The potential of neurotensin secreted from neuroendocrine tumor cells to promote gelsolin-mediated invasiveness of prostate adenocarcinoma cells

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Neuroendocrine (NE) cells in prostate cancer have been shown to be associated with the progression of prostate cancer. However, little is known about the molecular basis of this association. We have previously demonstrated that NE cells promote metastasis of a human prostate cancer cell line (LNCaP) with overexpression of the *gelsolin* gene. The purpose of this study was to investigate the interactions between NE cells and LNCaP cells and the involvement of gelsolin in contributing to the invasive potential of LNCaP cells. In addition, we examined whether neurotensin induced gelsolin-mediated invasion. We used the NE cell line NE-CS that was established from the prostate of the LPB-Tag 12T-10 transgenic mouse. Small interfering RNA (siRNA) targeting *gelsolin* or not targeting it was transfected into LNCaP cells. Cell invasion was assessed by Matrigel invasion assay. The supernatant of NE-CS cells and neurotensin induced the trans-formation of LNCaP cells. Neurotensin was observed in the supernatant of NE-CS cells but not in LNCaP cells. The siRNA targeting of *gelsolin* resulted in inhibition of invasion of LNCaP cells in the culture medium with neurotensin added, and in the supernatant of NE-CS cells with epidermal growth factor. The invasive potential of LNCaP cells enhanced by neurotensin or the supernatant of NE-CS cells through neurotensin receptor 1 (NTSR1) was blocked by a phospholipase C γ inhibitor and an intracellular calcium chelator, with concomitant gelsolin suppression. This study indicates that NE cells and neurotensin induce gelsolin-mediated invasion of LNCaP cells through NTSR1 activation.

Laboratory Investigation (2015) **95**, 283–295; doi:10.1038/labinvest.2014.165; published online 12 January 2015

Prostate cancer is the most common noncutaneous malignancy in men in industrialized countries.¹ A global statistics study showed that the estimated number of new cases worldwide was 914 000, with 258 000 deaths in 2008.¹ Androgen deprivation therapy is the mainstay of treatment for advanced prostate cancer. In most cases, however, the disease progresses despite a castration level of serum testosterone, and results in castration-resistant prostate cancer (CRPC). In the past decade, improvement of docetaxel-based chemotherapy has reduced the risk of progression and death from CRPC.² Nonetheless, the clinical benefits of these treatment options are transient.³ Death is associated with the development of metastasis of the disease. The characterization of new triggers of metastasis initiation and progression

processes is needed to adjust the treatment and its aggressiveness.

Recent studies suggest that neuroendocrine (NE) cells may play an important role in the development of castration resistance.⁴ NE cells are negative for prostate-specific antigen (PSA) and androgen receptor (AR). They are present in the normal prostate and regulate surrounding prostate cells by secreting growth-modulating neuropeptides such as chromogranin A, serotonin, bombesin/gastrin-releasing peptide (GRP), and parathyroid hormone-related protein (PTHrP).⁴ Although the proportion of NE cells present in cancer tissue varies depending on the case, several reports have shown that NE cells and their secretory products (NE factors) are increased in patients with CRPC.^{5,6} It is suggested that

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Received 31 March 2014; revised 23 November 2014; accepted 24 November 2014

inappropriate regulation of NE cells might exist in cancer tissue and, particularly, in CRPC.⁷

NE tumors of the prostate are heterogeneous, including adenocarcinoma with focal differentiation, carcinoid tumors, and small cell carcinomas (SCCs).⁸ SCCs are clinically distinct tumors associated with poor prognosis. Their histological features are scant cytoplasm, a high nucleus/cytoplasm ratio, a fine chromatin pattern, no nucleoli, frequent mitotic figures, and areas of necrosis.⁹ In biology, they have aggressive properties with increased expression of genes involved in cellular proliferation, the cell cycle, antiapoptosis, and mitosis.^{10,11} A recent study used next-generation sequencing for molecular characterization of NE tumors of the prostate.¹² The cell cycle kinase *AURKA* (*aurora kinase A*) and *MYCN* are overexpressed and amplified in NE tumors. These two proto-oncogenes cooperate to drive aggressiveness and the NE phenotype. Inhibition of *AURKA* by PHA-739358 treatment can effectively impede the growth of NE tumor cells *in vitro* and *in vivo*. This implies that *AURKA* inhibitors may potentially provide benefits for patients with SCCs. SCCs, which may or may not coexist with adenocarcinoma, increase by up to 25% in patients with CRPC, suggesting that the NE tumors are driven by the hostile environment created by androgen deprivation therapy.¹³ It is also suggested that NE tumors can affect adenocarcinoma with regard to the metastasis initiation and progression processes in CRPC. To elucidate the mechanism involving progression in CRPC, it is important to clarify the relationship of NE tumors to adenocarcinoma using an ideal SCC model.

Previously, we developed an NE allograft (NE-10) and its cell line (NE-CS) from the prostate of the LPB-Tag 12T-10 transgenic mouse.^{14–16} NE-10 maintains the expression of Tag and exhibits NE features defined histologically by SCCs and immunohistologically by chromogranin A.¹⁵ The NE-10 allograft is negative for AR and shows androgen-independent growth. When subcutaneously implanted into athymic mice, it metastasizes to the liver and the lung at week 12 after implantation. NE-CS cells have dendritic-like extensions with dense core granules in the cytoplasm and produce serotonin and somatostatin in conditioned medium.¹⁶ The cells express neither Tag nor AR. They show androgen-independent growth and a hypotetraploid karyotype similar to the original NE-10 allograft. The doubling time of NE-CS cultured with charcoal-stripped fetal bovine serum (FBS) is 33 h. NE-CS cells subcutaneously inoculated into athymic mice form tumors with the NE phenotype. The NE-CS tumors are composed of poorly differentiated cells and exhibit accelerated growth compared with the original NE-10 allograft. We demonstrated that secretions from NE cells induced androgen-independent growth of human prostate cancer cell line LNCaP and promoted pulmonary metastasis.¹⁷ DNA microarray analysis showed that expression of mRNA of *gelsolin* in LNCaP cells was increased by the supernatant of NE-CS cells. Gelsolin is one of the most potent actin-severing

proteins that are required in remodeling of the cytoskeleton. Its molecular weight is 82 kD, and it works depending on calcium.¹⁸ Gelsolin plays an important role in actin dynamics. Our previous results led us to speculate that the upregulation of gelsolin in LNCaP cells by secretions from NE cells was involved in facilitation of migration of LNCaP cells.

