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Growth of Androgen-Independent Prostate Cancer

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<b>13. ABSTRACT (Maximum 200 Words)</b> Prostate cancer is the most common primary cancer among men and the second leading cause of cancer deaths in males in the United States. While withdrawal of male hormones is the primary treatment for patients who develop advanced disease, most patients will show evidence of disease progression within 2 years. Secondary therapies are unsuccessful once tumors become hormone refractory, and most patients will die of their disease within 12 months resulting in 37,000 anticipated deaths in 1999. Understanding the mechanisms involved in the development of hormone resistance is crucial to developing therapies to treat hormone-refractory prostate cancer. We propose that growth of hormone-refractory prostate cancer is aided in part by the decreased presence of a cell surface enzyme, neutral endopeptidase, which normally functions to inactive growth factors which stimulate the prostate cancer cells to grow. The aim of this project is to delineate the role of neutral endopeptidase in regulating the growth of hormone refractory prostate cancer, and to define the mechanisms by which neutral endopeptidase can inhibit prostate cancer growth. Understanding the involvement of NEP in the progression to hormone refractory prostate cancer and in inhibiting prostate cancer growth will lead to the development of a clinical strategy for the use of neutral endopeptidase as therapy to treat this disease.				
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## **Introduction**

Numerous studies implicate the neuropeptides neurotensin, bombesin and endothelin-1 in the growth and development of hormone-refractory (androgen-independent) prostate cancer (PC). Neutral endopeptidase (NEP) is a cell-surface peptidase normally expressed by prostate epithelial cells and hormone-naïve (androgen-dependent) PC cells which inactivates a variety of physiologically active peptides, including neurotensin, bombesin and endothelin-1, thereby reducing local concentrations of peptide available for receptor binding and signal transduction. We have shown that expression of NEP is decreased in androgen-independent PCs in vitro in cell lines, and in vivo in metastatic biopsy specimens from patients with androgen-independent disease. Growth of androgen-independent PC cells is significantly inhibited by recombinant NEP added to media and by overexpression of cell-surface NEP using an inducible vector system in PC cells. Furthermore, our data indicate that in androgen-dependent PC cells, expression of the NEP gene is transcriptionally regulated by androgen and decreases with androgen-withdrawal. Consequently, PC cells which survive androgen-withdrawal can emerge with reduced NEP. These data are consistent with a model in which androgen-withdrawal allows neuropeptide-mediated actions by downregulating NEP and facilitating the development of a neuropeptide-stimulated androgen-independent PC cell population, suggesting one reason why standard therapy for patients with advanced PC leads to androgen-independent progression. The goals of this proposal are to explore the mechanisms of NEP regulation of PC cell growth with the intent of developing a clinical strategy for the use of NEP as therapy for androgen-independent PC. The specific aims of this proposal are: (1) to explore the mechanism by which NEP inhibits prostate cancer cell growth by characterizing the biological effects of NEP on androgen-independent PC cells, establishing that recombinant NEP and overexpression of cell-surface NEP induces apoptosis in androgen-independent PC cells, and by determining if growth inhibition induced by NEP results from PC cells arresting in a specific phase of the cell cycle; (2) to determine the effect of NEP on integrin mediated signaling pathways by confirming that NEP can inhibit the phosphorylation on tyrosine of focal adhesion kinase (FAK), by assessing the affect of NEP on the invasiveness and migration of androgen-independent PC cells, and by determining the effect of NEP on neuropeptide-mediated signal transduction; and (3) to assess the antitumor effects of NEP in an animal model of prostate cancer. These studies will help delineate biological significance of NEP loss in the development and maintenance of androgen-independent prostate cancer, and indicate the potential for recombinant NEP or NEP directed gene therapy as therapy in patients with androgen-independent PC.

**Body**

*Note: Dr. Dai left the lab in January of 2001 and Dr. Iwase joined the lab in May 2001. The summary represents work by both researchers.*

**a. Statement of Works.**

The objective of this research proposal is to elucidate and to understand the mechanism of neutral endopeptidase 24.11 (NEP), a cell-surface peptidase which inactivates neuropeptide growth factors at the cell surface, inhibits neuropeptide mediated growth of androgen-independent prostate cancer. The specific aims are:

*Task 1.* To explore the mechanism by which NEP inhibits prostate cancer cell growth.

- a. characterize the biological effects of NEP androgen-independent PC cells.
- b. establish that recombinant NEP and overexpression of cell-surface NEP induces apoptosis in androgen-independent PC cells.
- c. determine if growth inhibition induced by NEP results from PC cells arresting in a specific phase of the cell cycle.

*Task 2.* To determine the effect of NEP on integrin mediated signaling pathways

- a to confirm that NEP can inhibit the phosphorylation on tyrosine of focal adhesion kinase (FAK).
- b to assess the affect of NEP on the invasiveness and migration of androgen-independent PC cells.
- c to determine the effect of NEP on neuropeptide-mediated signal transduction.

