Neurotransmitter Substance P Mediates Pancreatic Cancer Perineural Invasion via NK-1R in Cancer Cells

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Abstract

Pancreatic cancer significantly affects the quality of life due to the severe abdominal pain. However, the underlying mechanism is not clear. This study aimed to determine the relationship between Substance P (SP) and pancreatic cancer perineural invasion (PNI) as well as the mechanism of SP mediating pancreatic cancer PNI, which causes pain in patients with pancreatic cancer. Human pancreatic cancer cells and newborn dorsal root ganglia (DRG) were used to determine the expression of SP or NK-1R in pancreatic cancer cells and DRGs by qRT-PCR and Western blotting. The effects of SP on pancreatic cancer cell proliferation and invasion were analyzed using MTT assay and Transwell Matrigel invasion assay, respectively. Alterations in the neurotropism of pancreatic cancer cells were assessed by coculture system, which mimics the interaction of tumor/neuron in vivo. SP is not only widely distributed in the neurite outgrowth from newborn DRGs but also expressed in MIA PaCa-2 and BxPC-3 cells. NK-1R is found to be overexpressed in the pancreatic cancer cell lines examined. SP induces cancer cell proliferation and invasion as well as the expression of matrix metalloproteinase (MMP)-2 in pancreatic cancer cells, and NK-1R antagonists inhibit these effects. Furthermore, SP promotes neurite outgrowth and the migration of pancreatic cancer cell cluster to the DRGs, which is blocked by NK-1R antagonists in the coculture model. Our results suggest that SP plays an important role in the development of pancreatic cancer metastasis and PNI, and blocking the SP/NK-1R signaling system is a novel strategy for the treatment of pancreatic cancer.


Introduction

Pancreatic cancer is a deadly disease with a mortality rate very near to the incidence rate. A lack of early symptoms, explosive outcomes, short survival, and resistance to therapy are hallmarks of this type of cancer (1). The treatment of pancreatic cancer has not improved substantially during the past 30 years. Perineural invasion (PNI) in pancreatic cancer is a common pathologic phenomenon. It has been reported that all of pancreatic tumors would reveal PNI if enough sections are evaluated (2), of which up to 90% of patients have intrapancreatic nerves infiltrated by tumor cells and 69% have involvement of the extra-pancreatic nerve terminations. The presence of tumor cells in the perineurium space of local peripheral nerves in the pancreas may be associated with a higher risk of retropancreatic tumor extension, precluding curative resection, and promoting local recurrence after tumor resection.

Pancreatic cancer significantly affects the quality of life due to the chronic symptoms and severe abdominal pain (3, 4). Substance P (SP) is an undecapeptide, released from primary sensory nerve fibers that belongs to the tachykinin family, which has been implicated in a myriad of physiologic processes (5, 6). SP is widely expressed in the central and peripheral nervous system as well as in peripheral tissues such as B and T cells in an autocrine or paracrine manner (7) and macrophages (8). NK-1R, a receptor of SP, is overexpressed in several normal (9–11) and neoplastic cell types (12–18). SP regulates many biologic functions and has also been implicated in neurogenic inflammation, pain, and depression (6, 19). Upon the binding of SP to its high affinity receptor, NK-1R, SP initiates multiple biologic functions including tumor cell proliferation, angiogenesis (9), and migration, which are critical for tumor cells invasion and metastasis (7, 10). These biologic functions can be reversed by the NK-1R antagonists. These reports suggest that SP/NK-1R signaling may play an important role in the cancer progression and metastasis, as SP may be a mitogen in NK-
1R-expressing tumor cell types (10, 11). Therefore, targeting the NK-1 receptor could be a promising approach for treating patients with cancer, and NK-1 receptor antagonists could improve cancer treatment.

