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Action of Antiproteases on Pancreatic Cancer Cells

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Summary

Tumor-associated trypsinogen, urokinase-type plasminogen activator, matrix metalloprotease-2 (MMP-2), and MMP-9 each play a dominant role in the degradation of the extracellular matrix (ECM) during the invasion process of pancreatic cancer. Transforming growth factor beta1 (TGF-beta1) is a multifunctional polypeptide that regulates cell growth and differentiation, extracellular matrix deposition, cellular adhesion properties, angiogenesis and also immune functions. The protease-activated receptor-2 (PAR-2) is a G protein-coupled receptor which is cleaved and activated by trypsin and tryptase. PAR-2 activated by trypsin plays an important role in promoting the proliferation of pancreatic cancer.
We previously reported that TGF-beta1 up-regulated vascular endothelial growth factor (VEGF) production, and the protease production of both MMP-2 and urokinase-type plasminogen activator in the highly metastatic pancreatic cancer cell lines SW1990 and CAPAN-2. We had examined the inhibitor effects of a protease inhibitor, gabexate mesilate, on cell invasion, cell proliferation, growth factor production, and ECM degradation. We also examined the effect of gabexate mesilate on the production of growth factor and ECM degradation by these cell proteases and enzymatic activities. Gabexate mesilate down-regulated the invasiveness, the proliferation and liver metastasis potential of SW1990 and CAPAN-2 cells. Gabexate mesilate inhibited not only the enzymatic activities of tumor-associated trypsinogen and urokinase-type plasminogen activator but also the production of MMP-2, all of which have been known to be secondarily up-regulated by TGF-beta1. These findings suggested that gabexate mesilate is potentially useful in the treatment against invasion, proliferation, and metastasis of pancreatic cancer.

Introduction

Cancer metastasis results from several complex processes including: a) detachment of cancer cells from their original location; b) cancer cell migration; c) invasion of cancer cells into the surrounding tissue, requiring adhesion to and degradation of extracellular matrix components; d) access of cancer cells to blood and lymphatic vessels; and e) adhesion to and invasion through the endothelium, allowing colonization at distant sites in the organism [1]. Tumor cell invasion is a key step of this process which is believed to be related to the tumor cell-associated extracellular matrix (ECM) and basement membrane [2]. Pancreatic cancer is characterized by local invasion, early metastasis and a strong desmoplastic reaction. Proteolytic degradation of extracellular matrix components is an essential process for tumor invasion and metastasis. Several classes of proteases may be involved in the proteolytic events which occur during pancreatic cancer

cell invasion; these include serine proteases and matrix metalloproteinases (MMPs), such as urokinase-type plasminogen activator (u-PA) [3, 4, 5, 6, 7] and tumor-associated trypsinogen (TAT) [3, 7, 8], MMP-2, MMP-9, and membrane type (MT)-MMPs [3, 4, 6, 9, 10, 11, 12, 13]. We herein introduce the mechanism of the ECM protease network in pancreatic cancer, and the inhibition of protease inhibitor action in pancreatic cancer invasion and metastasis.

The Role of Proteases in Pancreatic Cancer Invasion and Metastasis

We used the three human pancreatic cancer cell lines which we had used in our previous experiments: SW1990 (well- to moderately-differentiated adenocarcinoma), CAPAN-2 (moderately-differentiated adenocarcinoma), and PANC-1 (poorly-differentiated adeno-carcinoma). SW1990 and CAPAN-2 demonstrated high invasive and metastatic ability [3, 4, 7] (Table 1).

The activator u-PA is a highly restricted serine protease which converts the zymogen plasminogen to active plasmin, a broad-spectrum serine proteinase capable of degrading most of the major protein components of the extracellular matrix. The binding of u-PA to its receptor and subsequent u-PA-mediated pericellular proteolysis are involved in many processes, including cell migration and tissue remodeling in angiogenesis and metastasis [1]. Recent studies have shown that the overexpression of u-PA increases the invasive and metastatic rates of pancreatic cancer [3, 4, 5], breast cancer [14] and melanoma [15, 16].