Neurotensin is a 13 amino-acid neuropeptide that is largely distributed along the gastrointestinal tract.¹⁹ Its functions include stimulation of pancreatic and biliary secretions, inhibition of small bowel and gastric motility, and facilitation of fatty acid translocation via specific neurotensin receptors (NTSR1) belonging to the G protein-coupled receptor (GPCR) family. Several reports have shown that neurotensin and NTSR1 are implicated in the progression of pancreas, prostate, colon, and lung cancers.^{20–22} Activation of NTSR1 is known to be involved in phosphatidyl inositol hydrolyzation. This pathway leads to the release of calcium ions into the cytoplasm from intracellular stores within the endoplasmic reticulum (calcium mobilization).²³ Therefore, it is suspected that calcium mobilization by neurotensin might induce activation of gelsolin-mediated invasion of cancer cells.

The purpose of this study was to investigate the interactions between NE cells and LNCaP cells and the involvement of gelsolin in contributing to the invasive potential of LNCaP cells. In addition, we examined whether neurotensin induced this gelsolin-mediated invasion.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human prostate adenocarcinoma cell line LNCaP was obtained from the American Type Culture Collection and used with passage numbers between 53 and 59. NE-CS is a murine prostate neuroendocrine cancer cell line established in our institute.¹⁶ It was derived from an NE-10 tumor.¹⁵ Passage numbers between 14 and 20 were used in the study. The NE-CS cells were maintained in the culture medium described below in 5% CO₂ in a humidified incubator. The medium consisted of RPMI-1640 (Gibco BRL, Breda, The Netherlands) supplemented with MEM nonessential amino acid (10 ml/l, Gibco BRL), MEM sodium pyruvate, penicillin–streptomycin (10 ml/l, Gibco BRL), and 7.5% (w/v) NaHCO₃. According to the experiment, 10% (v/v) FBS (ICN Biomedicals, Costa Mesa, CA, USA) or 0.1% (w/v) bovine serum albumin (BSA, WAKO, Osaka, Japan) was contained in the medium. The supernatant of NE-CS cells that were 80% confluent and incubated in culture medium with 10% FBS or 0.1% BSA in a humidified incubator for 48 h was filtered with a 0.22 μ m pore-sized filter. Then, it was used as NE medium with FBS or NE medium with BSA. Depending on the experiment, NE-CS cells were incubated in culture medium with 0.1% BSA supplemented with epidermal growth factor (EGF), nerve growth factor (NGF),

transforming growth factor- β (TGF- β), or insulin-like growth factor (IGF).

Reverse Transcription-PCR (RT-PCR) Analysis

RT-PCR was carried out to determine the expression of *neurotensin* in NE-CS. Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A total of 2 μ g of total RNA was reverse transcribed in a thermal cycler (Perkin-Elmer, Norwalk, CT, USA) using SuperScript III (Invitrogen, Carlsbad, CA, USA) and oligo (dT) 12–18 primers according to the manufacturer's instructions for 1 h at 50 °C in a 40 μ l reaction mixture. The resulting cDNA (1 μ l) was amplified with Taq polymerase and one set of oligonucleotide primers. Samples were denatured for 5 min at 94 °C and then amplified for 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. Aliquots (9 μ l) from each PCR sample were then analyzed by agarose gel electrophoresis. Forward and reverse primer sequences were as follows: *neurotensin* (5'-GTGTGGACCTGCTTGTCAGA-3' and 5'-TGCTTTGCTGATCTTGGATG-3') and *GAPDH* (5'-TACAGCAACAGGGTGGTGA-3' and 5'-ACCACAGTCCATGCCATCAC-3').

Real-Time RT-PCR (Real-Time PCR) Analysis

Expression of *gelsolin* and *NTSR1* in LNCaP cells incubated with conditioned medium for 6 h was determined using real-time PCR analysis. Total RNA was extracted as described in the Materials and Methods with regard to RT-PCR. All reagents for TaqMan PCR, including the primers for *gelsolin*, *NTSR1*, and *GAPDH*, were purchased from Applied Biosystems (Foster City, CA, USA). The reactions were run in 96-well plate format. The cycling conditions included an initial phase at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, and 55 °C for 60 s. The quantitative real-time PCR results were analyzed using Applied Biosystems Sequence Detection System software to determine the expression levels of the genes of interest relative to *GAPDH*.

Enzyme Immunoassay for Neurotensin in the Supernatant

We examined the concentrations of neurotensin in the supernatants of NE-CS cells and LNCaP cells by using a Neurotensin EIA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). The sensitivity of this kit was 0.2 ng/ml. NE-CS cells or LNCaP cells were incubated in culture medium with 0.1% BSA with or without 10 ng/ml EGF in a humidified incubator for 48 h. In addition, LNCaP cells were incubated in NE medium containing 0.1% BSA with or without 10 ng/ml EGF for 48 h. Then, the supernatant was carefully retrieved and filtered with a 0.22 μ m pore-size filter. The supernatant was concentrated by freeze drying. The prepared supernatant was added to the designated wells in duplicate according to the manufacturer's instructions. The immunoplate was measured for absorbance at 450 nm on a microplate reader.

