Glial cell line-derived neurotrophic factor enhances the invasive ability of pancreatic cancer cells by regulating urokinase-type plasminogen activator and matrix metalloproteinase

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Abstract

Introduction: Tumour invasion and metastasis consist of multiple steps involving changes in proliferation, motility, adhesion, invasion, and secretion of certain enzymes. Cancer cell invasion calls for the action of several proteinases that degrade the extracellular matrix and basement membranes. For several decades, matrix metalloproteinases (MMP) have been thought to be cancer-related proteinases, and recent reports have revealed that the plasminogen activator (uPA) system plays an important role in cancer cell viability.
Material and methods: The casein zymography procedure was used to determine uPA activity in the human pancreatic cancer cell lines BxPC-3, HPAF-II, and MIA PaCa-2. Gelatinase activity of cell culture supernatants was determined by gelatin zymography. Proliferation of pancreatic cancer cells was monitored using an MTT assay and cell counting. Invasive behavior was examined using a Matrigel double-chamber assay.
Results: The uPA activity of HPAF-II cells was the strongest of all the cells examined, and HPAF-II only showed both high molecular weight (HMW) and low molecular weight (LMW) uPA. GDNF enhanced the invasive ability of pancreatic cancer cells that expressed the components of the plasminogen activator system. This effect was inhibited by a specific uPA inhibitor. Furthermore, GDNF increased MMP activities, and the uPA inhibitor partially inhibited MMP activities.
Conclusions: The present study suggests that the plasminogen activator system plays an essential role in pancreatic cancer cell invasion. Activation of uPA by GDNF enhanced the invasive ability of pancreatic cancer cells and also increased the expression of active MMPs.

Key words: GDNF, uPA, MMP, pancreatic cancer.

Introduction

Pancreatic cancer is one of the gastrointestinal malignancies that has a miserable prognosis. The five-year survival rate is about 20% and median survival time is approximately 5 to 6 months, even with surgical reduction and/or chemotherapy [1]. This poor prognosis is caused by the specific tendency for pancreatic cancer to frequently invade into adjacent structures and metastasize to distant organs at an early stage. Despite intensive therapy for this disease, it is difficult to achieve sufficient results because most patients die within several months as a result of rapid local invasion or metastatic

dissemination. Many investigations have been performed to improve therapy or to elucidate the malignant potential of pancreatic cancer, but most of the biological characteristics of this disease remain a mystery.

Tumour invasion and metastasis consist of multiple steps involving changes in proliferation, motility, adhesion, invasion, and secretion of certain enzymes. Many proteolytic enzymes seem to play a critical role in these steps [2-4]. These proteases can be categorized into three families: matrix metalloproteinases (MMPs), serine proteinases, and cysteine proteinases. The aggressive activity of human pancreatic cancer is characterized by the interaction of these proteases, including urokinase-type plasminogen activator (uPA) and MMPs. Recent studies have indicated the important role of the serine proteinase uPA and its receptor (uPAR) in many cancers, including pancreatic cancer [5-8]. In addition, recent studies have shown that overexpression of uPA increases the invasive and metastatic rates of pancreatic cancer [9], breast cancer [10], colon cancer [11], and melanoma [12, 13]. uPA is a 52-kDa serine protease that is secreted by tumour cells and binds to uPAR [14]. The uPA-uPAR complex has many functions, including degradation of extracellular matrix (ECM). The other important role of the uPA-uPAR complex is the conversion of inactive zymogen plasminogen to the active proteinase plasmin, another serine protease with broad substrate specificity that is directly capable of degrading ECM protein and converting inactive MMP to the active form [15]. MMPs are a group of enzymes that degrade the connective tissue, ECM proteins, and basement membranes (BM). The MMPs constitute a large family of structurally related matrix-degrading proteases that play pivotal roles in development, tissue remodelling and cancer [16-18]. The gene family of MMPs includes the interstitial collagenases (MMP-1 and MMP-13), gelatinase A (MMP-2), gelatinase B (MMP-9), the stromelysins (MMP-3, -10, and -11), and the membrane type-matrix metalloproteinases (MT-MMPs) [18]. Of these, MMP-2 and MMP-9 are believed to play important roles in tumour metastasis, invasion and angiogenesis, and these two enzymes have been the most consistently detectable in malignant tissues [19-21].