MMPs are a group of enzymes which degrade the macromolecules of connective tissue, ECM and basement membrane. These enzymes are believed to play important roles in tumor metastasis, invasion and angiogenesis [17, 18, 19]. In general, MMP-2 and MMP-9 have been most consistently detected in malignant tissue and are associated with tumor growth and malignant tumor progression [18, 19]. MT1-MMP, the first member of a more recently established group of MMPs containing a membrane-spanning sequence, has been shown to have an important role in MMP-2 activation in cell membranes [20].

Trypsinogen is one of the major serine proteases and is produced by the acinar cells of the pancreas. Pancreatic acinar trypsinogen (PAT) is generally secreted in an inactive zymogen form and is activated by an intestinal enzyme, enterokinase in the duodenum [21]. Extra-pancreatic production of trypsin, i.e., TAT, has been reported to be produced by several cancer cell lines, through not by acinar cells [22, 23]. We previously reported that not only TAT and PAT have been shown to play an important role in pancreatic cancer invasion and metastasis. Both PAT and TAT are activated by u-PA as the first step in invasion by cancer cells. Once activated, TAT and PAT can degrade extracellular matrix components and they can both also directly further activate TAT, PAT, pro-u-PA, and pro-MMPs (except for pro-MMP-2), even at a neutral pH. These activated proteases also further degraded the ECM. This ECM-protease network may constitute a vicious cycle, which would include the activation of latent ECM protease and degradation of the ECM, thereby

Table 1. Characteristics of pancreatic cancer cell lines (*in vitro* and *in vivo*).

Cell Lines	Percentage of invasion [a]	Liver metastasis in nude mice [b]
SW1990	39.8±2.5	10/10 (100%)
CAPAN-2	25.3±2.4	2/10 (20%)
PANC-1	15.8±4.8	0/10 (0%)

Data show mean±SD

[a] MTT invasion assay using a Transwell® Double Chamber (Corning Inc., Corning, NY, USA) and Matrigel™ (BD Biosciences, San Jose, CA, USA). The proportion of invaded cells to the total number of cells was calculated as the percentage of invasion.

[b] *In vivo* liver metastasis by splenic injection. The number of nude mice having metastatic nodules in the liver/total number of nude mice.

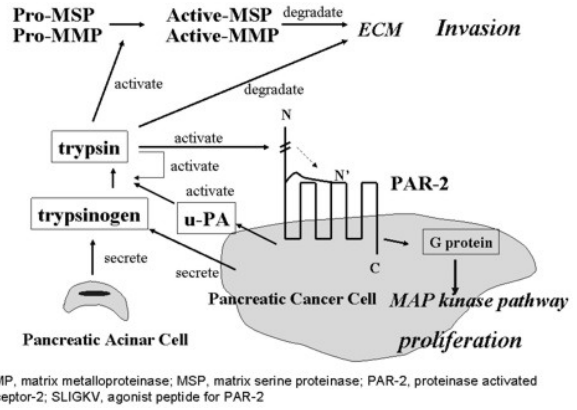


Figure 1. The mechanism of cell proliferation by PAR-2 activation in the microenvironment of pancreatic cancer. TAT produced by pancreatic cancer and PAT are both activated by u-PA which is produced by pancreatic cancer. Trypsin activates PAR-2 on the surface of cancer cells and stimulates cell proliferation through MAP kinase.

promoting tumor cell invasion and metastasis [7] (Figure 1).