Friess and colleagues (12), in their pioneer work on the expression of NK-1R, showed that the expression levels of NK-1R mRNA and protein in human pancreatic cancer samples were increased 36.7- and 26.0-fold, respectively, compared with normal controls. Enhanced NK-1R levels in the tumor tissues were associated with advanced tumor stage and poorer prognosis. SP analogs stimulate pancreatic cancer cell growth, depending on the NK-1R expression level, and this effect could be blocked by a selective NK-1R antagonist. These findings suggest that there may be a neuro-cancer cell interaction in vivo (12).

The pancreas is an organ with rich innervations that are associated with PNI in pancreatic cancer (13). We reason that SP stimulating NK-1R, which is overexpressed in tumor cells and in the tumor and peritumoral tissue (7), may be a molecular mechanism for tumor cells to develop PNI.

To date, the relationship between SP and pancreatic cancer metastasis and PNI has not been reported. The purpose of the present study was to test whether SP/NK-1R signaling could influence the progression of pancreatic cancer. Our data suggest that SP plays an important role in the development of pancreatic cancer by inducing cell proliferation, metastasis, and PNI; and blocking the SP/NK-1R signaling may be a novel strategy for the treatment of pancreatic cancer.

Materials and Methods

Cell lines, animals, and reagents

The human pancreatic tumor cell lines Mia PaCa-2, BxPC-3, CFPAC-1, HAPC, Panc-1, and SW1990 were obtained from American Type Culture Collection (14). Newborn rats were purchased from the laboratory animal center of the Xi’an Jiaotong University (Xi’an, China). Dulbecco’s modified Eagle’s medium (DMEM) and FBS were obtained from Invitrogen Life Technologies. Polyclonal anti-NK-1R and polyclonal anti-Sp antibody were bought from Sigma-Aldrich. A polyclonal anti-human matrix metalloproteinase (MMP)-2 antibody was obtained from Santa Cruz Biotechnology. SP acetate salt (Sigma-Aldrich) was dissolved in distilled water and different concentrations of SP (5, 10, 50, 100 and 120 nmol/L) were evaluated. (2S, 3S) 3-[(3, 5-Bis (trifluoromethyl)phenyl) methoxy)-2-phenylpiperidine hydrochloride (L-733,060) was procured from Tocris Cookson. N-acetyl-1-tryptophan-3,5-bis(trifluoromethyl)-benzyl ester (L-732,138) was purchased from Tocris Cookson. Unless otherwise specified, all other reagents were acquired from Invitrogen. All experiments were carried out in triplicate.

Cell lines and rat dorsal root ganglions

Pancreatic cancer cells were maintained in DMEM supplemented with 10% heat-inactivated FBS (Invitrogen), 50 U/mL penicillin G, 50 µg/mL streptomycin sulfate, and 25 mmol/L glucose at 37°C in a humidified 5% CO2, 95% air atmosphere. Newborn rats were euthanized with carbon dioxide and sterilized with 75% ethanol. Dorsal root ganglions (DRG) from the lumbar areas were dissected, collected into medium (DMEM), stripped of meninges and nerve stumps, and plated into a drop of liquid Matrigel (BD Biosciences). After solidification, medium (DMEM containing 0, 5, 10, 50, or 100 nmol/L SP) was carefully added and renewed every 2 days (15, 16).

Immunocytochemical staining

For fluorescence immunocytochemistry, pancreatic tumor cell lines and neurite outgrowths from newborn DRGs were fixed for 30 minutes in 4% paraformaldehyde in PBS and the endogenous peroxidase activity was quenched by 3% hydrogen peroxide. The specimens were preblocked for 30 minutes with bovine serum albumin (BSA) at 37°C and incubated with primary antibody against SP (1:100) or NK-1R (1:100) overnight at 4°C. Then staining was detected with fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit immunoglobulin G (IgG) antibody or CY3-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research). Slides were mounted and then examined by using a Nikon Instruments confocal microscope (Nikon Instruments Inc.).