Fluorescence Immunohistochemistry

LNCaP cells (3×10^4) were suspended with 100 μ l of culture medium with 10% FBS in a 96-well plate for 24 h. Then, the culture medium was replaced with culture medium containing 0.1% BSA with or without 10 ng/ml EGF, 0.1% BSA containing 0.5 ng/ml neurotensin (Peptide Institute, Osaka, Japan), NE medium with BSA containing 10 ng/ml EGF, or NE medium with BSA alone for 24 h. The cells were fixed with 4% (v/v) paraformaldehyde, permeabilized with 0.1% (v/v) Triton X-100, and labeled with a rabbit monoclonal anti-gelsolin antibody (Epitomics, Burlingame, CA, USA), a mouse monoclonal anti- β -actin antibody (Sigma Aldrich, St Louis, MO, USA) in a 1:200 dilution. The anti-gelsolin antibody was detected with an Alexa Fluor 488 donkey anti-rabbit antibody (Invitrogen) and the anti- β -actin antibody was detected with a Cy3 donkey anti-mouse antibody (Jackson ImmunoResearch, Baltimore, PA, USA). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Immunohistochemistry was observed using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

Gelsolin siRNA Construction and Transfection

The siRNAs for human *gelsolin*, siTrio cocktail: (i) 5'-CCAACGAGGUGGUGGUGCA[dT]-3', 5'-UGCACCACCACCU CGUUGG[dT]-3', (ii) 5'-GGAAAGGCAAGCAGGCAAA[dT]-3', 5'-UUUGCCUGCUUGCCUUUCC[dT]-3', and (iii) 5'-CAACAAGAUUGGACGUUUU[dT]-3', 5'-AAAACGUCC AAUCUUGUUG[dT]-3', and control siRNA targeting 5'-ATCCGCGCGATAGTACGTA-3' were prepared according to the manufacturer's instructions (B-Bridge International, Cupertino, CA, USA). LNCaP cells were transiently transfected with *gelsolin* siRNA or control siRNA using transfection reagent (Dharmacon, Thermo Fisher Science, Waltham, MA, USA). Stable transfectants were screened at 24 and 72 h using *gelsolin* real time-PCR or at 24 and 72 h using western blot assay. Two LNCaP-siGSN clones and LNCaP-siCtr clones were chosen for further experiments.

Invasion Assays

Cell invasion analyses were performed as described previously.¹⁷ In a Transwell culture chamber (Costar Science, Cambridge, MA, USA), a polyvinylpyrrolidone-free polycarbonate filter with an 8.0 μ m pore size was precoated with 5 μ g of fibronectin (Biomedical Technologies, Stoughton, MA, USA) on the lower surface and 10 μ g of the reconstituted basement membrane material Matrigel (Becton Dickinson, Sunnyvale, CA, USA) on the upper surface. The cells that invaded across the pores at 24 h were counted under a microscope after hematoxylin and eosin staining. The experiments were carried out in triplicate. The invasion index was calculated as the number of cells invading per 1000 cells.

To examine whether the proliferative potential of LNCaP-siGSN cells or LNCaP-siCtr cells affected the invasion assay, proliferation assays were performed. LNCaP-siGSN cells (1×10^4) or LNCaP-siCtr cells (1×10^4) were suspended with

100 μ l of culture medium containing 10% FBS in a 96-well plate for 24 h. Then, the culture medium was replaced with culture medium with 0.1% BSA and 0.5 ng/ml neurotensin with or without 10 ng/ml EGF, or NE medium containing BSA with or without 10 ng/ml EGF for 24 h. Cell proliferation was assessed using a WST-8 (modified tetrazolium salt) cell proliferation kit (Cell Counting Kit-8, Dojin, Japan). Changes in absorbance at 450 nm were measured with a microplate reader.

Western Blot Assays

LNCaP cells (1×10^5) were suspended with 2 ml of culture medium containing 10% FBS in a 6-well plate for 24 h. Then, the culture medium was replaced with 0.1% BSA containing 0.5 ng/ml neurotensin or NE medium with BSA in the presence or absence of the indicated effectors for 48 h. Subsequent to the incubation, the cells were washed three times with ice-cold PBS and solubilized in lysis buffer (RIPA buffer, 100 mM PMSF, 500 mM Na_3VO_4 , 1 M NaF, 2 M Sigma 104 phosphatase substrate, Protease Inhibitor Mini Cocktail). The total protein content of the cell lysates was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA).

The cell lysates obtained were boiled in SDS sample buffer containing 0.5 M 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and immunoblotted with a goat polyclonal anti-NTSR1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse monoclonal anti-gelsolin and anti- β -actin antibodies (Sigma Aldrich). Separated proteins were visualized using horseradish peroxidase with enhancement by chemiluminescence (GE Healthcare Bio-Sciences, NJ, USA). Quantitative analysis of bands in western blots was performed using the ImageJ program.

Statistical Analysis

We used the computer program StatView 5.0 for Windows (SAS Institute, Cary, NC, USA). Mann-Whitney *U*-test was applied to compare results between two different groups. One-way ANOVA was used when comparing the cell invasion in an individual group. Statistical significance was assigned at $P < 0.05$.

RESULTS

Gene Expression of Gelsolin in LNCaP Cells with NE-CS Medium

To determine whether gelsolin expression in LNCaP cells was affected by the NE medium or neurotensin, it was compared using real-time PCR. LNCaP cells (1×10^5) were suspended with 2 ml of culture medium with 10% (v/v) FBS in a 6-well plate for 24 h. After preculture, three different experiments were performed. In experiment 1, we investigated whether NE medium with FBS or BSA influenced the expression of *gelsolin* in LNCaP cells. The culture medium was replaced with NE medium with FBS or NE medium with BSA for 6 h, and then LNCaP cells were harvested for analysis (Figure 1a).