In our previous study, RT-PCR analysis could detect TAT, u-PA, MT1-MMP, MMP-2, and MMP-9 expression in the three pancreatic cancer cell lines (SW1990, CAPAN-2, and PANC-1) tested. We observed TAT expression at the mRNA level in SW1990 and CAPAN-2 cells. These cell culture supernatants had high TAT activities and u-PA activities. We also investigated MT1-MMP, MMP-9 and u-PA expression at the mRNA level in all cells and found MMP-2 expression and MMP-2 activity in SW1990 and PANC-1 cells, respectively. SW1990 cells showed invasiveness *in vitro* and *in vivo* and a hepatic metastatic capacity greater than those of CAPAN-2 and PANC-1 cells. We hypothesized that, in pancreatic cancer, the expression of TAT and u-PA were correlated with invasion and metastasis more than MMPs [3] (Figure 2).

The Role of Protease-Activated Receptor-2 in Pancreatic Cancer Cell Proliferation

Protease-activated receptors (PARs) are G protein coupled, seven transmembrane domain receptors divided into four classes, PAR-1 to -4, according to their distinct N-terminal cleavage sites and tethered ligand pharmacology. Among these classes, PAR-2 has been reported to be expressed in the gastrointestinal tract, pancreas, liver, kidney, and sensory neurons [24, 25, 26]. In the pancreas, PAR-2 stimulates amylase secretion and mediates the activation of pancreatic duct epithelial cell ion channels [25, 26, 27, 28]. The activation of PAR is achieved by the proteolytic unmasking of the cryptic N-terminal receptor-activating sequence (SLIGKV) which binds to the body of the same receptor molecule. PAR-2 is activated by trypsin and tryptase [24]. In the microenvironment of pancreatic cancer carcinogenesis, pancreatic acinar cells secrete trypsinogen which could activate PAR-2 function. We reported that PAR-2 played an important role in the cell proliferation of pancreatic cancer. TAT and PAT may contribute to the continuous stimulation of cancer cell proliferation by activating PAR-2 [29] (Figure 1).

The expression of PAR-2 mRNA and protein was recognized in all cell lines (SW1990, CAPAN-2, and PANC-1). The proliferation of these cells was stimulated by the PAR-2

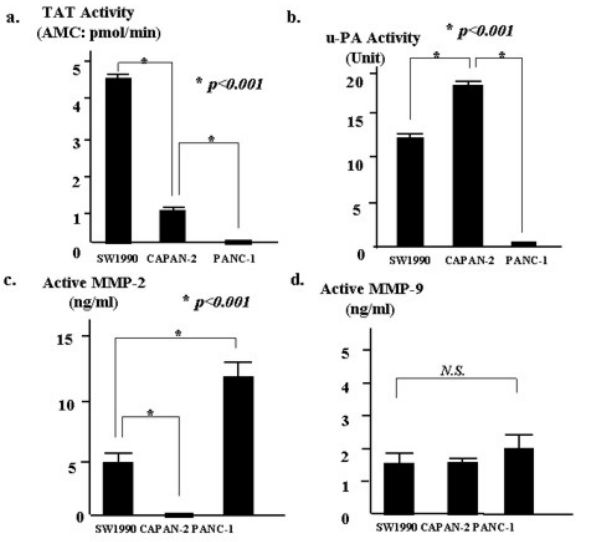


Figure 2. The constitutive expression of TAT, u-PA, MMP-2, and MMP-9 in human pancreatic cancer cell lines. Pancreatic cancer cells were cultured almost to confluence in DMEM with 10% FCS and were washed with PBS, followed by culture in 8 mL serum-free DMEM for 48 h. **a.** TAT activity in the control supernatants was determined by the substrate assay, as described in the text. **b.** u-PA activity in the control supernatants was determined by using the u-PA activity kit. **c.** and **d.** MMP-2 and MMP-9 activity in the control supernatants was determined by using the MMP-2 and MMP-9 activity kit.

agonist SLIGKV and also by trypsin. These stimulating effects were inhibited by anti-PAR-2 antibody, so these effects were derived from the activation of PAR-2 in cancer cells. Moreover, enhanced cell proliferation by SLIGKV or trypsin was caused by the activation of the MAP kinase cascade, and *in vivo* SLIGKV inoculation enhanced tumor growth in xenograft nude mice [29] (Figure 3).

