cells are apoptosis-resistant in response to etoposide (6–8), several groups reported that etoposide could induce cell cycle arrest followed by marked growth inhibition in PC-3 cells (5, 9, 10). These findings suggest that DNA damage-induced cell cycle arrest (growth inhibition) and apoptosis induction are independent from each other, and that cytostatic anticancer drugs may have limited impact on androgen-independent PC cell survival (2). Moreover, these studies suggest that activation of apoptosis signaling may be required to improve the effects of anticancer drugs on androgen-independent PC cells.

Neutral endopeptidase 24.11 (NEP; CD10) is a cell-surface peptidase, expressed by prostate epithelial cells, that inactivates a variety of neuropeptides implicated in PC, including bombesin, endothelin-1, and neurotensin. The loss of NEP expression has been implicated in the progression to androgen-independence (11). We demonstrated that NEP increased the protein expression of the protein kinase C (PKC) isoform PKCα in PC cells by inhibiting neuropeptide-induced Src signaling which leads to the degradation of PKCα protein (12). Recently, we and others reported that PKCα activity is required for etoposide-induced mitochondrial apoptosis in various cell types including PC cells (5, 13, 14). These findings led us to hypothesize that NEP could promote drug-induced apoptosis by increasing the protein expression and kinase activity of PKCα. We report here that NEP plays an important role in promoting drug-induced mitochondrial apoptosis through affecting PKCα activity in PC cells, but not in normal prostate epithelial cells.
MATERIALS AND METHODS

Reagents. Etoposide and paclitaxel were provided by Bristol-Myers Squibb KK (Tokyo, Japan). Recombinant NEP (rNEP) was provided by Arris Pharmaceutical, Inc. (South San Francisco, CA). The specific PKC inhibitors rottlerin and Gö6976 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). CGS24592, a competitive inhibitor of NEP, was supplied by Novartis Pharmaceuticals (Summit, NJ).

Cell Lines. PC cell lines were maintained in RPMI 1640 (LNCaP) or MEM (PC-3) supplemented with 2% glutamine, 1% nonessential amino acids, 100 units/ml streptomycin and penicillin, and 10% FCS. The human prostate epithelial cells PrEC (Clonetics, Walkersville, MD) were maintained in PrEBM medium (Clonetics) supplemented with the PrEGM BulletKit (Clonetics).

Apoptosis Assay. Apoptotic cells were detected using annexin V apoptosis detection kit (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) as described previously (12) by flow cytometry using a Becton Dickinson FACS system. A total of 10,000 events were recorded for each treatment. All of the assays were performed on three separate occasions.

Western Blot Analysis. Total cell lysates and cytosolic fractions were prepared with radioimmunoprecipitation assay buffer as described previously (5, 15). For the detection of PKCδ, total cell lysates were separated on an 8% SDS-PAGE, transferred to nitrocellulose and incubated in 1% BSA for 2 h. Membranes were immunoblotted with anti-PKCδ (Santa Cruz Biotechnology, Inc.; 1:1000) and detected using ECL chemiluminescence system (Amersham Pharmacia Biotech.). For the detection of cytochrome-c (cyt-c) in cytosolic fractions, cytosolic fractions were separated on 15% SDS-PAGE with an equal amount of protein loaded onto each lane. The gels were then transferred to nitrocellulose. Membranes were immunoblotted with anti-cyt-c (Santa Cruz Biotechnology, Inc.; 1:500) and detected. To monitor the loading equality of each lane, we also immunoblotted membranes with anti-actin (Chemicon International, Inc., Temecula, CA; 1:3000) for total cell lysates or cytosolic fractions.

Pulse-Chase Assay. The PKCδ protein turnover was detected by pulse-chase assay as described previously (12). Briefly, Cells prelabeled with 300 μCi/ml [35S]methionine were treated and lysed in radioimmunoprecipitation assay buffer. For immunoprecipitation, 300 μg of lysates were incubated 1 h with 1 μg of anti-PKCδ antibody, and then for 1 h with 40 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech., Piscataway, NJ) at 4°C. Immunoprecipitates were collected by centrifugation and resuspended in 2× Laemmli sample buffer. Samples were resolved on an 8% SDS-PAGE and transferred to nitrocellulose. Autoradiography and immunoblotting were performed using the same membrane. The relative intensity of each band obtained by autoradiography was measured by NIH image.