The expression of *gelsolin* was compared with that of culture medium with 10% FBS or 0.1% BSA incubated without NE-CS cells. In experiment 2, we investigated whether NE medium with BSA and growth factors influenced the expression of *gelsolin* in LNCaP cells. The culture medium was replaced with NE medium containing BSA supplemented with 10 ng/ml EGF, 10 ng/ml 2.5S-NGF, 10 ng/ml TGF- β , or 100 ng/ml IGF for 6 h (Figure 1b). Then, the LNCaP cells were harvested for analysis. Expression of *gelsolin* in these cells was evaluated compared with that of culture medium with 0.1% BSA incubated without NE-CS cells. In experiment 3, we investigated whether neurotensin influenced the expression of *gelsolin* in LNCaP cells. The culture medium was replaced with culture medium containing BSA supplemented with 20 ng/ml neurotensin for 6 h. Then, the LNCaP cells were harvested for analysis. Expression of *gelsolin* in these cells was evaluated compared with that of culture medium with 0.1% BSA alone (Figure 1c).

We compared expression of *gelsolin* in LNCaP cells incubated with NE medium containing FBS or BSA in experiment 1. When LNCaP cells were incubated with NE medium containing FBS, expression of *gelsolin* was 1.8-fold that of the culture medium with FBS incubated without NE-CS cells (Figure 1a). On the other hand, it was not changed in LNCaP cells incubated with NE medium containing BSA. Next, we examined whether expression of *gelsolin* in LNCaP cells was changed by NE medium containing BSA supplemented with various growth factors. Expression of *gelsolin* in LNCaP cells incubated with NE medium containing BSA was increased compared with that of culture medium with 0.1% BSA incubated without NE-CS cells only under addition of 10 ng/ml EGF (Figure 1b). When using neurotensin, expression of *gelsolin* in LNCaP cells was increased compared with that of culture medium containing BSA (Figure 1c).

Expression of Neurotensin in NE-CS Cells and Effect of Neurotensin on NTSR1 and Invasion in LNCaP Cells

To investigate the expression of neurotensin in NE-CS, RT-PCR was carried out using NE-CS cultured for 48 h in culture media containing 0.1% BSA with and without 10 ng/ml EGF. Gene expression of *neurotensin* was observed in NE-CS cells incubated with medium containing 0.1% BSA with EGF (Figure 2a).

It was clear that neurotensin was present in the supernatant of the NE-CS cells incubated with culture medium with 0.1% BSA containing 10 ng/ml EGF (Figure 2b). This concentration was 145.4 ± 62.5 pg/ml. However, it was not present in NE-CS cells incubated in the absence of EGF, or LNCaP cells in the presence or absence of EGF. Neurotensin was also present in the supernatant of no cells and LNCaP cells incubated with NE medium containing 0.1% BSA with 10 ng/ml EGF (Figure 2c). Their concentrations were 51.2 ± 17.2 and 53.7 ± 20.3 pg/ml, respectively. However, it was not present in NE medium in the absence of EGF.

Figure 1 Expression of *gelsolin* in LNCaP cells. After LNCaP cells (1×10^5) were suspended in culture medium with 10% (v/v) FBS for 24 h, three different experiments were performed to investigate *gelsolin* expression in LNCaP cells by using real-time PCR analysis ($n = 3/\text{group}$). Data are means; bars indicate $\pm$ s.d. **(a)** In experiment 1, culture medium was replaced with NE medium plus FBS or NE medium plus BSA for 6 h, and then LNCaP cells were harvested for analysis. Expression of *gelsolin* was compared with that in culture medium with 10% (v/v) FBS or 0.1% (w/v) BSA incubated without NE-CS cells. When LNCaP cells were incubated with NE medium containing FBS, expression of *gelsolin* was 1.8-fold that of culture medium incubated without NE-CS cells but did not change in LNCaP cells incubated with NE medium plus BSA. **(b)** In experiment 2, culture medium was replaced with NE medium plus BSA supplemented with 10 ng/ml epidermal growth factor (EGF), 10 ng/ml 2.5S-nerve growth factor (NGF), 10 ng/ml transforming growth factor- β (TGF- β), or 100 ng/ml insulin-like growth factor (IGF) for 6 h. Expression of *gelsolin* in LNCaP cells was evaluated compared with that of culture medium with 0.1% (w/v) BSA incubated without NE-CS cells. Expression of *gelsolin* in LNCaP cells incubated with NE medium containing BSA was increased compared with that of culture medium with 0.1% (w/v) BSA incubated without NE-CS cells only with 10 ng/ml EGF. **(c)** In experiment 3, culture medium was replaced with culture medium plus BSA supplemented with 20 ng/ml neurotensin for 6 h. Expression of *gelsolin* in LNCaP cells was evaluated compared with that of culture medium with 0.1% (w/v) BSA alone. When using neurotensin, expression of *gelsolin* in LNCaP cells was increased compared with that using culture medium containing BSA.

The expression of the *NTSR1* gene and NTSR1 protein was studied in LNCaP cells with culture medium containing 0.1% BSA with various concentrations of neurotensin. The expression of the *NTSR1* gene in LNCaP cells was increased as the concentration of neurotensin increased (Figure 3a). In western blot analysis, the expression of NTSR1 was also examined in LNCaP cells with culture medium containing 0.1% BSA with 10 ng/ml EGF. The expression of NTSR1 in PC3 cells was used as a positive control. It increased with neurotensin (Figure 3b). The expression of NTSR1 in LNCaP cells was faint. Quantitative analysis of the results of western blotting also revealed that expression of NTSR1s was increased as the concentration of neurotensin increased (Figure 3c).

Next, we investigated the effect of neurotensin on the invasiveness of LNCaP cells. LNCaP cells (1×10^5) were placed in the upper chamber with 100 μ l of culture medium with 0.1% BSA. The lower chamber contained 600 μ l of culture medium with 0.1% BSA at the indicated concentrations (from 0.1 to 20 ng/ml) of neurotensin or 0.1% BSA with 10 ng/ml EGF alone for 24 h. The invasion index of LNCaP cells was significantly increased with the concentration of neurotensin (Figure 3d).

