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ROUND TABLE

Antiproteases in Preventing the Invasive Potential of Pancreatic Cancer Cells

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Summary

The process of tumor progression and metastasis involves degradation of the extracellular matrix and is governed by an intricate balance of proteases, their activators and their inhibitors, in which malignant cells are permitted to infiltrate the adjacent structures and gain access to lymph and blood vessels. These proteases can be broadly categorized into three families: matrix metalloproteinases, serine proteinases and cysteine proteinases, all of which have all been implicated in these processes. The presence of neural invasion is often considered to be a poor prognostic sign; however, the cellular mechanisms underlying this propensity for perineural invasion are unknown. We recently researched the relationship between the glial cell line-derived neurotrophic factor and perineural invasion by human pancreatic cancer cells. We also confirmed that NF-kappa B is a part of the signaling pathway from the glial cell line-derived neurotrophic factor in human pancreatic cancer cells, and documented the inhibitory effect of gabexate mesilate, a well-known non-physiological synthetic serine protease inhibitor, for pancreatic cancer invasion. Recent studies on the role of proteases and protease inhibitors in pancreatic cancer invasion are also reviewed.

Introduction

Of all gastrointestinal carcinomas, pancreatic cancer has the most unfavorable prognosis, and many patients die from liver metastasis or local recurrence because pancreatic cancer frequently and rapidly invades the surrounding tissue, such as the lymph nodes and neural plexuses. In particular, the presence of neural invasion is widely considered to be a poor prognostic sign [1, 2, 3], but the cellular mechanisms underlying this propensity for perineural invasion remain unknown.

Recent studies report that transforming-growth-factor-beta (TGF-beta) is implicated in the metastatic and invasive potential of pancreatic cancer [4]. The glial cell line-derived neurotrophic factor (GDNF), a distantly related member of the TGF-beta family which was originally purified from the B49 glial cell line [5], is a potent survival factor for dopaminergic neurons and motoneurons [5, 6]. We recently investigated the relationship between GDNF and perineural invasion by human pancreatic cancer cells. The results revealed that the invasive potential was increased by GDNF in human pancreatic cancer cell lines; we confirmed that nuclear factor kappaB (NF-kappa B) is a part of the signaling pathway from GDNF in human pancreatic cancer cells [7, 8, 9]. These results indicate that the NF-

kappa B inhibitor has a marked influence on pancreatic cancer invasion and suggest that these drugs may have an application in the prevention and treatment of pancreatic cancer.
Gabexate mesilate is a well-known non-physiological synthetic serine protease inhibitor. Gabexate mesilate inhibits various serine proteases such as trypsin, plasmin and thrombin, and has been used in Japan for the treatment of acute pancreatitis and disseminated intravascular coagulation [10]. Gabexate mesilate is also commonly used after endoscopic retrograde cholangio-pancreatography and pancreatic cancer surgery to prevent post-procedure or postoperative pancreatitis [11].
It has recently been reported that gabexate mesilate inhibits NF-kappa B activation in human monocytes and human umbilical vein endothelial cells (HUVECs) [12, 13].The transcription factor NF-kappa B is a homodimer or heterodimer composed of members of the rel/NF-kappa B protein family, including Rel A, Rel B, c-Rel, p50/p105, and p52/p110. NF-kappa B has various functions in cancer cells, including the prevention of apoptosis [14] and the promotion of chemoresistance [15, 16], cell invasion and metastases [17, 18]. In several types of malignancies, NF-kappa B is activated constitutively [19, 20, 21]. The inhibition of NF-kappa B activity by one of its inhibitors, such as a proteasome inhibitor, sulfasalazine or salicylate, reduces NF-kappa B-dependent protection from apoptosis [15, 22, 23, 24].
While several reports describe the role of serine protease inhibitors in cancer, little is known about their influence on invasive potential in human pancreatic cancer cells. In the present study, we investigate the influence of serine protease inhibitors on NF-kappa B activation and invasive potential in human pancreatic cancer, and conduct a review of recent studies on the role of proteases and protease inhibitors in pancreatic cancer invasion.