Reverse transcription-PCR and real-time quantitative PCR

Total RNA from prostate cancer cells or DRGs were extracted using a Fastgen200 Kit RNA isolation system (Fastgen) according to the manufacturer’s protocol. Total RNA was reverse-transcribed into cDNA using the Fermentas RevertAidTM Kit (MBI Fermentas). The primer sequences were as follows:

**SP-F:** 5′-GACTCCTCTGACCGCTAC-3′
**SP-R:** 5′-AGACCTGCTGTGATC3′
**NK-1R-F:** 5′-AGGTTCGCTTGGCTGTTCAA-3′
**NK-1R-R:** 5′-TCCAAGCCGCTGACTTTGTA-3′
**MMP-2-F:** 5′-GATGATGCCTTTGCTCGTGC-3′
**MMP-2-R:** 5′-CAAAGGGGTATCCATCGCCA-3′
**β-Actin-F:** 5′-GACTTATGTTGGTTAACACCTTCT-3′
**β-Actin-R:** 5′-GAACCGTGAAGGTCACAGCAGT-3′

Reverse transcription-PCR (RT-PCR) products were resolved using a 1.5% agarose gel. After each real-time quantitative PCR (QT-PCR), a dissociation curve analysis was conducted. Relative gene expression was calculated using the 2−ΔΔCT method reported previously (17). Each measurement was carried out in triplicate.

Western blotting

Proteins were electrophoretically resolved on a denaturing SDS polyacrylamide gel and electrotransferred onto nitrocellulose membranes. The membranes were initially blocked with 5% nonfat dry milk in TBS for 2 hours and then probed...
with antibodies against SP, NK-1R, MMP-2, or β-actin. After coincubation with the primary antibodies at 4°C overnight, the membranes were hybridized with secondary goat anti-mouse antibody or goat anti-rabbit antibody (Sigma-Aldrich) for 2 hours at room temperature. Immunopositive bands were developed using an enhanced chemiluminescence (ECL) detection system (Amersham Bioscience). All analyses were done in duplicate.

MTT assay
MIA PaCa-2 and BxPC-3 cells were seeded in 96-well tissue culture plates at a density of 5,000 to 10,000 cells per well 24 hours before serum starvation. After serum starvation for 24 hours, cells were cultured in DMEM with different concentrations of SP (0, 5, 10, 50, 100, and 120 nmol/L), L-733,060 (0, 5, 10, 20, 30, and 40 μmol/L) or L-732,138 (0, 20, 40, 60, 80, and 100 μmol/L) and incubated at 37°C. After 24, 48, or 72 hours, the medium was removed, and MTT reagent was added to each well and incubated at 37°C for 4 hours. The optical densities (OD) at 490 nm were measured using a microplate reader (BIO-TEC Inc.). The proliferation rate was defined as OD (cells plate)/OD (control plate).

Transwell Matrigel invasion assay
An invasion assay was conducted with a Millicell invasion chamber (Millipore). The 8-μm pore inserts were coated with 25 μg of Matrigel (Becton Dickinson Labware). After serum starvation for 24 hours, MIA PaCa-2 and BxPC-3 cells (5 x 10^4) were seeded in the top chamber and medium with SP or NK-1R antagonists were also added to the bottom chamber to induce the cancer cell lines. The Matrigel invasion chamber was incubated for 48 hours in a humidified tissue culture incubator. Noninvasive cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells on the bottom surface of filters were fixed in methanol and stained with Crystal Violet (Boster Biological Technology Ltd.). The invasion ability was defined as the number of cells migrated to the bottom chamber.

