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Neuroendocrine-derived peptides promote prostate cancer cell survival through activation of IGF-1R signaling

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Abstract

Background—Neuroendocrine (NE) cells promote the progression of prostate cancer to a castration-resistant state through the production of paracrine growth factors. We have demonstrated this principle using *in vitro* and *in vivo* proliferative endpoints; however, the contributions of NE-derived pro-survival factors and anti-apoptosis to this phenomenon have not been thoroughly investigated.

Methods—Here, we utilized conditioned-medium (CM) from LNCaP cells, engineered to undergo NE differentiation, and examined its effects on PC3 and LNCaP cell survival.

Results—Statistically significant changes in clonogenic survival, Annexin V staining, PARP cleavage and trypan blue positivity of approximately 2-fold were observed in the presence of NE-derived CM relative to control-CM for both LNCaP and PC3 cells. These changes were partially abrogated by antagonists of the neuropeptides neurotensin, bombesin, and PTHrP. Selective inhibitors of IGF-1R, EGFR or Src caused significant and nearly complete blockade of prostate cancer cell survival due to NE secretions. Similar increases in cell survival were observed for LNCaP or PC3 cells treated with NE-derived medium in the presence of docetaxel. Increased phosphorylation of IGF-1R, following treatment with NE-derived medium, was accompanied by decreased Protein Tyrosine Phosphatase, receptor type F (PTPRF) mRNA and protein levels. Overexpression of PTPRF decreased cell survival, the amplitude and duration of IGF-1R phosphorylation, and enhanced PARP cleavage in the presence of NE-derived medium.

Conclusions—These data support the hypothesis that NE-derived factors act upon prostate cancer cells to stimulate pro-survival signaling and describe a novel mechanism of cross-talk between NE-derived factors and IGF-1R, mediated in part by PTPRF.

Keywords

Neuroendocrine; EGFR; IGF-1R; PTPRF; apoptosis

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Introduction

Early stage prostate cancer (PCa) is responsive to androgen ablation therapy, but invariably recurs as castration-resistant and highly aggressive. The molecular mechanisms that promote PCa progression are not fully understood, but both continued androgen receptor-mediated signaling under conditions of low circulating ligand and upregulation of pro-survival pathways are implicated in this process (1, 2).

Neuroendocrine (NE) differentiation has been associated with progression of PCa to a castration-resistant phenotype. NE cells are more prevalent in castration-resistant disease, occurring in 30–100% of tumors studied (3, 4). The low proliferative capacity of NE cells allows them to resist treatment with most chemotherapeutic agents, as well as endocrine and radiation treatments (5, 6). Paracrine factors produced by NE cells bind to G-protein-coupled receptors (GPCR) and activate signaling pathways that stimulate tumor cell growth and may promote survival (7–9). Among these pathways is the EGFR/Src/STAT5b axis, which is transactivated upon NE factors binding their cognate receptors and subsequent metalloproteinase (MMP) liberation of membrane-bound EGFR ligands (9, 10). This hypothesis is supported by work demonstrating that NE-like cells enhance the growth of LNCaP xenografts, with the greatest effects seen under conditions of androgen deprivation (11, 12).

Accumulating evidence suggests that insulin-like growth factor 1 (IGF-1) signaling pathways also contribute to PCa progression. Studies in murine models have correlated altered IGF-1 or IGF-1 receptor (IGF-1R) levels with prostatic dysplasia, tumor growth, or metastasis (13–16). Human metastatic PCa specimens exhibit increased IGF-1R expression relative to primary tumors (17–19). Experimental approaches using lineage-derived PCa progression models demonstrate that increased IGF-1R signaling results in protection from apoptotic stress and enhanced mitotic activity that correlates with an androgen independent state (19). Furthermore, long-term androgen-ablation has been shown to cause increased resistance to phosphatidylinositol 3'-kinase (PI3K)/Akt inhibition (20), while reduction of IGF-1R levels in androgen independent DU145 cells results in increased chemotherapeutic sensitivity (21).