We previously reported that GDNF altered invasive and adhesive abilities by alteration of integrin expression in pancreatic cancer cells [22]. Recent studies have revealed that the interaction between uPA and integrin had an effect on cancer cell invasion [23-25]. Therefore, we presumed that GDNF increased invasion by modulating uPA and MMPs. In this study, we describe the importance of uPA and MMPs in the invasion of pancreatic cancer cells. We further found that GDNF increased pancreatic cancer cell invasion by GDNF-mediated regulation of uPA and MMPs. Our results may help in further understanding of the biological processes and construction of a new therapeutic strategy for patients with pancreatic cancer.

Material and methods

Reagents

Recombinant human GDNF was provided by Wako (Osaka, Japan). The anti-uPA antibody was from American Diagnostica (Temecula, CA). The MMP antibody GM6001, which inhibits MMP-1, -2, -3, -8, and -9, was purchased from Chemicon (Temecula, CA).

Cell culture

The human pancreatic cancer cell lines BxPC-3, HPAF-II, and MIA PaCa-2 were obtained from the American Type Culture Collection (Rockville, MD). The BxPC-3 and HPAF-II lines were maintained in RPMI 1640 (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 units/ml), and streptomycin (100 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The MIA PaCa-2 line was maintained in Dulbecco's Modified Eagle Medium (Gibco BRL) supplemented with 10% FBS, penicillin G (100 units/ml), and streptomycin (100 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

Culture medium concentration

The culture medium was concentrated using centrifugal filter devices from Millipore (Bedford, MA). The cells were incubated until confluent in 150-mm dishes and then starved in medium containing 0.5% FBS for 24 hrs. Cells were then washed twice with phosphate-buffered saline (PBS), and fresh serum-free medium containing 100 nM GDNF was added. The cells were pretreated with 10 mM antibody. After 24 hrs of incubation, the supernatant was harvested and concentrated 200 times by centrifugation at 5,000 rpm. The protein level in the concentrated supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Casein zymography

To determine uPA activity, a modification of the casein zymography procedure described by Roche et al. [26] was used. Briefly, samples were prepared as described above and an equal volume of sodium dodecyl sulphate (SDS) sample buffer was added to the samples (20 µg). The sample mixture was characterized by 10% SDS-polyacrylamide gel electrophoresis (PAGE) containing 12% casein (Invitrogen, Carlsbad, CA) at 125 V for 90 min. After electrophoresis, gels were washed twice in 2.5% Triton-X for 30 min to remove SDS. Then the Zymogram Developing Buffer (Invitrogen) was added and gels were incubated at room temperature for 60 min with gentle shaking. The gels were then incubated in a humidified atmosphere of 5% CO_2 at 37°C for 20 hrs. After incubation, the gels were

stained using 0.5% Coomassie Blue R-250 (Bio-Rad, Hercules, CA) in destaining solution (methanol:acetic acid:water =50:10:40) for 1 hr. The gels were destained in destaining solution until a clear band was visualized against the blue background. The band, which indicated where casein degradation occurred, revealed uPA activity.

Gelatin zymography

Gelatinase activity of cell culture supernatants was determined by gelatin zymography as previously reported with slight modifications [27]. Briefly, 30 µg samples in an SDS buffer, which were prepared as described above, were run on 10% SDS-PAGE containing 10% gelatin. After the gels were washed in 2.5% Triton-X for 30 min at room temperature, the gels were incubated for 20 hrs at 37°C in the Zymogram Developing Buffer. The gels were subsequently stained in 0.5% Coomassie Blue R-250 in destaining solution for 30 min and destained for approximately 1 hr. Proteolytic activity appeared as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Cell proliferation assay

Cell proliferation was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay and cell counting. In the MTT assay, pancreatic cancer cells were seeded at a density of 5×10^3 cells per well in 96-well plates with complete medium containing 10% FBS and incubated for 24 hrs. The cells were then cultured with and without GDNF (100 nM) and inhibitors (10 mM) in fresh medium without FBS. After 24 hrs, cells were cultured for 4 hrs with the metabolic substrate tetrazolium salt MTT at a final concentration of 0.5 mg/ml. Living cells metabolized tetrazolium to formazan, which was detected spectrophotometrically at 540 nm with a multiwell spectrophotometer (ELISA Reader; BioTek Instruments, Burlington, VT).