Coculture assay
Coculture experiments were carried out using a modified method based on previously described methods (15, 16). The DRGs were kept on ice after collection in DMEM medium (Invitrogen) and subsequently seeded on 24-well Petri dishes in 25 μL of Matrigel gel as described earlier. BxPC-3 cells were suspended in 25 μL of solidified Matrigel and placed next to the DRG suspension. To exclude the possibility of a nonspecific guided migration of BxPC-3 cells, an additional 25 μL of Matrigel containing no neural cells was positioned on the opposite side. The Petri dishes were then placed for 20 minutes in an incubator at 37°C saturated with 5% CO2 in a humid atmosphere to allow polymerization of the Matrigel. After solidification, medium (DMEM containing SP or/and NK-1R antagonists) was added and renewed every 2 days. Photographic documentation of the 2 adjacent sides of the cell suspensions was conducted with an inverted light microscope imaging system (Ti-E; Nikon Instruments Inc.) and analytic system (NIS BR3.0; Nikon Instruments Inc.).

Statistical analysis
Statistical analysis was done with SPSS software (version 17.0, SPSS Inc.). Data were presented as the mean ± SEM of 3 replicate assays. Differences between the groups were analyzed by ANOVA, followed by the Bonferroni correction for multiple comparisons. P < 0.05 was considered statistically significant. All experiments were repeated independently at least 3 times.

Results
SP is mainly expressed in DRGs, whereas NK-1R is expressed in pancreatic cancer cells
To determine whether SP or NK-1R is expressed in pancreatic cancer cells, we tested 6 pancreatic cancer cell lines: MIA PaCa-2, BxPC-3, CFPAC-1, HAPC, Panc-1, and SW1990. As shown in Fig. 1A, the expression of SP in pancreatic cancer cells at mRNA level was low. Among the 6 cell lines, the expression levels of NK-1R from high to low are in the following order: Panc-1 > BxPC-3 > CFPAC-1 > SW1990 > HAPC > MIA PaCa-2 (Fig. 1B).

The expression of NK-1R and SP was also tested by Western blotting (Fig. 1C) and immunofluorescence (Fig. 2) in BxPC-3, MIA PaCa-2, and DRGs. In the 2 pancreatic cancer cell lines MIA PaCa-2 and BxPC-3, the NK-1R was visualized as a single band 45 kDa. A higher expression level of the NK-1R was present in BxPC-3. The expression of SP was also detected in these cell lines, whereas the expression level of SP was much higher in the neurite outgrowth from newborn DRG.

SP induces proliferation of pancreatic cancer cells
To determine the effects of SP on pancreatic cancer cell growth, we cultured BxPC-3 and MIA PaCa-2 cells in the media containing increasing concentrations (5–120 nmol/L) of SP and the effects on cell proliferation were determined using MTT assay. The results showed that SP induced cell proliferation in BxPC-3 and MIA PaCa-2 cells in a dose-dependent manner after incubation for 24, 48, or 72 hours with 5 to 100 nmol/L SP, whereas proliferation rate in 120 nmol/L begins to drop (Fig. 3A). A similar effect was observed in MIA PaCa-2 cancer cells.

NK-1R antagonists inhibit pancreatic cancer growth
We next treated both BxPC-3 and MIA PaCa-2 cells with 2 different NK-1R antagonists: L-733,060 that showed high affinity for the human NK-1R in vitro and L-732,138 that showed a competitive antagonism for the receptor (18). Treatment of pancreatic cancer cells with the antagonists resulted in a concentration-dependent cytotoxicity in both cell lines. The IC_{50} of L-733,060 in both pancreatic cancer cell lines BxPC-3 and MIA PaCa-2 was approximately 10 μmol/L. Furthermore, the IC_{50} of L-732,138 was approximately 60 μmol/L in BxPC-3, whereas it is 40 μmol/L in MIA PaCa-2 (Fig. 3B and C).
SP/NK-1R signaling system promotes invasion of pancreatic cancer cells

Next, we tested the effect of SP on pancreatic cancer cell invasion in vitro. The results show that the number of invasive BxPC-3 and MIA PaCa-2 cells increased with the addition of 100 nmol/L SP compared with the control and this effect was significantly inhibited by NK-1R antagonists (Fig. 4A).