Serine Protease Inhibitors and Our Findings

Serine protease inhibitors can be categorized into physiological inhibitors which are naturally present in tissues and non-physiological inhibitors which are either produced by micro-organisms or chemically synthesized. Physiological serine protease inhibitors belong to the superfamily of serpins which includes antithrombin III, PAI-1 and PAI-2 [25], among others. Serine proteases are known to have various effects on cancer cells. One such serine protease is the urokinase-type plasminogen activator (uPA). Plasmin has the ability of activating the matrix metalloproteinases necessary for basement membrane degradation [26]. uPA, which activates plasmin, is overexpressed in pancreatic cancer cells and has been shown to be involved in tumor invasion and metastasis [27]. The concomitant overexpression of uPA and uPA receptor (uPAR) was found to be associated with shorter survival in pancreatic cancer patients [28]. Sawai *et al*. demonstrated that IL-1alpha can induce selective upregulation of uPA/uPAR in pancreatic cancer cells and that inhibitory antibodies against uPAR can reduce the invasive potential of pancreatic cancer cells [29]. Sawai *et al*. reported that peroxisome proliferators-activated receptor (PPAR)-gamma ligands, which are currently in clinical use as antidiabetic drugs, decrease pancreatic cancer invasion via modulation of the plasminogen activator system [30].
We previously reported that pancreatic cancer cell lines have both GDNF receptors (GFRalpha-1 and Ret), and that the invasive capacity of human pancreatic cancer cell lines is increased by GDNF or co-cultivation with human glioma cells T98G or A172 [7, 8]. We also reported that NF-kappa B is a part of the signaling pathway from GDNF in human pancreatic cancer cells and that NF-kappa B activity is strongly correlated with its invasive potential [9]. In agreement with our results, Zhang *et al*. reported that the epidermal

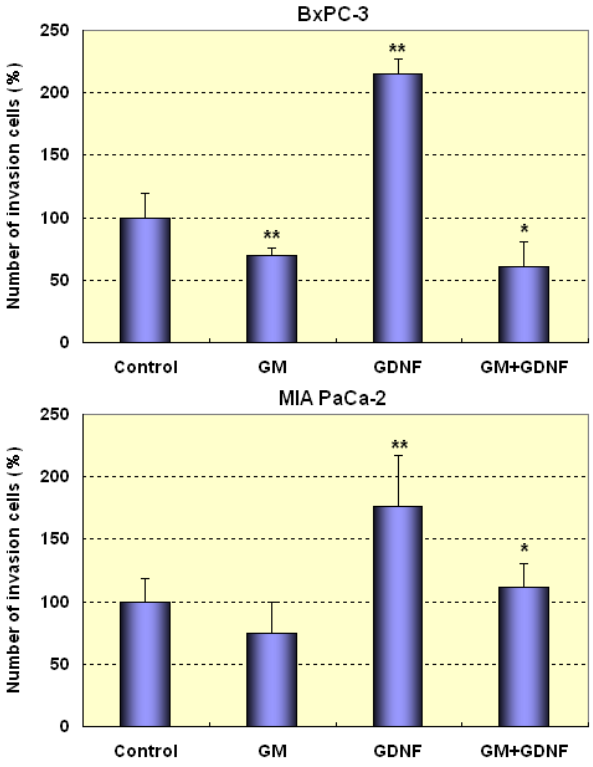

Figure 1. The effect of gabexate mesilate and GDNF on pancreatic cancer invasion. Using the Biocoat Matrigel Invasion Chamber system (BD Biosciences, Bedford, MA, USA), cells (1×10^5/well) were incubated on the upper component with gabexate mesilate (GM: 100 μM) and GDNF (100 ng/mL) for 24 hours. Statistical significance was analyzed by non-repeated ANOVA with an SNK test. Bars indicate SD, **P<0.01 *vs*. controls, and *P<0.01 *vs*. GDNF only.

growth factor (EGF) gives rise to matrix metalloproteinase-9 (MMP-9), uPA induction and the invasiveness of pancreatic cancer through the NF-kappa B pathway [31].

We recently investigated the influence of the synthetic serine protease inhibitor gabexate mesilate on GDNF-induced NF-kappa B activation and invasive potential in two human pancreatic cancer cells (BxPC-3, MIA PaCa-2) using a dual-luciferase reporter assay and an *in vitro* invasion assay. The results demonstrated that gabexate mesilate prevents GDNF-induced NF-kappa B activity in a dose-dependent manner and significantly inhibits invasive ability in two pancreatic cancer cell lines (Figures 1, 2). It is unclear, however, how gabexate mesilate inhibits NF-kappa B activation. In human monocytes and HUVECs, gabexate mesilate inhibits I-kappaB phosphorylation and degradation as well as NF-kappa B localization to the nucleus [12, 13]. Gabexate mesilate may inhibit the degradation of I-kappaB in a manner similar to that of other proteasome inhibitors such as MG132 or PS-341 by inhibiting the 26S proteasome [15, 22, 32]. Uchima *et al*. reported that gabexate mesilate could inhibit pancreatic cancer invasion by directly antagonizing the activities of uPA and tumor-associated trypsinogen (TAT). The uPA-promoter contains an NF-kappa B binding site which directly mediates the induction of uPA expression [33]; therefore, we consider that gabexate mesilate could inhibit pancreatic cancer invasion not only by antagonizing the activities of uPA and TAT, but also by suppressing uPA production