For cell counting, pancreatic cancer cells were seeded at a density of 1×10^6 cells per well in 150-mm dishes in culture media containing 10% FBS. After 24 hrs, cells were starved in media with 0.5% FBS. After another 24 hrs, the culture media was changed for fresh serum-free media with added GDNF at a concentration of 10 ng/ml. This lowest effective concentration was determined as previously described [22]. Pancreatic cancer cells were incubated for 24 hrs, and cells were washed twice with PBS, trypsinized, and centrifuged for 5 min at 1,500 rpm. The cell pellet was resuspended in 1 ml of PBS and counted using a light microscope. Cells were pretreated with antibodies for 1 hr before adding GDNF for the inhibitory experiments.

Cell invasion assay

The invasion activity of the pancreatic cancer cells that reacted with GDNF was determined using a Matrigel Invasion Chamber (BD Biosciences Discovery Labware, Bed) as described previously [22]. Briefly, pancreatic cancer cells at 2×10^5 cells/well were seeded in the upper chamber in serum-free media. Complete medium containing 20% FBS served as a chemoattractant in the lower chamber. After 24 hrs, cells were removed from the upper surface of the membrane by gently rubbing using cotton swabs. Invading cells on the lower membrane surface were fixed and Giemsa stained and the invading cells in five random microscope fields were counted. All invasion experiments were done a minimum of three times using separate cultures and the same lot of Matrigel chambers. In the inhibition experiments, cells were pretreated with antibodies for 1 hr.

Statistical analysis

Data are presented as the mean ± SD. Differences in the mean of two samples were analyzed by an unpaired *t* test. Comparisons of more than two groups were made using one-way ANOVA with post hoc Holm-Sidak analysis for pairwise comparisons and comparisons versus control. An α value of 0.05 was used to determine significant differences.

Results

Condition of uPA in pancreatic cancer cells

Many reports have revealed the existence of uPA, plasminogen activator inhibitor (PAI)-1, and PAI-2 in pancreatic cancer cells [28-30]. Therefore, we first investigated the activity of uPA in pancreatic cancer cells without stimulation. In the steady state, the uPA activity of HPAF-II cells was the strongest of all the cells examined, and HPAF-II showed both high molecular weight (HMW) and low molecular weight (LMW) uPA. The BxPC-3 cells revealed only HMW uPA and the intensity was lower than HPAF-II. uPA was not recognized in the MIA PaCa-2 cells in the general state (Figure 1). Thus, we performed all subsequent experiments primarily with HPAF-II cells.

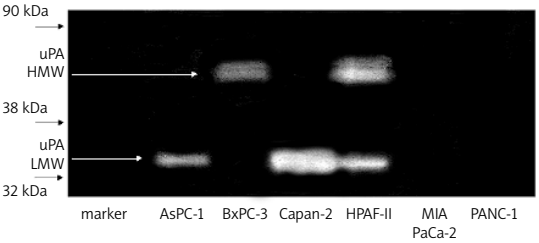

Figure 1. Detection of uPA activity in human pancreatic cancer cell lines by casein zymography as described in the *Material and methods*. Supernatant from the culture cells was concentrated 200 times by centrifugation at 5,000 rpm, and 20 µg of protein was used for zymography. The uPA activity was detected as clear bands on a blue background of Coomassie blue-stained gels

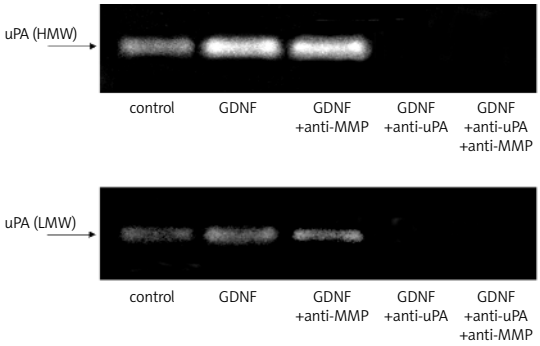

Figure 2. Effect of GDNF on uPA activity in HPAF-II pancreatic cancer cells. The HPAF-II cells were treated with 100 nM GDNF for 24 hrs in fresh serum-free medium. For inhibition experiments, cells were pretreated with anti-uPA and anti-MMP antibodies before adding GDNF. The samples were prepared as described in the Material and methods. Clear white bands indicate uPA activity