Ligand binding to the IGF-1R α -subunits leads to tyrosine phosphorylation of the β -subunits and activation of the receptor tyrosine kinase, resulting in subsequent activation of intracellular signaling pathways including Ras/MAPK and PI3K/Akt (2). Activation of PI3K/Akt signaling contributes to PCa progression by phosphorylation of Bcl-2 family member BAD and caspase-9 resulting in the inhibition of apoptosis and promoting cell survival (22). PI3K/Akt phosphorylation of BAD by Akt prevents the binding of BAD to BCL-XL, restoring the antiapoptotic function of BCL-XL (23). In addition, phosphorylation of pro-caspase 9 by Akt abrogates cytochrome c-induced proteolytic processing of pro-caspase 9, which in turn prevents the activation of downstream executor caspases (24).

GPCR agonists have also been shown to stimulate the phosphorylation of IGF-1R, suggesting that NE-derived paracrine factors promote crosstalk with the IGF-1 signaling pathway to modulate cell survival (25, 26). Activation of thrombin, angiotensin II, or GABA

receptors promotes phosphorylation of IGF-1R or PI3K/Akt (27–29). Furthermore, studies of the neuropeptides, endothelin-1 and bombesin, indicate they act as antiapoptotic factors and expression of neutral endopeptidase inhibits their ability to transactivate IGF-1R-mediated apoptosis (7, 8, 30).

Studies described in this report investigate the contribution of NE-derived factors to prostate cancer cell survival and resistance to chemotherapeutic agents, such as docetaxel, through the activation of IGF-1R. Our results demonstrate that CM derived from NE cells promotes PCa cell survival and protects from apoptotic stress through the transcriptional downregulation of the protein tyrosine phosphatase, receptor type F (PTPRF). PTPRF has been identified as a metastasis-associated gene in prostate cancer cell lines, and its activity is regulated through the interaction with EGFR (31, 32). In both LNCaP and PC3 PCa cell lines we observed that sustained EGFR and IGF-1R phosphorylation in response to NE-derived factors correlates with downregulation of PTPRF and decreased apoptosis, overexpression of PTPRF reversed these effects. To our knowledge, this is the first report demonstrating that IGF-1R activation is regulated downstream of NE signaling pathways by the modulation of protein tyrosine phosphatase function.

Materials and Methods

Reagents

Annexin-V-FITC flow cytometry kits were obtained from BD Biosciences (San Diego, CA). Anti-human IGF-1 antibody was purchased from R&D Systems (Minneapolis, MN). pCMV-PTPRF was obtained from Thermo Scientific (Huntsville, AL). Recombinant human des(1-3)IGF-1 was obtained from Cell Sciences (Canton, MA). The neurotensin receptor antagonist, SR48692, was a gift from Sanofi-Aventis (Malvern, PA). The bombesin receptor antagonist, (D-Phe⁶,Leu¹³-p-chloro-Phe¹⁴)-Bombesin(6-14), and PTHrP receptor inhibitor, (Nle^{8,18},Tyr^{3,4})-pTH(3-34) amide were obtained from Bachem (King of Prussia, PA). Inhibitors of EGFR (AG1478-100nM, Millipore, Billerica, MA), IGF-1R (PQ401-5μM, Tocris Bioscience, Ellisville, MO), c-Src (PP2-10μM, Millipore), or MMPs (GM6001-20μM, Millipore) were added to cells 1h prior to stimulation.

Cell culture

All cell culture media, fetal bovine serum (FBS), and Lipofectamine 2000 transfection reagents were obtained from Invitrogen (Carlsbad, CA). PC3 and LNCaP human prostate carcinoma cells were obtained from American Type Tissue Collection (ATTC; Rockville, MD) and grown as recommended.

Preparation of NE-CM

NE differentiation of LNCaP-D29 cells by doxycycline-induced expression of a constitutively active PKA catalytic subunit has been previously described (11). Neuroendocrine conditioned-medium (NE-CM) of LNCaP-D29 cells was collected following incubation for 5 days in serum-free medium with 1μg/ml doxycycline. Control CM (C-CM) was collected from LNCaP-D29 cells incubated in the absence of doxycycline.