*Task3.* To assess the antitumor effects of NEP in an animal model of prostate cancer.

- a. to establish that recombinant NEP can inhibit the tumorigenicity of androgen-independent PC cells in an orthotopic model of PC .
- b. to establish that overexpression of NEP in androgen-independent PC cells inhibits the tumorigenicity of androgen-independent PC cells in an orthotopic model of PC.

**b. Studies and Results**

During the second year of the fellowship, we have focused our efforts on defining the mechanisms by which NEP inhibits prostate cancer growth, and for the first time have shown that NEP has multiple effects on PC cells. We have described novel mechanisms of NEP action by delineating the effects of NEP on a variety of signaling pathways, including GPCR and tyrosine kinase pathways. In addition to showing that NEP inhibits neuropeptide signaling through catalytic inactivation of its substrates, we show that NEP directly interacts with lyn kinase to sequester the p85 subunit of phosphoinositide 3-kinase (PI3K), an important regulator of cell growth, survival and cell migration. Furthermore, we have shown that NEP can inhibit ligand-independent activation of the insulin growth factor-I receptor and of akt/protein kinase B. Using our animal model of PC, we have shown that overexpression of cell-surface NEP can inhibit tumor growth in an established tumor, and have begun analyzing this tumor tissue to assess the mechanisms of NEP action in tumor cells in vivo.

**The mechanism by which NEP inhibits cell growth (Task 1).** Using our PC cell lines and our constructed PC cell lines in which wild-type (WT-5 cells) and mutant (M22 cells) NEP cell-surface expression can be induced, we investigated the mechanisms of NEP regulation of cell migration in PC cells, including regulation of phosphorylation on tyrosine of focal adhesion kinase (FAK). Western analyses and cell migration assays revealed an inverse correlation between NEP expression and the levels of FAK phosphorylation and cell migration. Constitutively expressed NEP, recombinant NEP, and induced NEP expression using a tetracycline-repressive expression system inhibited bombesin and endothelin-1 stimulated FAK phosphorylation and cell migration. This results from NEP-induced inhibition of neuropeptide-stimulated association of FAK with cSrc protein. Expression of a mutated catalytically inactive NEP protein (M22) also resulted in partial inhibition of FAK phosphorylation and cell migration. Co-immunoprecipitation experiments show that NEP associates with

tyrosine-phosphorylated Lyn kinase, which then binds the p85 subunit of PI3-K resulting in NEP-Lyn-PI3-K protein complex. This complex competitively blocks FAK-PI3-K interaction, suggesting that the NEP protein inhibits cell migration via a protein-protein interaction independent of its catalytic function. These experiments demonstrate that NEP can inhibit FAK phosphorylation on tyrosine and PC cell migration through multiple pathways, and suggest that NEP plays a crucial role in regulating cell migration which contributes to invasion and metastases in PC cells.

We also have examined the effects of NEP expression on cell cycle and cell-cycle related proteins. Flow cytometric analysis show that induced NEP expression in WT-5 cells resulted in a G1 cell cycle arrest. Furthermore, NEP induced a 4-fold increase in the number of PC cells undergoing apoptosis, and increased expression of p21 tumor suppressor gene protein and in the level of unphosphorylated retinoblastoma protein, but did not affect expression of cyclin A, cyclin D, or p27 proteins. Recent studies also indicate that NEP expression results in decreased expression of the epidermal growth factor receptor.

G-protein coupled receptor (GPCR) agonists such as neuropeptides activate the insulin-like growth factor-1 receptor (IGF-1R) or the serine-threonine protein kinase Akt suggesting that neuropeptides-GPCR signaling can crosscommunicate with IGF-1R-Akt signaling pathways. We therefore investigated the effect of NEP on its neuropeptide substrates ability to induce phosphorylations of IGF-1R and Akt. Western analyses revealed endothelin-1 (ET-1) and bombesin treatment induced phosphorylation of IGF-1R $\beta$  and Akt independent of IGF-1 in TSU-Pr1, DU145 and PC-3 PC cells which lack NEP expression, but not in NEP-expressing LNCaP cells. Recombinant NEP and induced NEP expression in TSU-Pr1 cells using a tetracycline-repressive expression system inhibited ET-1-mediated phosphorylation of IGF-1R $\beta$  and Akt, and blocked the protective effects of ET-1 against apoptosis induced by serum starvation. These data show that ET-1 and bombesin stimulate ligand-independent activation of the IGF-1R which results in Akt activation, and that this crosscommunication between GPCR and IGF-1R signaling is inhibited by NEP.