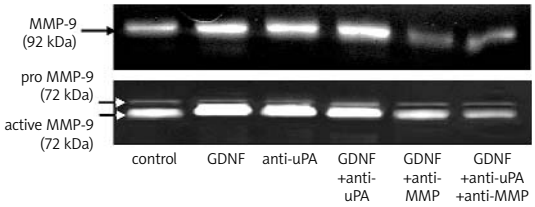

Figure 3. Effect of GDNF on the activation of MMPs. HPAF-II cells were treated with 100 nM GDNF for 24 hrs and the conditioned medium was collected and concentrated. Next, 20 µg protein was separated on a 10% polyacrylamide gel containing 1 mg/ml gelatin. Gels were soaked in renaturing buffer and incubated in developing buffer. Gels were stained on 0.5% Coomassie blue and MMP activity was detected as clear bands on a blue background

Alteration of uPA by GDNF in pancreatic cancer cells

We examined alterations of uPA and MMP activity on HPAF-II cells in reaction to GDNF for 24 hrs. As shown in Figure 2, the band showing uPA activity became stronger after GDNF stimulation. To investigate whether this augmentation effect of uPA by GDNF was due to GDNF alone, HPAF-II cells were treated with anti-uPA and anti-MMP antibody 1 hr prior to treatment with GDNF. Although the anti-uPA antibody blocked the activity of uPA by GDNF, the anti-MMP antibody had no effect on uPA activity (Figure 2).

Regulation of MMP activity by GDNF in pancreatic cancer cells

The MMP activity of the HPAF-II pancreatic cancer cells, stimulated with GDNF for 24 hrs, was examined using gelatin zymography. The results showed that GDNF enhanced the MMP activity, especially MMP-9. The pro- and active-MMP-2 band was also increased, but less than that of the MMP-9 band. Moreover, when we treated pancreatic cancer cells using anti-uPA and anti-MMP antibodies, the anti-MMP antibody negated the effect of GDNF and the anti-uPA antibody decreased the effect of GDNF to a slight degree (Figure 3).

Inhibitors of uPA and MMP decrease pancreatic cancer cell proliferation

We examined the proliferation of pancreatic cancer cells in response to GDNF stimulation for 24 hrs. GDNF increased cell growth in HPAF-II cells in comparison with untreated cells. The anti-uPA and anti-MMP antibodies blocked cell growth to an extent, but cell growth was inhibited to near baseline values when both antibodies were used simultaneously (Figure 4). These results indicated that uPA and MMPs are both involved in GDNF-induced pancreatic cancer cell proliferation.

Invasion of pancreatic cancer cells in response to GDNF

Having demonstrated that GDNF enhanced the activities of uPA and MMPs, we sought to investigate whether this effect converted to an augmentation of invasion ability. The invasion potential of pancreatic cancer cells stimulated with GDNF for 24 hrs was determined using a modified Boyden chamber assay with Matrigel-coated cell culture inserts. GDNF significantly increased invasion of the HPAF-II cells. This effect was partially inhibited by anti-uPA and anti-MMP antibodies. The inhibitory effect of anti-uPA antibody on invasion was weaker than that of the anti-MMP antibody. When we blocked both uPA and MMP using both antibodies, the invasion ability of HPAF-II cells was almost completely inhibited, but was not totally complete (Figure 5).

Discussion

Pancreatic cancer is known as one of the malignant diseases having the most terrible prognosis, since almost all cases are beyond cure when diagnosed. Invasion and metastasis in the early phases of the disease are a biological characteristic of pancreatic cancer. Management of invasion and metastasis may give us new therapeutic strategies for treatment of pancreatic cancer. Although there are some reports concerning tumour dissemination and metastasis formation by malignant tumours [31, 32], our knowledge about molecular mechanisms responsible for the dissemination of tumour cells and metastasis is relatively limited.