We further determined the expression of metastatic-related factor MMP-2 by QT-PCR (Fig. 4B) and Western blotting (Fig. 4C). Interestingly, SP was able to promote the expression of MMP-2, which was also counterbalanced by the NK-1R antagonists. Taken together, these results suggest that SP/NK-1R signaling system promotes the invasion of pancreatic cancer cells, which can be mediated by MMP-2.

SP promotes neurotropism in pancreatic cancer cells

To determine if SP contributes to the neurotropism of pancreatic cancer cell, BxPC-3 cancer cells, which express a higher level of NK-1R were used to coculture with newborn rat DRGs in Matrigel in the addition of SP or/and NK-1R antagonists for 8 days. We first measured the effect of SP levels on neurite regeneration (Supplementary Fig. S1A–S1E). Newborn rat DRGs were cultured in the medium containing various concentrations of SP (0, 5, 10, 50, or 100 nmol/L) for 8 days. The neurite regeneration exhibited a slow but significant increase when the concentration of SP increased from 5 to 100 nmol/L (Supplementary Fig. S1F). These results suggest that SP have a stimulating effect on neurites. We also observed that BxPC-3 cancer cells facing the DRGs formed peak-like clusters (Supplementary Fig. S1G–S1I). The neurite outgrowth (Supplementary Fig. S1J and S1K) extended to the clusters from the DRGs and provided an invasive pathway for the clusters.

In addition, our results showed that SP promotes pancreatic cancer cell clusters gradually migrating to the DRG and NK-1R antagonists could significantly reduce these effects (Fig. 5). These interesting phenomena in the new coculture environment indicate that the nerve might play an important role in the process of PNI.

Discussion

In the current study, we showed that SP is highly expressed in the neurite outgrowth from newborn DRGs. We found that NK-1R is overexpressed in the pancreatic cancer cell lines. Furthermore, we showed that SP induces pancreatic cancer cell proliferation and invasion with statistically significant and these effects could be counterbalanced by NK-1R antagonists. We then showed that the neurite regeneration slowly was increased in response to various SP concentrations from 5 to 100 nmol/L in the coculture system. Moreover, we showed that SP promotes pancreatic cancer cell clusters gradually migrating to the DRG and SP-induced neurite regeneration extended to the clusters from the DRGs, which provides an invasive pathway for the clusters. Taken together, to our knowledge, it is the first time to show that SP, which mediates the interaction between cancer cells and nerves, may promote the proliferation, invasion, and neurotropism of pancreatic cancer cells. SP-promoted PNI in pancreatic cancer may constitute a novel mechanism for how pain decreases the survival of patients with pancreatic cancer. Blocking the SP/NK-1R signaling system may provide a novel strategy for the pancreatic cancer therapeutics.
Traditionally, SP has been classified as a neurotransmitter that exerts its effects on the periphery only by being released from nerve endings. However, SP has been recently shown to be expressed in non-neuronal cell types, including endothelial cells, macrophages and monocytes, eosinophils, lymphocytes, and Leydig cells (7, 19). This suggests that it may not only act as a neurotransmitter but also as a functional regulator in an autocrine or paracrine manner.

It has been shown that SP exerts promigratory effects on many types of cancer cells such as colon carcinoma, small cell lung cancer (20), and breast carcinoma (21). In the U-373MG astrocytoma cell line, SP stimulates mitogenesis by activating the mitogen-activated protein kinase (MAPK) pathway through receptors of the NK-1 subtype, including extracellular signal–regulated kinases 1 and 2 (ERK1/2), which translocate to the nucleus to induce cell proliferation and protect the cell from apoptosis (7, 22). SP also stimulates cell proliferation via transactivation of the EGF receptor (EGFR; ref. 23) and activation of Akt, resulting in the suppression of apoptosis (24).