Morphological Changes in LNCaP Cells with NE Medium and Neurotensin

We examined how the supernatant of NE-CS cells and neurotensin influenced morphological changes in LNCaP cells. The proportion of LNCaP cells with obvious protrusions was increased in NE medium containing BSA and 10 ng/ml EGF compared with culture medium with BSA alone (Figure 4). The conversion of the actin filaments occurred at the bases of protrusions of LNCaP cells. This morphological change was also observed in LNCaP cells incubated with culture medium with 0.1% BSA and 0.5 ng/ml neurotensin added. Expression of *gelsolin* was increased at the bases of protrusions and at the peripheral lesions of LNCaP cells in NE medium containing BSA and 10 ng/ml EGF and in culture medium with BSA and neurotensin added.

LNCaP Cells with Knockdown of Gelsolin by siRNA

We developed LNCaP-siGSN and LNCaP-siCtr cells into which siRNA targeting *gelsolin* or not targeting it was transiently transfected. Silencing of the *gelsolin* gene of $> 60\%$ was achieved in LNCaP-siGSN₁ (24 h) and LNCaP-siGSN₂ (72 h) (Figure 5a), with concomitant protein knockdown

Figure 2 To investigate expression of neurotensin in NE-CS, RT-PCR was carried out for NE-CS cultured for 48 h in culture medium containing 0.1% (w/v) BSA supplemented with and without 10 ng/ml EGF. **(a)** Gene expression of *neurotensin* was observed in NE-CS cells by RT-PCR analysis (*neurotensin*: 177 base pairs; *GAPDH*: 452 base pairs). **(b)** The concentrations of neurotensin in the supernatants of NE-CS cells and LNCaP cells were examined using Neurotensin EIA kits ($n = 3/\text{group}$). Neurotensin was present in the supernatant of the NE-CS cells incubated with culture medium containing 0.1% (w/v) BSA plus 10 ng/ml EGF ($145.4 \pm 62.5 \text{ pg/ml}$), but not in that of NE-CS cells incubated without EGF, or LNCaP cells with EGF. Data are means; bars indicate $\pm$ s.d. $**P < 0.01$ for the Mann-Whitney *U*-test. **(c)** Neurotensin was present in the supernatant of no cells and LNCaP cells incubated with NE medium containing 0.1% (w/v) BSA plus 10 ng/ml EGF (51.2 ± 17.2 and $53.7 \pm 20.3 \text{ pg/ml}$, respectively). Data are means; bars indicate $\pm$ s.d. $**P < 0.01$ for the Mann-Whitney *U*-test.

(Figure 5b). Knockdown of the gelsolin protein by $> 60\%$ was achieved in LNCaP-siGSN₁ and LNCaP-siGSN₂ for 24 and 72 h (Figure 5c).

Neurotensin and NE Cells Involved Gelsolin in Contributing to Invasive Potential of LNCaP Cells

Using a Boyden chamber assay, we evaluated whether the supernatant of NE-CS cells or neurotensin influenced the gelsolin-mediated chemotaxis of LNCaP cells. Two different experiments were performed using LNCaP-siGSN and LNCaP-siCtr transfected for 48 h. In experiment 1, to stimulate production of neurotensin by NE-CS cells, the cells (3×10^5) were incubated with $600 \mu\text{l}$ of culture medium containing 0.1% BSA with 10 ng/ml EGF in the lower chamber of a Boyden chamber for 48 h, and then LNCaP-siGSN cells (1×10^5) or LNCaP-siCtr cells (1×10^5) suspended in $100 \mu\text{l}$ of culture medium with 0.1% BSA were placed in the upper chamber (Figure 6a). This assay was compared with NE-CS cells incubated with culture medium containing 0.1% BSA without 10 ng/ml EGF in the lower

chamber. Invasion of LNCaP-siCtr cells was significantly promoted when NE-CS cells were incubated with culture medium containing 0.1% BSA and EGF in the lower chamber, indicating that this condition induced neurotensin production by NE-CS cells compared with NE-CS cells incubated without EGF in the lower chamber (Figure 6b). When NE-CS cells were incubated with culture medium containing 0.1% BSA without EGF in the lower chamber, there was no significant difference between the invasion indices of LNCaP-siGSN cells and LNCaP-siCtr cells (Figure 6b). However, when NE-CS cells were treated with EGF in the lower chamber, the invasion index of LNCaP-siGSN cells was significantly decreased compared with LNCaP-siCtr cells.

In experiment 2, LNCaP-siGSN cells (1×10^5) or LNCaP-siCtr cells (1×10^5) were placed in the upper chamber with $100 \mu\text{l}$ of culture medium containing 0.1% BSA. The lower chamber contained $600 \mu\text{l}$ of culture medium with 0.1% BSA and 0.5 ng/ml neurotensin with or without 10 ng/ml EGF (Figure 6c). Using the culture medium containing 0.1% BSA

Figure 3 Expression of NTSR1 was studied in LNCaP cells with culture medium containing 0.1% (w/v) BSA ($n=3$ /group). (a) Real-time PCR showed that expression of the *NTSR1* gene in LNCaP cells was increased as the concentration of neurotensin increased. (b) Western blot assay showed that expression of NTSR1 (54 kD) in LNCaP cells was increased with neurotensin. (c) Quantitative analysis revealed that expression of NTSR1 in LNCaP cells was increased as the concentration of neurotensin increased. Data are means; bars indicate $\pm$ s.d. * $P<0.05$, ** $P<0.01$ for one-way ANOVA test. (d) The association between neurotensin and the chemotaxis of LNCaP cells was evaluated using a Boyden chamber assay. In a Transwell culture chamber, a polyvinylpyrrolidone-free polycarbonate filter with an 8.0 μ m pore size was precoated with 5 μ g of fibronectin on the lower surface and 10 μ g of the reconstituted basement membrane material Matrigel on the upper surface. The cells that invaded across the pores at 24 h were counted under a microscope after hematoxylin and eosin staining. The invasion index was calculated as the number of cells invading per 1000 cells. LNCaP cells (1×10^5) were placed in the upper chamber with 100 μ l of culture medium with 0.1% (w/v) BSA. The lower chamber contained 600 μ l of culture medium with 0.1% (w/v) BSA with the indicated concentrations of neurotensin or 0.1% BSA with 10 ng/ml EGF alone for 24 h. The invasion index in LNCaP cells was increased with the concentration of neurotensin. Data are means; bars indicate $\pm$ s.d. * $P<0.05$, ** $P<0.01$ for one-way ANOVA test.

with neurotensin in the lower chamber, invasion enhanced by neurotensin was suppressed in LNCaP-siGSN cells compared with LNCaP-siCtr cells regardless of the presence of EGF (Figure 6d).