In the present study, we found that GDNF enhanced the invasive potential of pancreatic cancer cells by up-regulating not only uPA but also the

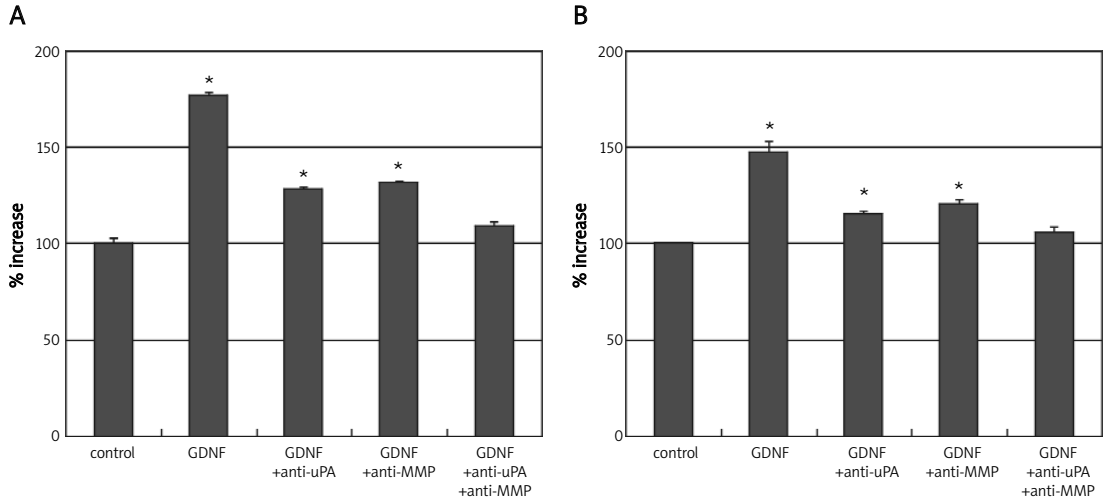

Figure 4. Effect of GDNF on the proliferation of HPAF-II pancreatic cancer cell lines. (A) Cancer cell viability as a parameter of cell proliferation was assessed by cell counting. HPAF-II cells were treated with 100 nM GDNF pretreated with/without 10 mM anti-uPA or anti-MMP antibody for 24 hrs in serum-free medium. Bars indicate the s.d. Experiments were performed in triplicate and repeated three times. *p<0.05 vs. control. (B) The effect of GDNF on HPAF-II cell growth assessed by MTT assay. HPAF-II cells were treated with 100 nM GDNF pretreated with/without 10 mM anti-uPA or anti-MMP antibody for 24 hrs in serum-free medium. Bars indicate the s.d. Experiments were performed in triplicate and repeated three times. *p<0.05 vs. control

MMP-2 and -9 systems. All of these have been known to be secondarily up-regulated by transforming growth factor (TGF)-β1 [33, 34]. GDNF, a distantly related member of the TGF-β family that was originally purified from the B49 glial cell line [35], is a potent survival factor for dopaminergic neurons [36] and motoneurons [37]. We hypothesized that GDNF enhanced both uPA and the MMP system, and elucidated their relationship to the increased invasive properties of GDNF.

The functional receptor for GDNF has recently been identified as a heterotetrameric complex of the RET proto-oncogene and the novel glycosylphosphatidylinositol-linked protein GFRα-1. We previously reported on the expression of GDNF receptors on pancreatic cancer cells [22]. Moreover, we demonstrated the occurrence of an RET receptor polymorphism in human pancreatic cancers, and provided evidence that the G691S RET polymorphism amplifies the proinvasive and proliferative properties of GDNF on pancreatic cancer cells by enhancing mitogen-activated protein kinase (MAPK) signalling [38]. Recent studies have revealed that pancreatic cancer has the uPA and MMP systems [39, 40]. We also demonstrated that pancreatic cancer has uPA and the inhibitor PAI-1, and that MIA PaCa-2 and PANC-1 cells have high uPA levels. Based on our previous results, we decided to elucidate the relationship between uPA and GDNF. The present study showed that HPAF-II had the strongest activity of high molecular weight (HMW) and low molecular weight (LMW) uPA over BxPC-3 and MIA PaCa-2 cells. The BxPC-3 cells had only weak activity of HMW uPA