The Role of TGF-beta1 in Pancreatic Cancer Progression

We previously reported that the presence of transforming growth factor-beta1 (TGF-beta1) in the microenvironment produced by tumors or surrounding tissue may play an important role in enhancing liver metastasis in pancreatic cancer by promoting the tumor's capacity for invasion and modulating the capacity for angiogenesis and immunogenecity [3, 4]. TGF-beta is a strong inhibitor of cell growth in normal epithelial cells and acts as a tumor suppressor in early tumor stages [30]. However, tumor cells frequently escape from growth regulation by TGF-beta and, once the negative growth response to TGF-beta is attenuated, other responses to TGF-beta can manifest themselves, leading to a more malignant phenotype [31, 32]. It has been reported that TGF-beta plays an important role in modifying the invasive and metastatic potential of malignant tumor cells, and several investigators have also reported that TGF-beta might act as a strong mediator of pancreatic cancer cell invasion by up-regulating MMP-2 and the u-PA system [3, 4, 6, 33]. Furthermore, TGF-beta1 is usually secreted in an active form, and it can be activated by means of many mechanisms. Among these mechanisms, plasmin is known to enhance the activation of latent TGF-beta1 [34]. When u-PA levels were up-regulated by active TGF-beta1, they may also have activated latent TGF-beta1 which had been produced by tumors or surrounding tissue. Together, these effects may form a vicious cycle of u-PA and TGF-beta1, thereby promoting tumor cell invasion through the enhancement of pericellular proteolysis.

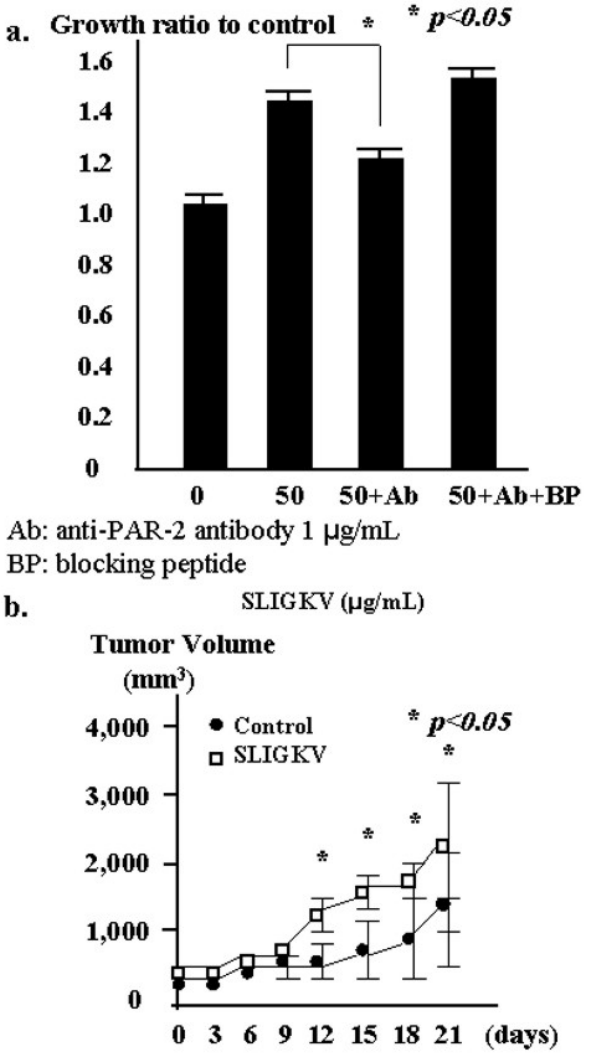


Figure 3. a. The inhibitory effect of anti-PAR-2 antibody on cell proliferation. The enhancement of cell proliferation by SLIGKV was inhibited by anti-PAR-2 antibody in SW1990. **b.** *In vivo* effect of SLIGKV in SW1990 xenografts. The growth of subcutaneous xenograft tumors in mice treated by SLIGKV (100 μg x 5 times) was significantly enhanced on days 12-21 after SLIGKV inoculation as compared to the controls (PBS 100 μL x 5 times).