These NE media with BSA, EGF, and neurotensin did not influence the proliferation of LNCaP-siCtr cells and LNCaP-siGSN cells for 24 h (data not shown).

Neurotensin and NE Cells Induced Gelsolin-Mediated Invasion of LNCaP Cells Through NTSR1 Activation

We evaluated the signal pathway through which neurotensin and NE medium with BSA were involved in gelsolin-mediated invasion of LNCaP cells (Figure 7a). We used calcium chelators, a phospholipase C γ (PLC γ) inhibitor, or Rac1 inhibitor to determine whether gelsolin affects calcium mobilization or cellular motility signaling. LNCaP cells (1×10^5) were placed in the upper chamber with 100 μ l of culture medium containing 0.1% BSA. The lower chamber contained 600 μ l of culture medium with 0.1% BSA containing 0.5 ng/ml neurotensin, or NE medium with BSA containing 10 ng/ml EGF was used in the presence and absence of the indicated effectors, including PLC γ inhibitor

(U73122: 10 μ M), Rac1 inhibitor (NSC23766: 10 μ M), intracellular calcium chelator (BAPTA/AM: 30 μ M), and extracellular calcium chelator (EGTA: 30 μ M). Activation of invasion in LNCaP cells enhanced by neurotensin and NE medium with BSA and EGF was blocked by selective pharmacologic inhibitors, including U73122 and BAPTA/AM, but not by NSC23766 or EGTA (Figure 7a). Expression of gelsolin was also suppressed by U73122 and BAPTA/AM, but the expression of NTSR1 remained in the presence of U73122 and BAPTA/AM (Figure 7b). Quantitative analysis revealed that expression of gelsolin was inhibited by U73122 and BAPTA/AM in NE medium and culture medium containing BSA with neurotensin in the lower chamber (Figure 7c).

DISCUSSION

This study showed that NE-CS cells secreted neurotensin, a neuroendocrine factor, in the presence of EGF. The supernatant of NE-CS cells with EGF promoted morphological changes and invasion of LNCaP cells involving increased expression of gelsolin. This action in LNCaP cells could be reproduced by neurotensin. It was noted that inhibitors

Figure 4 Fluorescence immunohistochemistry was performed to examine morphological changes in LNCaP cells. LNCaP cells (3×10^4) were suspended with $100 \mu\text{l}$ of culture medium with 10% (v/v) FBS in a 96-well plate for 24 h. Then, the culture medium was replaced with culture media containing 0.1% (w/v) BSA alone, BSA plus 10 ng/ml EGF, BSA plus 0.5 ng/ml neurotensin, NE medium with BSA plus 10 ng/ml EGF, and NE medium with BSA alone for 24 h. An anti-gelsolin antibody, anti- β -actin antibody, and 4,6-diamidino-2-phenylindole (DAPI) were used in immunofluorescence. The proportion of LNCaP cells with obvious protrusions was increased in NE medium with EGF compared with that of culture medium with BSA alone. The conversion of the actin filaments occurred at the bases of protrusions of LNCaP cells. This morphological change was observed in LNCaP cells incubated with culture medium with BSA plus neurotensin. Expression of gelsolin was increased at the bases of protrusions and at the peripheral lesions of LNCaP cells in NE medium with EGF and in culture medium with BSA plus neurotensin.

that regulated the neurotensin signal pathway and PLC γ activation leading to intracellular calcium mobilization suppressed the invasive potential of LNCaP cells via inhibition of expression of gelsolin (Figure 8). The novel findings of this study were that neurotensin from NE cells stimulated by EGF

affected actin dynamics in prostate adenocarcinoma cells and induced their acquisition of invasive properties. These findings are supported by those in our previous report that secretions from NE cells promoted gelsolin-induced pulmonary metastasis of LNCaP cells *in vivo*.¹⁷

Figure 5 LNCaP cells were transiently transfected with small interfering RNAs (siRNAs) targeting *gelsolin* or control siRNA (LNCaP-siGSN, LNCaP-siCtr) ($n = 3/\text{group}$). (a) Real-time PCR analysis: *gelsolin* gene silencing of $>60\%$ was achieved in LNCaP-siGSN₁ and LNCaP-siGSN₂ after 24 and 72 h of transfection. Data are means; bars indicate $\pm$ s.d. (b) Western blot assay: gelsolin protein knockdown was also achieved in LNCaP-siGSN₁ and LNCaP-siGSN₂ after transfection. (c) Quantitative analysis: gelsolin protein knockdown of $>60\%$ was also achieved in LNCaP-siGSN₁ and LNCaP-siGSN₂ after 24 and 72 h of transfection. Data are means; bars indicate $\pm$ s.d.

Cancer invasion is a critical step leading to the lethality associated with the metastatic spread of malignant tumors. Although the proportion of SCCs in prostate cancer tissue varies from absent to found focally with adenocarcinoma, it can increase in patients with late CRPC. It is suggested that SCCs are associated with prostate cancer progression, and inappropriate NE regulation can facilitate metastatic progression.^{7,13} Thus, it is important to consider inappropriate NE regulation in the progression process and clarify the mechanism of cellular interaction between NE cells and adenocarcinoma cells. We previously demonstrated that secretions from NE cells stimulated LNCaP cells to achieve pulmonary metastasis.¹⁷ In addition, by DNA microarray analysis, we found that expression of the *gelsolin* gene was increased in LNCaP cells incubated with NE-CS medium. This study demonstrated that neurotensin secreted from NE-CS cells could promote gelsolin-mediated invasion of LNCaP cells. Here, we propose a model to clarify the involvement of NE cells in the action of gelsolin contributing to the invasive potential of LNCaP cells.