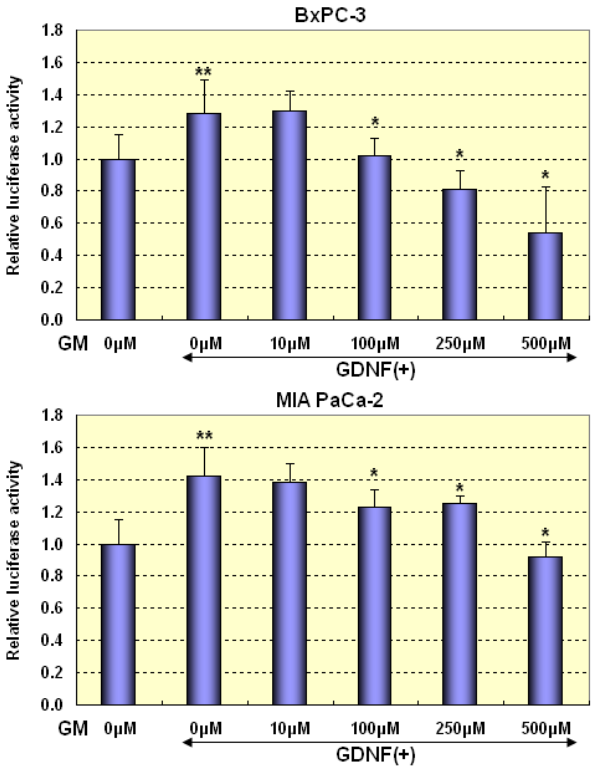

Figure 2. The effect of gabexate mesilate and GDNF on NF-kappa B activity. Cells (5×10^4/well) were exposed to GDNF (100 ng/mL) and gabexate mesilate (GM: 0-500 μM) for 6 hours, after which relative luciferase activity was measured. GDNF significantly increased the relative luciferase activity of both cell lines, and the increased NF-kappa B activity was suppressed by pre-treatment with gabexate mesilate at concentrations higher than 100 μM. Statistical significance was analyzed by non-repeated ANOVA with Dunnett's test. Bars indicate SD; **P<0.01 *vs*. controls; *P<0.01 *vs*. GDNF only.

(Figure 3). Furthermore, we reported that gabexate mesilate could prevent NF-kappa B activation and increase TNF-alpha mediated apoptosis in human pancreatic cancer cells by suppressing the NF-kappa B signaling pathway [34]. These findings further support the notion that serine protease inhibitors are potentially promising therapeutic targets in pancreatic cancer.

Matrix Metalloproteinases and Their Inhibitors

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes capable of degrading the extracellular matrix (ECM) which plays a critical role during cancer cell invasion [35, 36, 37]; 18 different subtypes have recently been identified [38]. Recent studies report that MMP-2 and/or MMP-9 were overexpressed in pancreatic cancer; this expression strongly correlated with the presence of the desmoplastic reaction and the lymphoid reaction [39, 40]. MMP-7 is also involved in cell dissociation and the subsequent invasion of pancreatic cancer [41]. A variety of stimuli (cytokines, growth factors, cellular stress) are reported to upregulate the expression of MMPs; for example, Okada *et al*. reported that GDNF upregulates the expression and activation of MMP-9 in human pancreatic cancer, mainly via the PI3-K/AKT signaling pathway [42]. As a consequence of these findings, it is thought that MMP inhibitors play an important role in human pancreatic cancer. Kilian *et al*. reported that low-dose therapy with a selective MMP inhibitor (Ro 28-2653) decreases liver metastasis due to an inhibition of MMP-2 and -9 concentrations in ductal pancreatic cancer [43]. BB-94 (batimastat), a bioactive synthetic MMP inhibitor, has been shown to inhibit MMP activity and invasive potential in pancreatic cancer cell lines [44]. Several clinical trials using BB-2516 and BAY 12-9566 have recently been undertaken; however, no significant overall survival advantage was seen in advanced pancreatic cancer patients [45, 46].