Western blotting

Immunoblotting was performed as previously described (9), using the following antibodies: anti-PTPRF (BD Biosciences), anti-EGFR^{Tyr992} and anti-EGFR (Invitrogen), Anti-phospho-IGF-1R^{Tyr1135/1136}, anti-IGF-1R, and anti-rabbit-HRP (Cell Signaling Technology, Beverly, MA).

Clonogenic assays

Survival assays were performed as previously described (51). Colonies were counted after fixation and staining with 0.5% crystal violet in methanol. The surviving fraction was calculated as follows: Surviving fraction (S.F.) = (mean # colonies)/(cells inoculated) (plating fraction), where the plating fraction represents (# colonies)/(cells inoculated). Data were normalized such that S.F. = 1 for C-CM samples.

Flow cytometry

Cells were trypsinized, counted, and processed with binding buffer and Annexin-V-FITC (BD Biosciences) according to the manufacturer's instructions. 10,000 cells/sample were analyzed on a Becton-Dickinson FACS Caliber flow cytometer (Franklin Lakes, NJ). Data analysis was performed using Flow-Jo software at the UVA Flow Cytometry Core Facility.

Trypan Blue Cell Death Assay

Floating and adherent cells obtained following trypsinization were incubated in 0.2% trypan blue (Sigma, St. Louis, MO), and counted with a hemocytometer.

Gene expression analysis

Gene expression analysis using GeneChip HGU133 Plus 2.0 (Affymetrix, Santa Clara, CA) was performed by the UVA Biomolecular Research Facility. mRNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Triplicates of C-CM-treated LNCaP RNA were compared to triplicates of NE-CM-treated LNCaP RNA. Affimetrix GeneChip Operating Software was used to calculate signal intensity values as well as p-values for each sample. Two group comparisons were carried out using the dChip software (<http://biosun1.harvard.edu/~cli/dchip>). Gene expression was deemed significantly different if the p-value was less than or equal to 0.05, the fold change in signal intensity was greater than 1.5 or less than -1.5, and the absolute difference in signal intensity was greater than 100.

Signal transduction pathway bioinformatics

The genes identified as significantly varied between the controls and the experimental samples were used for data mining with the Ingenuity software. Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules in the canonical pathway. 2) Fisher's exact test was used to calculate a p-value

determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Real-time reverse transcription-PCR

Real-time reverse transcription-PCR was performed as previously described (9). The PCR primers used were the following: PTPRF, 5'-GCATGTCTACTGGAAGCT-3' (forward) and 5'-CCTTGCCGAAATCTATGG-3' (reverse); and GUS, 5'-CCGACTTCTCTGACAACCGACG-3' (forward) and 5'-AGCCGACAAAATGCCGCAGACG-3' (reverse).

Statistical analysis

Error bars represent the standard error of the mean (SEM) from N=3 independent experiments, unless indicated. P values were obtained using a Student's t-test; P values less than 0.05 were considered statistically significant.

Results

NE-CM enhances survival of prostate cancer cells

To determine whether NE-CM confers a survival advantage to PCa cells clonogenic survival of LNCaP or PC3 cells was assayed following overnight serum deprivation and 24h treatment with C-CM or NE-CM. An increase of approximately 2 fold in the surviving fraction of LNCaP or PC3 cells was observed in the presence of NE-CM (Fig. 1A+B). Incubation with NE-CM reduced the number of apoptotic LNCaP or PC3 cells by 38% or 47%, respectively, as measured by Annexin V flow cytometry (Fig. 1C+D). Under similar conditions as described in Figure 1A, the number of LNCaP or PC3 cells staining positively for trypan blue decreased by 55% or 54%, respectively, following treatment with NE-CM relative to C-CM (Fig. 1E+F). These results indicate that paracrine factors secreted by NE cells contribute to pro-survival signaling in PCa cells.