Gelsolin serves preexisting actin filaments and caps them in the presence of micromolar calcium, and then is displaced by interactions with regulatory phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP₂).¹⁸ Under depolymerizing conditions, these gelsolin-capped ends allow the disassembly of populations of actin filaments by subunit loss from the pointed ends. In contrast, under polymerizing conditions, gelsolin exhibits calcium-dependent actin nucleating activity and stimulates actin filament formation from monomers. Thus, the activity of gelsolin is likely to play an important role in the actin dynamics leading to remodeling of the cytoskeleton.

The malignant potential of gelsolin in cancer tissue remains to be determined. Several reports showed that gelsolin expression was low in the poorly differentiated stages of bladder cancer,²⁴ ovarian cancer,²⁵ and colon cancer.²⁶ Lee *et al.*²⁷ examined gelsolin expression in 72 prostatectomy specimens with adenocarcinoma and 8 nonneoplastic prostates from autopsies. The expression was decreased in prostatic adenocarcinoma in comparison with nonproliferative tissue. *Gelsolin* may be considered to be a candidate tumor-suppressor gene. In contrast, we demonstrated that invasion of LNCaP cells was inhibited by silencing the *gelsolin* gene. This suggested that gelsolin might contribute to cell motility in CRPC. Similarly, recent studies have shown that gelsolin is associated with advanced stage or poor prognosis in breast cancer, uterine cervical cancer,²⁸ non-small-cell lung cancer,²⁹ breast cancer,³⁰ and urothelial cancer.³¹

In this study, the enhancement of invasion of LNCaP cells incubated with the medium of NE-CS cells was blocked by U73122 and BAPTA/AM but not by NSC 23766 and EGTA. This indicated that the invasive potential of LNCaP cells involving gelsolin was dependent on PLC γ and an intracellular calcium but not on the Rac1 signal or extracellular calcium. Lader *et al.*³² reported that gelsolin was downstream of the PLC γ pathway in a lung cancer cell line. Further studies are needed to fully clarify the association between these results and the family of small Rho-like GTPases, including Rho, Rac, and Cdc42, that are involved in epithelial cell migration and invasion.³³

We also found that NTSR1 existed in LNCaP cells and that neurotensin, a neuroendocrine factor, induced the

Figure 6 Invasion assay was performed by using a Boyden chamber as described in Figure 3b. Two different experiments were performed using LNCaP-siGSN and LNCaP-siCtr ($n=3$ /group). (a) In experiment 1, NE-CS cells (3×10^5) were incubated with 600 μ l of culture medium containing 0.1% (w/v) BSA with or without 10 ng/ml EGF in the lower chamber of a Boyden chamber for 48 h and then LNCaP-siGSN cells (1×10^5) or LNCaP-siCtr cells (1×10^5) suspended in 100 μ l of culture medium with 0.1% (w/v) BSA were placed in the upper chamber. (b) In experiment 1, invasion of LNCaP-siCtr cells was promoted when NE-CS cells were incubated in medium containing EGF in the lower chamber. There was no significant difference in the invasion index between LNCaP-siGSN cells and LNCaP-siCtr cells in the absence of EGF. However, when NE-CS cells were treated with EGF in the lower chamber, the invasion index of LNCaP-siGSN cells was significantly decreased compared with that of LNCaP-siCtr cells. Data are means; bars indicate $\pm$ s.d. $^{**}P<0.01$ for one-way ANOVA test. (c) In experiment 2, LNCaP-siGSN cells (1×10^5) or LNCaP-siCtr cells (1×10^5) were placed in the upper chamber with 100 μ l of culture medium containing 0.1% (w/v) BSA. The lower chamber contained 600 μ l of culture medium with 0.1% (w/v) BSA plus 0.5 ng/ml neurotensin with or without 10 ng/ml EGF. (d) Using the culture medium containing 0.1% BSA with neurotensin in the lower chamber, invasion enhanced by neurotensin was suppressed in LNCaP-siGSN cells compared with LNCaP-siCtr cells regardless of the presence of EGF. Data are means; bars indicate $\pm$ s.d. $^{**}P<0.01$ for one-way ANOVA test.

gelsolin-mediated invasion of LNCaP cells. Similar to the medium of NE-CS cells, this enhancement of invasive potential was inhibited by a PLC γ inhibitor and intracellular calcium chelator. Similarly, Souza *et al.*²¹ reported that NTSR1 agonist-induced motility of breast cancer cell line MDA-MB-231 was blocked by selective inhibitors, including drugs targeting mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and PLC γ . They also showed that neurotensin-induced cell invasion involved the activation of matrix metalloproteinase-9 (MMP-9). In this study, neurotensin was observed in the supernatant of NE-CS cells

but not that of LNCaP cells. Thus, the present results strengthen the argument for the involvement of neurotensin derived from NE cells in gelsolin-mediated invasion of prostate cancer cells.

Moreover, it is noteworthy that EGF is essential to stimulate secretion of neurotensin from NE-CS cells. Neurotensin is regulated by the Wnt/ β -catenin signaling pathway in human neuroendocrine tumors.³⁴ This pathway is a fundamental mechanism for cell proliferation, cell polarity, and cell-fate determination during embryonic development and tissue homeostasis.³⁵ In prostate cancer, the Wnt/ β -catenin

Figure 7 The invasion index of LNCaP cells was examined in the supernatant of NE-CS cells incubated with culture media containing 0.1% BSA with 10 ng/ml EGF (NE medium #), or culture medium with 0.1% (w/v) BSA plus 0.5 ng/ml neurotensin containing the indicated effectors, including a phospholipase C γ inhibitor (U73122: 10 μ M), Rac1 inhibitor (NSC23766: 10 μ M), intracellular calcium chelator (BAPTA/AM: 30 μ M), or extracellular calcium chelator (EGTA: 30 μ M) ($n=3$ /group). (a) Activation of invasion in LNCaP cells enhanced by NE medium plus EGF and neurotensin was inhibited by U73122 and BAPTA/AM. Data are means; bars indicate $\pm$ s.d. $**P<0.01$ for one-way ANOVA test. (b) Expression of gelsolin was also suppressed by U73122 and BAPTA/AM, but expression of the NTSR1 remained in the presence of U73122 and BAPTA/AM as evaluated by western blot assay. These results were observed in both NE medium and culture medium containing BSA with neurotensin. (c) Quantitative analysis revealed that expression of gelsolin was inhibited by U73122 and BAPTA/AM in NE medium and culture medium containing BSA with neurotensin. Data are means; bars indicate $\pm$ s.d.