Moreover, we had reported that TGF-beta1 up-regulated the production of VEGF in pancreatic cancer cell lines [35].

Inhibition of Human Pancreatic Cancer Cells and Mechanism of Action of Gabexate Mesilate

Gabexate mesilate, another name for ethyl 4-(6-guanidinohexanoyloxy) benzoate mono-methanesulfonate, antagonizes proteolytic enzymes in a manner similar to that of aprotinin, inhibiting the actions of trypsin, plasmin, kallikrein, thrombin, and C1-elastase

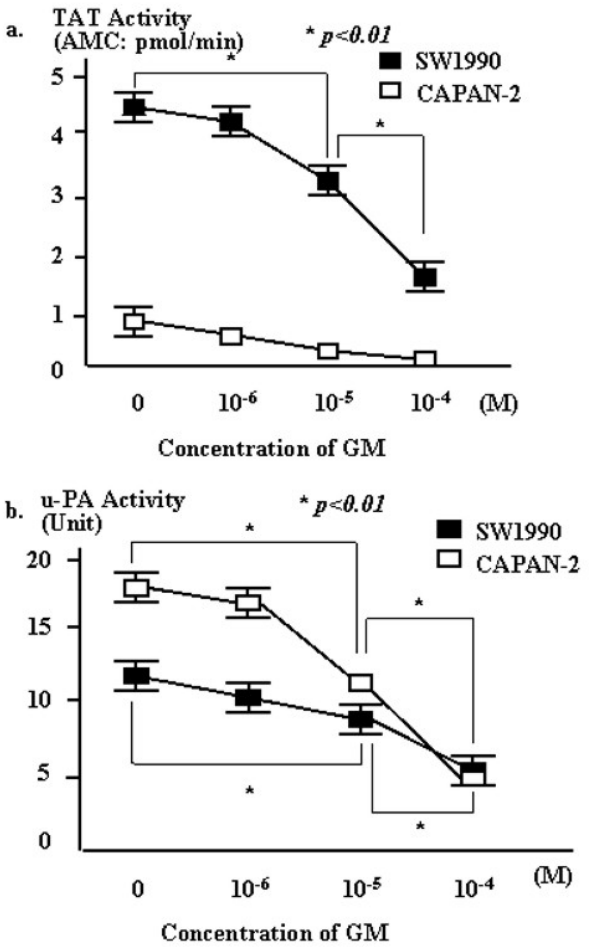


Figure 4. Inhibition of TAT and u-PA activity by gabexate mesilate *in vitro*. The control culture supernatants were incubated with various concentrations of gabexate mesilate at 37°C for 1 h. The resultant solutions were measured for TAT and u-PA activity by the substrate assay and the u-PA activity kit.

[36]. Gabexate mesilate has been used in the treatment of hyperenzymemia, disseminated intravascular coagulation, and acute pancreatitis in Japan, Italy, Korea, and Taiwan.

We explored whether pancreatic cancer cell culture supernatants with gabexate mesilate inhibited protease activity. Spontaneous TAT activities in the SW1990 and CAPAN-2 cell culture supernatants were significantly inhibited by the addition of 10^{-5} and 10^{-4} M gabexate mesilate, respectively (Figure 4a). The u-PA activities in the SW1990 and CAPAN-2 cell culture supernatants were also significantly inhibited by the addition of 10^{-5} and 10^{-4} M gabexate mesilate, respectively (Figure 4b).