Docetaxel-based regimens have become the standard of care in the management of castration-resistant PCa (33). To determine how NE-derived peptides influence the response of PCa cells to chemotherapeutics we analyzed the effect of NE-CM treatment on docetaxel-induced LNCaP or PC3 cell death. Treatment of LNCaP or PC3 cells with 10nM docetaxel in the presence of C-CM resulted in approximately 6-fold increase in the number of cells staining positive for trypan blue relative to no docetaxel in C-CM. This level of cell death was reduced by 50% in the presence of NE-CM (Fig. 2A+B). NE-CM treatment also decreased the extent of PARP cleavage, indicative of reduced apoptotic induction (Fig. 2C +D). Treatment of the PCa cells with docetaxel together with the small molecule inhibitors specific for EGFR, IGF-1R, Src or MMP's in the presence of NE-CM restored PARP cleavage to levels equivalent to docetaxel/C-CM conditions implicating the involvement of EGFR transactivation and IGF-1R function in the pro-survival effect of NE-CM (Fig. 2C +D).

Inhibition of IGF-1R or EGFR antagonizes pro-survival effects of NE-CM

To further investigate the role of paracrine factors produced by NE cells in promoting survival of PCa cells we performed global analysis of gene expression in LNCaP cells treated for 24h with NE-CM (Table S1). Canonical pathway analysis of gene expression data using Ingenuity Pathways Library revealed that expression of IGF-1R and EGFR pathway genes are most significantly different between NE-CM and C-CM treatments (Fig 3A).

Inhibition of IGF-1R or EGFR signaling confirmed the requirement of these signaling pathways in mediating the survival signals of NE-CM. Treatment of LNCaP or PC3 cells with AG1478, PQ401, or the Src inhibitor PP2 for 24h abolished the pro-survival effects of NE-CM as measured by trypan blue exclusion. In addition, statistically significant increases in the percentage of cell death were observed with combinatorial but not individual antagonism of GPCRs mediating the action of neuropeptides previously shown to be present in NE-CM (Fig. 3B+C, (34)). Antagonism of the PTHrP receptor significantly reduced the pro-survival effects of NE-CM in PC3 cells, suggesting that this GPCR pathway is a more critical mediator of NE-induced survival signaling in the PC3 cell model (Fig. 3C). Pharmacological inhibition of components mediating NE-induced signaling does not alter the percentage of surviving cells in the presence of C-CM, indicating that their effects on cell viability are specific to NE-secreted factors (Fig. S1).

NE-induced activation of IGF-1R signaling is independent of IGF-1 binding

To determine whether NE-CM promotes the activation of IGF-1R in LNCaP or PC3 cells, phosphorylation of IGF-1R Tyr^{1135/1136} was measured by immunoblotting with phosphospecific antibodies. Phosphorylation of Tyr^{1135/1136} was increased 1h after stimulation with NE-CM and persisted for 24h but was not induced by C-CM. Similarly, phosphorylation of EGFR-Tyr⁹⁹² was sustained throughout the time course of analysis in response to NE-CM (Fig. 4A+B). The kinetics of IGF-1R and EGFR activation in response to NE-CM differs from stimulation with the cognate growth factor. Upon stimulation of LNCaP or PC3 cells with IGF-1, the extent of IGF-1R tyrosine phosphorylation is significantly greater than with NE-CM treatment, peaking at 30 min and returning to basal levels after 3h (Fig. S2A+B). In response to EGF the kinetics of IGF-1R activation are similar to stimulation with IGF-1, however the amplitude of receptor phosphorylation is equivalent to NE-CM treatment (Fig. S2A+B and 4E+F). Treatment of LNCaP or PC3 cells with AG1478, PQ401, PP2, or GM6001 partially or completely inhibited IGF-1R phosphorylation in response to NE-CM. Interestingly, pIGF-1R inhibition had no effect on NE-CM-induced EGFR phosphorylation (Fig. 4C+D). In the AR-positive C4-2B model of CRPC, NE-CM-induced activation of IGFR, EGFR, or Src was also completely abrogated by the combined inhibition of the PTHrP, Neurotensin, and Bombesin receptors, the EGFR inhibitor Lapatinib, the Src inhibitor Dasatinib, or GM6001 (Fig S3). Together these observations suggest that the pro-survival advantage conferred by NE-derived factors is associated with low but sustained phosphorylation of IGF-1R and occurs in cancer cells that model both pre-castrate-resistance (LNCaP) and castrate-resistance (PC-3). In addition, pharmacological inhibition of NE-induced signaling pathways indicates that EGFR and Src activation are required for IGF-1R phosphorylation.