Figure 8 Schematic model for the interactions between NE cells and prostate adenocarcinoma cells in tumor progression of prostate cancer. EGF stimulates neurotensin release in NE cells. It might be enhanced by its autocrine loop involving cross-talk among the NTSR1, EGFR, and Wnt/ β -catenin signaling pathways. Neurotensin induces overexpression of gelsolin via PLC γ activation, leading to intracellular calcium mobilization, and promotes invasive properties of prostate adenocarcinoma cells involving changes in actin dynamics.

signaling pathway was reported to be associated with NE tumors.³⁶ Yang *et al*³⁷ showed that this pathway was associated with protocadherin-PC (PCDH-PC) and promoted NE transdifferentiation in prostate cancer. The Wnt/ β -catenin pathway is also known to cooperate functionally with the EGFR pathway.³⁸ Thus, the EGF signaling pathway can lead to the expression and release of neurotensin in NE cells. Neurotensin can stimulate expression of the EGF receptor (EGFR) and early growth response gene-1 (Egr-1) binding to its site within the EGFR promoter in human colonic epithelial cells.³⁹ Therefore, EGF-induced neurotensin release in NE-CS cells might be enhanced by its autocrine loop (Figure 8). This is supported by our finding that the concentration of neurotensin in the supernatant of the NE-CS cells incubated in culture medium containing 0.1% BSA with 10 ng/ml EGF was higher than in the supernatant of no cells and LNCaP cells incubated in NE medium containing 0.1% BSA with 10 ng/ml EGF.

Neurotensin signaling is implicated in the regulation of not only PLC γ leading to intracellular calcium mobilization,²³ but also ERK1/2, Rho GTPases (RhoA, Rac1, Cdc-42), NF- κ B, and focal adhesion kinase (FAK) activation.^{40–42} These signaling factors are correlated with cell and tissue growth, apoptosis, and progression. In prostate cancer, the treatment of PC3 cells with neurotensin has been shown to induce cellular proliferation.⁴³ LNCaP cells also represent an alternative growth pathway to enable continued tumor growth induced by neurotensin, particularly in the absence of an androgen.^{44,45} However, LNCaP bicalutamide-resistant (LNCaP-Bic) cells and a high dose of neurotensin (50 nM: almost 84 ng/ml) were used in these studies. In this study, it was not observed that neurotensin stimulated proliferative activation of LNCaP cells. Although the exact reason is unclear, the differences in the cells used and the concentrations of neurotensin might have influenced our results.

There are some limitations in this study. The NE-CS cell line was derived from the mouse prostate. The role of human NE cells in human prostate cancer may be different from that of mouse NE cells. In addition, the characteristics of the established cell line, NE-CS, could be different from those of the original NE-10 allograft because cells suitable for survival *in vitro* were selected during establishment of the cell line. However, there are no ideal human lines for which both *in vitro* and *in vivo* NE carcinoma models are available. In this study, the concentration of EGF that stimulated secretion of neurotensin from NE-CS cells was 10 ng/ml. This was relatively higher than that of physiological EGF that ranges from 0.1 to 1 nM (mostly from 0.6 to 6 ng/ml).⁴⁶ Moreover, the concentration of neurotensin in the supernatant of NE-CS cells was low compared with that causing an increase in LNCaP cell invasion. This difference might be because of the different number of cells and volume of the medium. Soft agar assay and colony-formation assay were not performed for transformation of LNCaP cells. We did not examine the other two NTSR subtypes (NTSR2 and NTSR3) because

NTSR1 has a higher affinity to neurotensin ($K_d = 0.56 \pm 0.1$ nM) than NTSR2 ($K_d = 3.7 \pm 0.2$ nM)^{47,48} and NTSR3 is predominantly present within intracellular compartments such as the trans-Golgi network.⁴⁹ Although there could be another pathway in gelsolin-mediated invasion of LNCaP cells, this is the first study to show the potential effects of neurotensin secreted from NE cells on invasion of prostate cancer cells. This proposed mechanism could contribute to benefits for patients with advanced prostate cancer. Regulating NE cells, potential triggers of prostate cancer leading to CRPC, may lead to betterment of its treatment. We previously reported the effects of zoledronic acid, a nitrogen-containing bisphosphonate, on NE cells.⁵⁰ Further studies will be needed to confirm effects of zoledronic acid on the association between neurotensin and gelsolin-mediated invasion of LNCaP cells, and to elucidate how the proposed mechanism is associated with different stages of cancer and different prostate cancer cell lines. In addition, it is necessary to determine whether NTSR1 or NTSR1 inhibitors affect the phospho-activated form of PLC γ to clarify the gelsolin signaling pathway.

CONCLUSIONS

We examined the interactions between NE cells and LNCaP cells and the involvement of gelsolin in contributing to the invasive potential of LNCaP cells. This study indicates that secretions from NE cells contribute to invasion of LNCaP cells involving gelsolin. In addition, we confirmed that neurotensin induced this gelsolin-mediated invasion. Our findings support the significance of controlling NE cells that may trigger progression of prostate cancer to CRPC.

ACKNOWLEDGMENTS

We thank Shiori Sato of the Department of Urology in Sapporo Medical University School of Medicine for technical assistance with cell culture and enzyme immunoassay for neurotensin. This work was supported by Japan Society for the Promotion of Science KAKENHI Grant Number 23791765.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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