Caspase-9 Colorimetric Assay. N-Acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA) cleavage was detected using a kit purchased from R&D Systems Inc. (Minneapolis, MN). On cleavage of the substrate by caspase-9, free pNA light absorbance can be quantified using a microtiter plate reader at 405 nm. The statistical analysis was performed using an unpaired t test. Ps of <0.01 were regarded as statistically significant. Caspase-9 cleavage was also evaluated by Western blot analysis using the anti-caspase-9 antibody (New England Bio Lab, Beverly, MA; 1:1000) for detecting both procasosase-9 and cleaved products, which was well correlated with the results obtained by the colorimetric assay.

Statistical Analysis. The statistical analysis was performed using an unpaired t test. Ps of less than 0.05 were regarded as statistically significant.

RESULTS

NEP Activity Regulates PKCδ Protein Expression in PC Cells. The androgen-dependent PC cell line LNCaP expresses NEP, whereas the androgen-independent PC cell line PC-3 does not (11), although both cell lines are derived from metastatic PC. To investigate whether the decrease in PKCδ protein expression results from the loss of NEP enzyme activity in metastatic PC, we evaluated PKCδ protein expression levels in LNCaP cells with or without the specific NEP inhibitor CGS24592 and PC-3 cells with or without recombinant NEP (rNEP). We confirmed that CGS24592 at a concentration of 100 nM effectively inhibited NEP enzyme activity in LNCaP cells as reported previously (15). Western blot analysis showed that LNCaP cells in medium containing 10% FCS (Fig. 1A, Lane 1) expressed a higher level of PKCδ protein compared with PC-3 cells (Lane 3). Treatment of LNCaP cells with 100 nM CGS24592 for 16 h resulted in a marked decrease in PKCδ protein expression compared with that in the untreated control (Lanes 1 and 2). In contrast, treatment of PC-3 cells with 50 μg/ml rNEP for 16 h resulted in a marked increase in PKCδ protein expression compared with that in the untreated control (Lanes 3 and 4). PKCδ expression was not affected by NEP enzyme activity in PC cells (Fig. 1A, middle panel). These results suggest that PKCδ protein expression levels in LNCaP and PC-3 cells are regulated in part by NEP enzyme activity.

We next used pulse-chase assays to determine whether NEP-induced effect on PKCδ protein resulted from increased production or delayed turnover of PKCδ protein. As shown in Fig. 1B, the level of labeled (initially produced) PKCδ proteins was decreased by about one-third at 6 h compared with that at time 0 in LNCaP cells cultured in medium containing 10% FCS (Fig. 1B, Lanes 1 and 2), whereas incubation with 100 nM CGS24592 for 6 h resulted in an 80% decrease in labeled PKCδ protein levels (Lanes 3 and 4). In PC-3 cells, the level of PKCδ protein production at time 0 was higher than in LNCaP cells (Lanes 1 and 5) but rapidly (90%) decreased within 6 h (Lanes 5 and 6). Incubation with 50 μg/ml rNEP for 6 h resulted in only a 30% decrease in labeled PKCδ protein level compared with that in untreated control cells (Lanes 7 and 8). These results suggest that NEP-induced increases in PKCδ protein expression in PC cells result from delaying PKCδ protein degradation.

NEP Promotes Etoposide-Induced Apoptosis in PC Cells. We next investigated whether NEP could promote etoposide-induced apoptosis in PC cells. Annexin V apoptosis assays demonstrated that incubation of LNCaP cells in 10 μM etoposide for 48 h resulted in a >6-fold increase in apoptotic cell number compared with untreated control (P < 0.01; Fig. 2A). Pretreatment with 100 nM CGS24592 16 h before the addition of etoposide resulted in a significant decrease in apo-
Apoptotic cell number \( (P < 0.05) \). In NEP-negative PC-3 cells, 10 \( \mu M \) etoposide treatment for 48 h failed to increase apoptotic cell number significantly, compared with untreated control (Fig. 2B). However, pretreatment with 50 \( \mu g/ml \) rNEP 16 h before the addition of etoposide resulted in a \( >4 \)-fold increase in apoptotic cell number compared with etoposide treatment \( (P < 0.01) \). These results suggest that NEP catalytic activity can promote etoposide-induced apoptosis in PC cells.