To uncover the mechanism of IGF-1R activation following NE-CM treatment we first asked whether, in addition to neuropeptide production, cells that undergo NE differentiation also increase the production of IGF-1. ELISA measurement of IGF-1 in C-CM or NE-CM revealed equivalent concentrations ranging from 0.012–0.013ng/ml (Fig. S4A). Similarly, statistically significant differences in IGF-1 concentration were not observed in the media of LNCaP, or PC3 cells following treatment with C-CM or NE-CM for 24h, indicating that the observed increase in IGF-1R phosphorylation does not result from enhanced autocrine production of IGF-1 following NE-CM treatment (Fig. S4B+C). To further rule out enhanced IGF-1-mediated activation of IGF-1R in NE-CM, PCa cells were treated with hIGF-1 blocking antibody. Neutralization of IGF-1 did not block IGF-1R phosphorylation in response to NE-CM, but did significantly reduce IGF-1R phosphorylation induced by IGF-1. Importantly, EGF-induced transactivation of IGF-1R was not neutralized by addition of IGF-1 blocking antibody (Fig. 4E+F), indicating that EGFR-mediated activation of IGF-1R is independent of IGF-1 binding to its cognate receptor. Together these data suggest that NE-CM-mediated activation of IGF-1R occurs via an intracellular mechanism.

Transcriptional downregulation of PTPRF in response to NE-CM results in decreased IGF-1R signaling

Analysis of global gene expression in response to NE-CM indicated that various components of IGF-1 signaling are significantly modulated by NE stimuli (Table 1), and identified PTPRF as a potentially important node in the activation of IGF-1R. This membrane-associated tyrosine phosphatase interacts with and regulates IRS-1, IRS-2, as well as Insulin receptor (IR), but its phosphatase activity is regulated by the interaction with EGFR and Src family kinases (32, 35). Using quantitative real-time PCR analysis we verified that PTPRF mRNA levels are reduced by approximately 2.5 fold in both LNCaP and PC3 cells following treatment for 24h with NE-CM relative to control media (Fig. 5A+B). Similarly, PTPRF protein levels were decreased upon treatment with NE-CM for 6h in both cell lines (Fig. 5C+D). To determine whether modulation of PTPRF levels alters IGF-1R activation we transiently overexpressed the protein in either LNCaP or PC3 cells and monitored the phosphorylation state of IGF-1R following treatment with NE-CM. Relative to vector transfection control conditions, PTPRF expression levels were not reduced following NE-CM treatment but IGF-1R phosphorylation was decreased in both amplitude and duration, returning to basal levels after 6h of stimulation (Fig. 5E+F). These results suggest that maintenance of appropriate levels of PTPRF expression influences both the extent and duration of IGF-1R signaling in response to NE stimuli.

To examine the role of PTPRF in mediating the pro-survival effects of paracrine factors produced by NE cells, we assayed the extent of PCa cell death under PTPRF overexpression conditions. Transient overexpression of PTPRF in LNCaP or PC3 cells reversed the survival effects of NE-CM, inducing equivalent percentages of cell death in the presence of NE-CM relative to C-CM treatment (Fig. 6A+B). The extent of PARP cleavage under conditions of PTPRF overexpression in the presence of NE-CM was also equivalent to C-CM alone or together with PTPRF overexpression (Fig. 6C+D). Together these observations indicate that the pro-survival effects of NE-derived factors are achieved in part through the

downregulation of PTPRF, and the inability of cells to reduce PTPRF expression abrogates these effects.