Tissue inhibitors of metalloproteinases (TIMPs) are small proteins capable of binding and inactivating MMPs; four TIMPs have been identified [47, 48]. Boomston *et al*. reported that TIMP-1 overexpression reduces the invasive potential of pancreatic cancer [49] and that TIMP-1 antisense gene transfection cells showed marked reductions in cell invasion and MMP-2 activity [50].

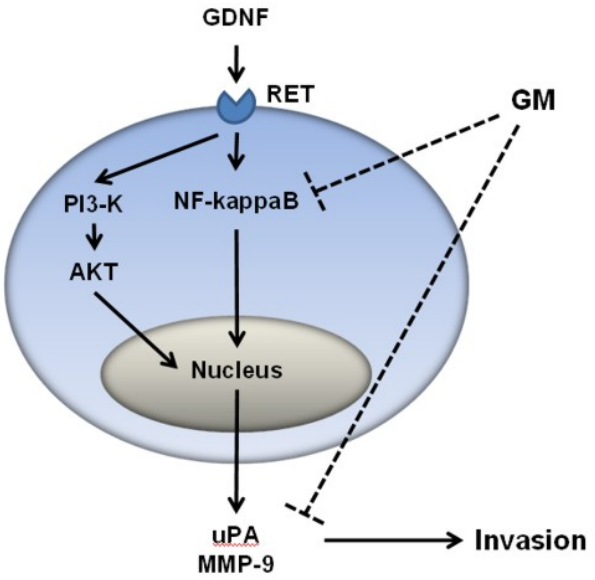

Figure 3. Gabexate mesilate (GM) inhibits the invasive ability of pancreatic cancer cells. GDNF binds the RET tyrosine kinase receptor, and then the NF-kappa B and PI3-K/AKT pathways are activated. We consider gabexate mesilate to be capable of inhibiting pancreatic cancer invasion by antagonizing the activities of uPA and suppressing uPA production.

Cysteine Proteases and Their Inhibitors

Cathepsin cysteine proteases have been implicated in processes important for tumor development and progression, including angiogenesis, cell proliferation, apoptosis and invasion [51]. There are 11 cysteine cathepsins present in the human genome and 19 in the mouse genome [52]. It has been noted that invasion is facilitated by a membrane or secreted form of cathepsin B which acts outside the cell to degrade extracellular matrix components at or adjacent to the surface of the invading cell [53]. Cathepsin B is able to degrade the components of the extracellular matrix and basement membrane either directly or indirectly by activating other proteases such as pro-uPA [54]. In pancreatic cancer, it is reported that cathepsin B and cathepsin L are

strong and independent prognostic markers [55]. Interruption of their expression, either by antisense RNA [56] or RNA interference [57], was found to reduce tumor cell invasion, angiogenesis and tumor growth. Stefin A is an endogenous inhibitor of cathepsin B; overexpression of stefin A (as with TIMP) inhibits tumor cell growth, angiogenesis, invasion, and metastasis [58]. The specific cysteine cathepsin B inhibitors in a previous study were purified [59]; however, the biological effects of these drugs on pancreatic cancer are not completely understood. Further investigation about the role of cysteine proteases and their inhibitors in the prevention of pancreatic cancer invasion and metastasis is needed.

Conclusion

In conclusion, we have demonstrated that GDNF increases NF-kappa B activity in human pancreatic cancer cell lines and that the invasive potential is regulated by NF-kappa B activation. Furthermore, the serine protease inhibitor gabexate mesilate may play an important role in the inhibition of neural invasion in human pancreatic cancer cells. Many protease inhibitors strongly influence pancreatic cancer invasion and metastasis. We foresee protease inhibitors eventually becoming part of the paradigm of treatment for pancreatic cancer, thus improving the prognoses of those with resectable and unresectable disease.

Keywords Gabexate; Neoplasm Invasiveness; NF-kappa B; Pancreatic Neoplasms; Protease Inhibitors

Abbreviations GDNF: glial cell line-derived neurotrophic factor; HUVEC: umbilical vein endothelial cell; MMP: matrix metalloproteinases; NF-kappa B: nuclear factor kappaB; PPAR: peroxisome proliferators-activated receptor; TAT: tumor-associated trypsinogen; TIMP: tissue inhibitors of metalloproteinases; uPA: urokinase-type plasminogen activator; uPAR: urokinase-type plasminogen activator receptor

Conflict of interest The authors have no potential conflicts of interest

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