Patients with cancer usually do not succumb to the primary tumor but to the development of metastases. The active migration of tumor cells is a crucial requirement for metastasis and cancer progression. Entschladen and colleagues (25) suggested that neurotransmitters are key players in the regulation of tumor-cell migration. It has also been shown that SP induces the migration of tumor cells in...
Our results show that SP/NK-1R signaling system also induces pancreatic cancer cell line invasion. We showed that the proliferative and invasive abilities of BxPC-3 and MIA PaCa-2 pancreatic cancer cells were increased with the addition of SP. NK-1R antagonist could inhibit these effects of SP. SP may induce the advancement of pancreatic cancer via a dual mechanism involving both proliferative and invasive properties.

Friess and colleagues (12) showed new evidence on neuro-cancer cell interaction. Moreover, it has been shown that tumor samples from patients with advanced stages exhibit significantly higher NK1-R levels and that such patients have poorer prognosis. We also showed that the pancreatic cancer cells BxPC-3 and MIA PaCa-2 express NK-1R. SP is higher expressed in the surrounding cells (normal and/or stromal tumors, etc.) or nerve endings, and then interacts with pancreatic cancer cells. In our experiments, we found that the proliferative and invasive ability of pancreatic cancer cells, especially in the SP nerve endings, may stimulate cancer cell migration. Our observations suggest that SP promotes the growth of neurite regeneration as well as pancreatic cancer cell invasion to the DRG and there is a SP–neuro-cancer cell interaction. Our coculture study shows that the high-SP tumor microenvironment enhances PNI. Taken together, these data suggest that SP and NK-1R could play an important role in the development of metastasis.

**Figure 3.** The effect of SP and/or NK-1R antagonists on cancer cell proliferation. A, induction of cell proliferation of human pancreatic tumor cell lines MIAPaCa-2 and BxPC-3 by SP at several nanomolar concentrations (0, 5, 10, 50, 100, and 120 nmol/L). In both cases, SP induced cell proliferation. Using the ANOVA test, a significant difference between each group and the control group was found. P < 0.05 as compared with control group. The percentage of growth inhibition of human pancreatic cancer BxPC-3 and MIA PaCa-2 cells treated by increasing concentrations (0–40 μmol/L) of L-733,060 (B) or (0–100 μmol/L) of L-732,138 (C) at 24, 48, and 72 hours. The concentrations of NK-1R antagonists less than IC50 were selected [L-733,060 (10 μmol/L) and L-732,138 (60 μmol/L in BxPC-3 and 40 μmol/L in MIA PaCa-2)].
The degree of pain control is an important endpoint of therapy, along with clinical outcome following surgical and medical treatment of pancreatic cancer (27). Pain is a main complaint from patients with pancreatic cancer. SP is involved in peripheral pain generation and is associated with tumor formation. Specific SP antagonists (11, 28) have shown clinical efficacy in patients, acting as the following: analgesics (29), antidepressants (30), antiemetics (29), and neuroprotectors (31). Our results may in part explain that pancreatic cancer patients suffer severe pain and the expression of SP is associated with pancreatic cancer patients with advanced diseases and poor outcomes.

In conclusion, our results show that SP, which mediates the interaction between cancer cells and nerves, may

Figure 4. The effect of SP and/or NK-1R antagonists on cancer cell invasive ability. A, SP promotes the invasion of pancreatic cancer cells, which can be inhibited by NK-1R antagonists L733, 060 and L-732, 138. *, P < 0.05 as compared with control group; , P < 0.05 as compared with SP group. SP increases the expression of MMP-2 at the mRNA level (B) and protein level (C) that can be counterbalanced by the NK-1R antagonists. *, P < 0.05 as compared with control group; , P < 0.05 as compared with SP group.
promote the proliferation, invasion, and neurotropism of pancreatic cancer cells. These results suggest that SP promotes PNI in pancreatic cancer, constituting a novel mechanism for how pain decreases the survival of patients with pancreatic cancer. These findings suggest that blocking the SP/NK-1R signaling system may provide a novel strategy for the pancreatic cancer therapeutics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Li, G. Ma, Q. Ma, J. Liu, L. Han, W. Duan, Q. Xu, Z. Wang
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