Discussion

In this study, we demonstrate that NE signaling pathways promote prostate cancer progression by stimulating pro-survival pathways through activation of IGF-1R. We describe the transcriptional downregulation of the IGF-1R phosphatase, PTPRF, as a consequence of GPCR-initiated signaling transduced through the activation of EGFR/Src, resulting in sustained activity of IGF-1R and decreased apoptotic stress. Results from different experimental approaches indicate that activation of IGF-1R in response to NE-derived factors occurs independently of IGF-1 binding to its receptor. Cytokine array analysis of NE-CM did not reveal the increased presence of any other factors that may directly activate IGF-1R (unpublished data). Although other yet unidentified factors may be present in NE-CM, this observation lends support to the hypothesis that increased phosphorylation of IGF-1R results from neuropeptide-induced crosstalk with the EGFR/Src signaling pathway.

IGF-1R has previously been implicated in the progression of PCa, but few studies have addressed the role of NE differentiation in modulating IGF-1R signaling. We and others have demonstrated that EGFR and Src kinase activities are enhanced by neuropeptide stimulation of PCa cells and these activities are essential to promote cell proliferation and migration (9, 10, 36). Our studies demonstrating that EGFR/Src activities modulate IGF-1R activity supports observations made in both Rat aortic smooth muscle and PCa cells that Src activation leads to IGF-1R phosphorylation on the same autophosphorylation sites as induced by IGF-1 (8, 30). These data suggest that paracrine factors that stimulate EGFR/Src activity can induce ligand-independent IGF-1R signaling. In agreement with this hypothesis, we observed that EGF-induced signaling results in IGF-1R phosphorylation in both LNCaP and PC3 cells. In the context of PCa progression, increased NE-derived peptides that activate EGFR/Src signaling contribute to tumor cell proliferation and may also enhance their survival through ligand-independent transactivation of IGF-1R. The effect of NE-CM was observed in both androgen-dependent and androgen-independent PCa models and thus may reflect an early event that precedes the acquisition of castration-resistant properties but could also enhance survival of castrate-resistant cells.

It is well established that temporal regulation of EGFR-mediated signaling pathways gives rise to unique patterns of gene expression resulting in differential phenotypic cellular responses. In PC-12 or MCF-7 cells, for example, transient activation of ERK in response to EGF results in a proliferative response, but sustained ERK signaling promotes cellular differentiation (37, 38). Distinct cell-fate decisions downstream of IGF-1R activation are thought to occur primarily through differential recruitment of substrate proteins such as IRS-1 or IRS-2, but temporal regulation of ligand bioavailability mediated by IGF binding proteins may also be involved (39, 40). We observed that modulation of PTPRF levels in response to NE-derived factors alters the temporal response of IGF-1R signaling and influences apoptotic induction. These results suggest that cell fate decisions downstream of

IGF-1R activation also involve a novel regulatory mechanism of temporal signaling controlled by receptor phosphatase activity.

Many protein tyrosine phosphatases have been shown to interact with and modulate various receptor tyrosine kinase (RTK) signaling pathways. The protein tyrosine kinases IGF-1R, IR, EGFR, HGFR, RET, LCK and FYN have been reported to be substrates of PTPRF (35, 41–44). In PTPRF overexpressing mice phosphorylation of IRS-2, a signaling mediator IGF-1R and IR, is reduced and PI3K function associated with IRS-1 and IRS-2 is also decreased (45). Overexpression of PTPRF in our PCa cell model systems resulted in reduced IGF-1R phosphorylation. This result is consistent with observations made in vascular smooth muscle cells demonstrating that IGF-1R phosphorylation is enhanced in the absence of PTPRF (44). EGFR association with PTPRF induces tyrosine phosphorylation and TACE-mediated proteolytic processing of the phosphatase (32). This post-translational mechanism of PTPRF regulation downstream of EGFR suggests that NE-induced modulation of IGF-1R signaling may be multilayered.