**The effect of NEP on neuropeptide mediated signaling pathways (Task 2).** Neuropeptide growth factors such as bombesin are implicated in progression to androgen-independent PC. To examine the impact of bombesin on androgen receptor (AR) mediated gene expression, we co-transfected the AR with the AR-responsive probasin ARR<sub>3</sub>tk-luc or PSA-pPUE-ELB-luc promoter into Swiss 3T3 and PC-3 cells, both of which express high affinity bombesin receptors, incubated the cells with bombesin (0-50 nM) and dihydrotestosterone (DHT; 0-10 nM), and measured luciferase activities. DHT increased transcription ~40-fold at doses of 1 and 10 nM but had no effect at 10 pM. Bombesin alone, or with 1 or 10 nM DHT did not further increase transcription. However, 5 nM bombesin and 10 pM DHT, doses that by themselves had no effect, resulted in a ~20 fold increase in transcription (p<0.005). This synergistic effect was blocked by bombesin receptor antagonists and recombinant neutral endopeptidase which hydrolyzes bombesin. Bombesin and DHT together also increased binding of nuclear extracts from PC-3 cells transfected with AR to a consensus androgen response element in mobility shift assays, and increased the level of secreted prostate specific antigen in LNCaP cell supernatant compared with DHT or bombesin alone. Immunoprecipitation of AR from <sup>32</sup>P-labeled LNCaP cells revealed that 5 nM bombesin + 10 pM DHT induced AR phosphorylation comparable to 1 nM DHT, while bombesin or 10 pM DHT alone did not. These data indicate that bombesin can synergize with low (castrate) levels of DHT to induce AR mediated transcription, and suggest that neuropeptides promote AR-mediated signaling in androgen-independent prostate cancer.

**To establish that recombinant NEP can inhibit the tumorigenicity (Task 3).** We have completed our study on the effects of NEP expression on tumorigenicity in an animal model of PC. We showed that expression of NEP inhibits tumor formation in the prostate. We also have completed an experiment in which WT-5 cells are injected into the flanks of animal fed with tetracycline (NEP not expressed), allow to grow to 0.5 cm in diameter, and then tetracycline removed from the feed of half the animals. The tumors in the animals not receiving tetracycline either stabilize or decrease in size compared to control. These tumor tissues were resected and are currently being analyzed to determine if the observations we have made on NEP mechanisms in vitro also are true in vivo (i.e., induction of apoptosis, inhibition of growth factor signaling pathways, etc.).

**c. Plans.**

Our continuing objective is to elucidate and to understand the involvement of NEP in the development and maintenance of androgen-independent prostate cancer, and to understand the mechanisms of NEP action. We will continue our studies aimed at understanding the mechanisms of NEP induced growth inhibition, and the proteins with which NEP interacts to inhibit cell growth. We will continue to determine the involvement of neuropeptide in the androgen receptor mediated transactivation and assess whether NEP can regulate it. In addition, we will continue in vivo studies to prove that NEP can inhibit tumor growth in transplanted tumor model.

**Key Research Accomplishments**

- Delineate NEP mechanisms of NEP-mediate growth inhibition
- Determining NEP effect on migration
- Defining the effect of NEP on neuropeptide mediated signaling
- Assessing anti-tumor effect of NEP

**Reportable Outcomes**

Sumitomo M, Shen R, Walburg M, Dai J, Geng Y, Navarro D, Boileau G, Papandreou CN, Giacotti FG, Knudsen B, Nanus DM. Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase (FAK) signaling. J Clin Inv 2000;106;1399-1407.

Sumitomo M, Shen R, Goldberg JS, Dai J, Navarro D, Nanus DM. Neutral endopeptidase promotes phorbol ester-induced apoptosis in prostate cancer cells by inhibiting neuropeptide-induced protein kinase C  $\delta$  degradation. Cancer Res 2000;60;6590-6596.

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Sumitomo M, Milowsky M, Shen R, Navarro D, Dai J, Asano T, Hayakawa M, Nanus DM. Neutral endopeptidase inhibits neuropeptide-mediated transactivation of the insulin-like growth factor receptor-Akt cell survival pathway. Cancer Res 2001;61;3294-3298.

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Dai J, Shen R, Sumitomo M, Stahl R, Navarro D, Gershengorn MC, Nanus DM. Synergistic activation of the androgen receptor by bombesin and low-dose androgen. Clin Cancer Res 2001, submitted.

Dai J, Shen R, Sumitomo M, Navarro D, Nanus DM. Inhibitory effect of overexpression of neutral endopeptidase on androgen-independent prostate cancer xenograft in athymic mice. ProcAACR 2001;42:655.

Dai J, Shen R, Xu D, Navarro D, Nanus DM. Detection of Protein Complexities Involved in Bombesin Mediated Androgen Receptor Transactivation by Protein Biochip Technology: Proteinchip Surface Enhances Laser Desorption /Ionization (SELDI) Mass Spectrometry. ProcAACR 2001;42:783.

**Conclusions**

Our results are consistent with a model in which androgen-withdrawal allows neuropeptide-mediated actions by downregulating NEP and facilitating the development of a neuropeptide-stimulated androgen-independent PC cell population, suggesting one reason why standard therapy for patients with advanced PC leads to hormonal refractoriness. Understanding the molecular events involved in the escape from hormonal regulation which lead to androgen-independent cell growth is critical since androgen-independent cells are the primary cell population responsible for the mortality associated with PC.