**NEP Promotes Anticancer Drug-Induced Apoptosis in PC-3 Cells through PKC\( _{\delta} \) Activity.** NEP increases PKC\( _{\delta} \) protein expression and promotes etoposide-induced apoptosis. Therefore, to determine whether these two processes are interconnected, we incubated PC-3 cells with the PKC\( _{\delta} \)-specific inhibitor rottlerin \( (IC_{50} = 3–6 \mu M \) for PKC\( _{\delta} ; 40 \mu M \) for PKC\( _{\alpha} \), PKC\( _{\beta I} \), PKC\( _{\gamma} \)) and measured etoposide-induced apoptosis. We had previously shown that rottlerin has a specific inhibitory effect on PKC\( _{\delta} \) activity in PC-3 cells (5). Apoptosis assays revealed that apoptosis resulting from the combination of 10 \( \mu M \) etoposide and 50 \( \mu g/ml \) rNEP in PC-3 cells was partially reversed by pretreatment with increasing concentrations of rottlerin 2 h before the addition of etoposide (Fig. 3, Lanes 4–7). Pretreatment with rNEP (50 \( \mu g/ml \) 16 h before treatment with 100 nM paclitaxel for 48 h in PC-3 cells also resulted in an \( \sim3 \)-fold increase in apoptotic cell number compared with paclitaxel alone (Fig. 3, Lanes 9 and 10). Similar to experiments using etoposide, apoptosis, induced by the combination of rNEP

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**Fig. 1** Neutral endopeptidase (NEP) activity regulates protein kinase C (PKC\( _{\delta} \)) protein expression in prostate cancer (PC) cells. A, PC cells were cultured in medium with the addition of the reagents indicated [100 nM CGS24592 (CGS) and 50 \( \mu g/ml \) recombinant NEP (rNEP) for 16 h. Total cell lysates were analyzed by Western blot analysis using anti-PKC\( _{\delta} \) antibody (Ab), anti-PKC\( _{\alpha} \) Ab, and anti-actin Ab. The intensity of each PKC\( _{\delta} \) band is shown as a value relative to that of LNCaP control, set to 1, in the bottom graph. B, the turnover of PKC\( _{\delta} \) was evaluated by pulse-chase assay, as described in “Materials and Methods.” The upper data are representative of three experiments. The intensity of each labeled PKC\( _{\delta} \) is shown as a value relative to that of each control, set to 100% (Lanes 1 and 4) in the bottom graph. The presented data are representative of two independent experiments with similar results.

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**Fig. 2** Neutral endopeptidase (NEP) promotes etoposide-induced apoptosis in prostate cancer (PC) cells. LNCaP (A) and PC-3 (B) cells were cultured in medium with the addition of the complex indicated [10 \( \mu M \) etoposide, 100 nM CGS24592 (CGS), 50 \( \mu g/ml \) recombinant NEP (rNEP)] for 48 h. Cells were stained with annexin V-FITC (annexin V binds to membrane phosphatidylserine that accumulates to the extracellular surface in early apoptotic cells). Apoptotic cells (annexin V-positive and propidium iodide-negative) were enumerated by flow cytometry. The upper data are representative of three experiments. In bottom graphs, means \( \pm \) SD from three separate occasions are shown. Bars, SD.
Neutral endopeptidase (NEP) promotes anticancer drug-induced apoptosis in PC-3 cells through protein kinase C (PKC)δ activity. A. PC-3 cells were pretreated with the complex indicated (50 μg/ml recombinant NEP (rNEP) for 16 h and rottlerin or G66976 at indicated concentrations for 2 h) and were further treated with anticancer drugs (10 μM etoposide or 100 nM paclitaxel) for 48 h. Apoptotic cells were enumerated by flow cytometry as described in Fig. 2A. The data show means ± SD from triplicate samples. Experiments were repeated twice with similar results.

Fig. 3 Neutral endopeptidase (NEP) promotes anticancer drug-induced apoptosis in PC-3 cells through protein kinase C (PKC)δ activity. A. PC-3 cells were pretreated with the complex indicated (50 μg/ml recombinant NEP (rNEP) for 16 h and rottlerin or G66976 at indicated concentrations for 2 h) and were further treated with anticancer drugs (10 μM etoposide or 100 nM paclitaxel) for 48 h. Apoptotic cells were enumerated by flow cytometry as described in Fig. 2A. The data show means ± SD from triplicate samples. Experiments were repeated twice with similar results.