We show that NE-derived factors enhance PCa cell viability in the presence of docetaxel, suggesting that increased NE differentiation observed in advance stage tumors may modulate the chemotherapeutic response. Docetaxel has become the treatment of choice for management of castration-resistant PCa. Although implementation of docetaxel-based therapeutic regimens has significantly improved patient survival, the response rate has been disappointing (33, 46). In addition, docetaxel is dose-limited due to various adverse side effects (33). Novel combinations to reduce dose-limiting toxicity of docetaxel and increase its efficacy could have a significant impact on disease-related morbidity and mortality. Docetaxel directly binds to the β -subunit of tubulin and alters microtubule dynamics, which ultimately leads to mitotic arrest and apoptosis (47). However, chemosensitivity to docetaxel is also influenced by other pro-survival signaling pathways (48–50). We found that combinatorial treatment with docetaxel and inhibitors of NE signaling mediators, including EGFR and IGF-1R, restores PARP cleavage and abrogates the pro-survival effects of NE-derived soluble paracrine factors. This approach may provide clinical benefit for patients with tumors that exhibit NE differentiation and warrants further investigation.

We conclude from these studies that secreted paracrine factors produced by NE cells contribute to pro-survival signaling of prostate cancer cells and provide a survival advantage under hormone-deprived and/or chemotherapeutic conditions. Binding of NE peptides to cognate GPCR's induces Src- and MMP-dependent EGFR transactivation that in turn mediates IGF-1R phosphorylation. Concomitantly, NE-initiated signaling down-regulates transcription of PTPRF, allowing moderate but sustained activation of IGF-1R. These signaling events enhance the ability to prostate cancer cells to survive apoptotic stress. The data suggest that the clinical benefit of docetaxel- or hormone deprivation-based regimens may be enhanced in prostate cancer patients exhibiting NE differentiation by combinatorial inhibition of EGFR or IGF-R signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NE-CM enhances survival of prostate cancer cells

A+B, LNCaP or PC3 cells were treated for 48h with NE-CM or C-CM, prepared as described in Materials and Methods. Cells were trypsinized, diluted, and plated for colony formation. After 10 days, colonies were stained with crystal violet and counted; the surviving fraction was calculated as described in Materials and Methods. **C+D**, Detection of apoptotic cell death by Annexin V-FITC staining using flow cytometry following 48h treatment with NE-CM or C-CM. **E+F**, Trypan blue exclusion assay of LNCaP or PC3 cell death following 48h treatment with NE-CM or C-CM. Data are presented as the mean $\pm$ SEM of 3 independent experiments, each performed in triplicate; *P < 0.05 compared to C-CM.

Figure 2. NE-CM reduces docetaxel-induced cell death

A+B, Cell death of LNCaP or PC3 cells treated with NE-CM or C-CM in the presence or absence of docetaxel (10 nM) for 48h was determined by trypan blue staining. Results are expressed as the mean $\pm$ SEM of 3 independent experiments; ** $P < 0.05$ relative to NE-CM/doc; * $P < 0.05$ compared to NE-CM. **C+D**, LNCaP or PC3 cells were incubated with NE-CM or C-CM $\pm$ inhibitors of EGFR (AG1478-AG), IGF-1R (PQ401-PQ), Src (PP2), or MMP's (GM6001-GM) for 48h. Apoptotic induction was determined by extent of PARP cleavage with immunoblotting using anti-PARP antibodies.

Figure 3. Inhibition of NE-induced signaling antagonizes the pro-survival effect of NE-CM
A, Global gene expression analysis of LNCaP cells treated with NE-CM for 24h was performed as described in Materials and Methods. Fisher's exact test was used to calculate a p-value determining the probability of association between the genes in the dataset and canonical signaling pathways within the Ingenuity library. The four signaling pathways most significantly altered by NE-CM treatment are depicted. **B+C**, LNCaP or PC3 cells were incubated with C-CM, NE-CM $\pm$ inhibitors of the neurotensin (NT), bombesin (Bom), PTHrP receptors, or all three inhibitors combined (comb), as well as inhibitors of EGFR (AG1478-AG), IGF-1R (PQ401-PQ), Src (PP2), or metalloproteases (GM6001-GM). Cell death was analyzed by trypan blue exclusion. Results are expressed as the mean $\pm$ SEM of 3 independent experiments; *P < 0.05 relative to NE-CM