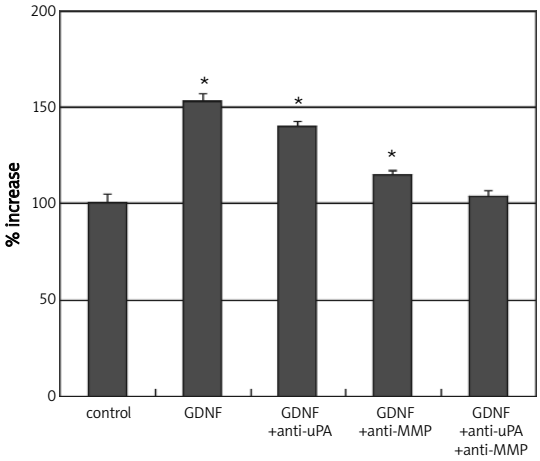

Figure 5. Effect of GDNF on HPAF-II pancreatic cancer cell invasion using the Matrigel Double Chamber Assay. HPAF-II cells were cultured with 100 nM GDNF pretreated with/without 10 mM anti-uPA or anti-MMP antibody for 24 hrs in the inner chamber coated with Matrigel. Bars indicate the s.d. Experiments were performed in triplicate and repeated three times. *p<0.05 vs. control

and there was no uPA activity in the MIA PaCa-2 and PANC-1 cells. The reason for these inconsistent results may be that these cells have high PAI-I levels and that PAI-I inhibited the uPA activity. The strong uPA activity in HPAF-II may depend on the absence of PAI-I in this cell line. We continued our experiments using the HPAF-II cells, which have both HMW and LMW uPA activity. We found that GDNF enhanced the activity

of HMW uPA but had no effect on LMW uPA. This result may be supported by a recent study that revealed that HMW uPA affected cell proliferation more than LMW uPA [15].

Matrix metalloproteinases belong to a family of proteolytic enzymes capable of degrading components of the ECM. Today, over 25 members of this family have been identified. MMP-2, -3, -7, -9, -11 and MT1-MMP have been evaluated in pancreatic cancer [41-43]. The expression of MMP-2 and MMP-9 especially correlated with pancreatic cancer cell invasion [44] and local recurrence rate [45]. Yang et al. reported that MMP-2 and MMP-9 expression correlated with the degree of pancreatic cancer cell invasion [44]. Another report revealed that the expression of MMP-2 and MMP-9 correlated with lymph node invasion and desmoplastic reaction [46]. In this study, gelatin zymography showed that GDNF enhanced the MMP-2 and MMP-9 activities. The enhancement of MMP-9 by GDNF was more intense than that of MMP-2. Our findings suggest that the effects of GDNF on pancreatic cancer cells, such as proliferation and invasion, may depend on MMP-9. Mrena et al. reported that MMP-2 regulated metastasis in gastric cancer cells mediated by COX-2 [47], but other reports showed that MMP-9 and uPA play an important role in invasion and metastasis of malignant tumour cells [48-50]. It is well known that uPA converts pro-MMP to active-MMP, which is mediated by turning plasminogen into plasmin [14]. Interestingly, in our study, pretreatment with anti-uPA antibody could not block MMP activity to the same degree as the control. Our findings suggest that the activation of pro-MMP may not be mediated by uPA alone, but that the interaction between uPA and MMP may play an important role in pancreatic cancer cell invasion.

In this study, we attempted to elucidate the expression of molecules regulated by GDNF in order to understand the invasion and metastasis of pancreatic cancer. We demonstrated that GDNF increased cell proliferation and invasion. To investigate the role of uPA and MMP in pancreatic cancer invasion and proliferation, we performed inhibitory experiments using anti-uPA and anti-MMP antibodies. We found that each antibody inhibited pancreatic cancer activity, but did not block it to the same level as the control. Interestingly, the invasion and proliferation abilities of the pancreatic cancer cells were not completely inhibited using both anti-uPA and anti-MMP antibodies. These results suggest that uPA and MMP play an important role in pancreatic cancer cell invasion that is mediated by GDNF. Although we did not investigate them at this time, other factors such as integrin may also have an important part in this process.

In conclusions we found that the urokinase activator system plays a major role in baseline invasion of pancreatic cancer cells. We demonstrated the alteration of invasive ability on pancreatic cancer cells mediated by enhancement of uPA and MMP activated by GDNF, and the inhibition of invasion in part by blocking uPA and MMP. However, further study is necessary on the inhibition of uPA and MMP as novel therapeutic agents in pancreatic cancer.

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