NEP Promotes Anticancer Drug-Induced Mitochondrial Events in PC-3 Cells through PKCδ Activity. PKCδ activity is implicated in anticancer drug-induced mitochondrial events such as cyt-c release and caspase-9 activation (13, 14). We recently reported that etoposide induced PKCδ mitochondrial translocation followed by cyt-c release and caspase-9 activation in LNCaP cells (5). We, therefore, investigated whether NEP could promote drug-induced mitochondrial events through PKCδ activity in PC-3 cells. Western blot analysis of cytoplasmic fractions in PC-3 cells with anti-cyt-c antibody demonstrated that pretreatment with rNEP (50 μg/ml) 16 h before etoposide (10 μM) or paclitaxel (100 nM) for 12 h (Fig. 4B, top panels, Lanes 4 and 7) resulted in a marked increase in cyt-c release compared with either drug alone (Lanes 3 and 6). Pretreatment of PC-3 cells with rottlerin (10 μM) for 2 h resulted in a marked decrease in cyt-c release induced by the combination of rNEP and anticancer drugs (Fig. 4B, top panels, Lanes 5 and 8), suggesting that PKCδ activity is required for cyt-c release. Similar results were obtained using a caspase-9 colorimetric assay. The combination of rNEP with anticancer drugs (Fig. 4B, bottom graph, Lane 4 and 7) resulted in a 2–3-fold increase in caspase-9 activity compared with drug treatment alone (Lanes 3 and 6; P < 0.01). Pretreatment with rottlerin significantly reversed caspase-9 activation induced by the combination of rNEP and anticancer drugs (Lanes 5 and 8; P < 0.01). These results suggest that NEP promotes anticancer drug-induced mitochondrial events followed by apoptotic cell death dependent on PKCδ activity.

NEP Predominantly Increases PKCδ Protein Expression in PC-3 but not in PrEC. Earlier studies demonstrated that decreased PKCδ protein expression resulted from rapid protein degradation (16, 17). This observation suggests the possibility that rNEP can effectively increase PKCδ expression in PC cells. We, therefore, compared PKCδ protein expression levels in PC-3 cells with normal prostate epithelial cells PrEC, in the presence and absence of rNEP. Enzyme assays showed high levels of NEP-specific activity in total cell lysates from PrEC (data not shown). Western blot analysis showed that PrEC cells expressed higher amounts of PKCδ protein than did PC-3 cells (Fig. 5A, Lanes 1 and 3). However, treatment of PC-3 cells with 50 μg/ml rNEP for 16 h resulted in a >2-fold increase in PKCδ protein expression compared with PrEC cells treated with rNEP (Lanes 2 and 4). We next compared PC-3 and PrEC (with or without rNEP) for differences in PKCδ protein turnover. Pulse-chase assays revealed that the level of labeled PKCδ proteins in PC-3 cells was 2.6-fold higher at time 0 but more rapidly decreased within 6 h than that in PrEC cells (Fig. 5B, Lanes 1, 2, 5, and 6). Incubation with 50 μg/ml rNEP for 6 h blocked the decrease in labeled PKCδ protein level in PC-3 cells (Lanes 7 and 8). PKCδ protein turnover levels were similar in PrEC and PC-3 cells treated with rNEP (30–40% decrease compared with time 0; Lanes 3, 4, 7,
These results indicate that rNEP selectively increases PKC protein expression by protein stabilization in PC-3 cells compared with benign prostate cells.