Figure 4. Treatment of prostate cancer cells with NE-CM activates IGF-1R

A+B, LNCaP or PC3 cells were serum-starved and treated with C-CM or NE-CM for the indicated time. Immunoblotting of cell extracts was performed with anti-phospho-IGF-1R, anti-IGF-1R, anti-phospho-EGFR, or anti-EGFR antibodies. **C+D**, Immunoblotting of cells treated as in (A+B) along with the small molecule inhibitors of EGFR (AG1478-AG), IGF-1R (PQ401-PQ), Src (PP2) or MMPs (GM6001-GM). Quantitation of phospho-receptor/total receptor was performed for 3 independent experiments and expressed as a ratio relative to NE-CM(3hr). **E+F**, LNCaP or PC3 cells were serum-starved, pre-incubated with or without hIGF-1 blocking antibody (5ug/ml), and stimulated with NE-CM or control-CM for 12h, as well as des(1-3)IGF-1 (5ng/ml) or EGF (10ng/ml) for 15min. IGF-1R phosphorylation was assessed by immunoblotting as described in (A+B). Quantitation of phospho-IGF-1R/IGFR was performed for 3 independent experiments and expressed as a ratio relative to IGF-1 stimulation. Representative blots are shown of 3 independent experiments.

Figure 5. Downregulation of PTPRF in response to NE-CM activates IGF-1R

A+B, RNA extracted from LNCaP or PC3 cells treated for 24h with NE-CM or C-CM was analyzed by quantitative real-time PCR using primers specific to PTPRF. Experiments were performed in duplicate and repeated 3 times. The results were normalized to β -glucuronidase reference gene and are expressed as fold change from control treatment. * $P < 0.05$. **C+D**, Cell extracts prepared following treatment with NE-CM or C-CM for the indicated times were immunoblotted with anti-PTPRF antibodies. **E+F**, LNCaP or PC3 cells were transiently transfected with vector or pCMV-PTPRF, and immunoblot analysis of IGF-1R phosphorylation was performed following treatment with NE-CM for the indicated times. Representative blots are shown of 3 independent experiments.

Figure 6. Overexpression of PTPRF abrogates the anti-apoptotic effect of NE-CM
A+B, LNCaP or PC3 cells were transiently transfected with vector or pCMV-PTPRF. Following NE-CM or C-CM treatment for 48h cell death was determined by trypan blue staining. Results are expressed as the mean $\pm$ SEM of 3 independent experiments; * $P < 0.05$ relative to NE-CM. **C+D**, Apoptotic induction of cells treated as described in (A+B), was determined by extent of PARP cleavage with immunoblotting using anti-PARP antibodies. Representative blots are shown of 3 independent experiments.

Table 1
IGF-1 signaling genes modulated by NE-CM

IGF-1 signaling pathway genes up-regulated or down-regulated in LNCaP cells following 24h NE-CM treatment, as identified by Affymetrix microarray and Ingenuity Pathways analysis.

Gene	Accession	Fold Change	P value
protein tyrosine phosphatase, receptor type, F	AU158443	−1.47	0.041649
triple functional domain (PTPRF interacting)	NM_007118	−1.71	0.036082
phosphoinositide-3-kinase, class 2, alpha polypeptide	NM_002645	−1.41	0.03238
phosphoinositide-3-kinase, catalytic, beta polypeptide	AA805318	−1.86	0.00372
mitogen-activated protein kinase kinase kinase kinase 4	NM_004834	1.2	0.005317
v-akt murine thymoma viral oncogene homolog 1	NM_005163	1.14	0.128342
ribosomal protein S6 kinase, 90kDa, polypeptide 2	BC002363	1.3	0.004726
ribosomal protein S6	AW205632	−1.42	0.018882
eukaryotic translation initiation factor 4E binding protein 1	AB044548	1.4	0.004413
eukaryotic translation initiation factor 4A, isoform 1	U79273	−1.58	0.015272
protein kinase, AMP-activated, beta 1 non-catalytic subunit	AW572402	−1.35	0.045728
phosphoenolpyruvate carboxykinase 2 (mitochondrial)	NM_004563	1.45	0.003347