**NEP Promotes Apoptosis Induced by the Low Concentration of Etoposide in PC-3 Cells but not in PrEC Cells.** Etoposide treatment for 48 h resulted in a ~4-fold increase in apoptotic cell number compared with untreated controls in PrEC (P < 0.01) but not in PC-3 cells, whereas the lower concentration of (0.1 μM) etoposide failed to promote apoptosis in either PrEC or PC-3 (Fig. 6A, Lanes 1, 3, and 5). Pretreatment with 50 μg/ml rNEP 16 h before the addition of 10 μM etoposide induced similar levels of apoptosis (45–50%) in both cell lines (Lane 4). However, pretreatment with 50 μg/ml rNEP 16 h before 0.1 μM etoposide resulted in a significant increase in apoptotic cell number (40%; P < 0.01 compared with 0.1 μM etoposide only) in PC-3 but not as significant increase in PrEC (Lane 6). Similar results were obtained by Western blot for detecting cyt-c release and caspase-9 cleavage (Fig. 6B). These results suggest that NEP can selectively promote PC cell apoptosis induced by lower concentration of etoposide with minimal damage to normal cells.

**DISCUSSION**

Recent studies indicate that expression of NEP is diminished in the majority of PCs (11, 18). We have previously shown that NEP has many biological functions. Reexpression of NEP inhibits PC cell growth, cell migration, and tumorigenicity (11, 15, 19). Although enzymatic inactivation of neuropeptides such as endothelin-1 and bombesin can account for some of the biological effects of NEP, we showed that NEP also inhibits the phosphorylation of focal adhesion kinase and PC cell migration...
by interacting with Lyn kinase and phosphatidylinositol-3-kinase independent of the catalytic activity of NEP (15). In the present study, we report on a previously unrecognized consequence of NEP loss. Through its ability to affect PKCδ expression, NEP plays an important role in anticancer drug-induced apoptosis. This observation provides a potentially effective therapeutic modality for the treatment of PC via modification of the protein expression of PKCδ, the mitochondrial translocation and kinase activity of which have been implicated in the mitochondrial apoptotic pathway (5, 13, 14, 20).

NEP neuropeptide substrates such as endothelin-1 and bombesin act as survival and antiapoptotic factors (21, 22), transactivators of epidermal growth factor receptor (23) and insulin-like growth factor receptor (24), and activators of Akt/phosphatidylinositol-3-kinase and transactivators of epidermal growth factor receptor (23) and activators of Akt/p130Cas signaling (22). Bombesin act as survival and antiapoptotic factors (21, 22), with neuropeptides inducing rapid PKCδ protein expression (25). Similar results were recently reported in which bombesin blocked etoposide-induced apoptosis in PC cells (27). NEP-negative PC cells are relatively resistant to anticancer drug-induced apoptosis because they express low levels of PKCδ protein and its kinase activity through rapid PKCδ degradation. The fact that rNEP is able to promote apoptosis induced by the low concentration of etoposide in PC-3 cells but not in PrEC is notable, suggesting that the combination of rNEP with anticancer drugs may be a potential therapeutic approach to treat advanced PC. Recombinant NEP potentially could decrease the dose of anticancer drugs with similar efficacy, lessening toxicity; and more importantly, rNEP may selectively sensitize apoptosis in PC cells resistant to conventional chemotherapy. Of note, PKCδ protein production levels are relatively higher in PKCδ-decreased PC-3 compared with PKCδ-highly expressing LNCaP and PrEC cells. Thus, NEP is capable of increase PKCδ protein expression predominantly in PC cells rather than normal cells as a result of higher production and an equal level of stabilization of PKCδ.

A preliminary analysis of metastatic PC tissue specimens suggests a high correlation of NEP and PKCδ expression,3 suggesting that decreased NEP expression in vivo leads to decreased PKCδ expression, similar to what has been observed in PC cell lines. This further supports the premise that NEP plus chemotherapy has a potential role in PC therapy and in PC-specific chemosenstitization. This could be accomplished through exogenous administration of recombinant NEP before chemotherapy, or through the up-regulation of cell-surface NEP, possibly using a demethylating agent, because, as we have previously shown, decreased NEP expression results in part from hypermethylation of the NEP promoter (28). Whereas we did not observe significant inhibition of tumorigenicity of PC xenographs in athymic mice receiving i.p. rNEP daily for 30 days (19), we did not explore whether rNEP, leading to increased PKCδ expression and followed by chemotherapy, would inhibit tumor growth. In this regard, PKCδ has been recently implicated in mitochondrial apoptosis induced by irradiation as well as by chemotherapy (29, 30). Ongoing in vivo studies using animal models are examining the effects of combining NEP and chemotherapy on PC tumor growth and should provide an additional rationale for the use of NEP to treat advanced PC.

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3 Unpublished data.