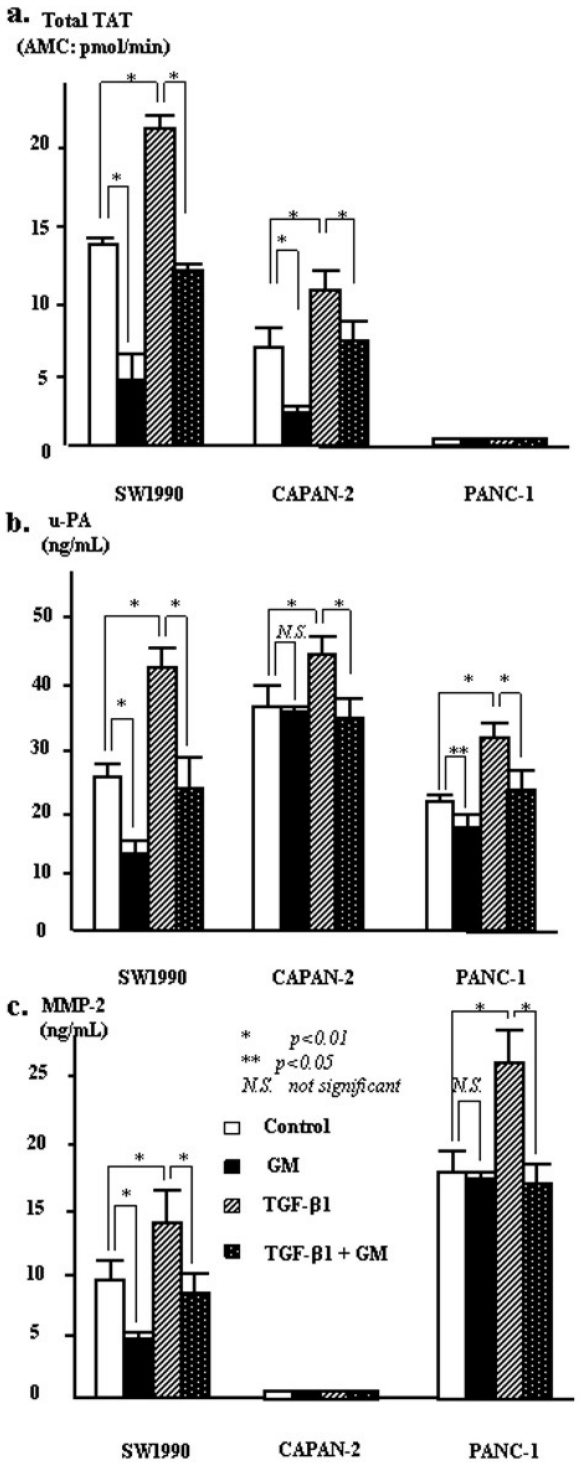
Gabexate mesilate may have acted by either of two mechanisms: suppressing the activation of TAT and u-PA, or suppressing the production of endogenous and exogenous TGF-beta1 [3] (Figure 5a, b, c). We together with Ellinrieder *et al.* reported that the up-regulation of TAT, MMP-2, and u-PA levels by TGF-beta1 resulted in the accelerated decomposition of the ECM and that this led to the progression of pancreatic cancer cell invasion [3, 4, 6]. Based on our results, it appears that gabexate mesilate could break the 'vicious cycle' of u-PA and TGF-beta1.

Gabexate mesilate did not have a significant effect on the proliferation of SW1990, CAPAN-2, and PANC-1 cells at gabexate mesilate concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M, respectively, after 48 h of treatment (Figure 6a). At gabexate mesilate concentrations of 10^{-3} M, a significant effect on the proliferation of all pancreatic cancer cells was observed. Decreased activation of PAR-2 by inhibiting the effect of trypsin or u-PA could be a new approach to inhibiting the proliferation of pancreatic cancer cell lines.

We evaluated the effect of gabexate mesilate on the invasion of SW1990, CAPAN-2 and PANC-1 cells using the Matrigel™ (BD Biosciences, San Jose, CA, USA) invasion assay. Gabexate mesilate demonstrated inhibitory effects on cancer cell invasion in a dose-dependent manner (Figure 6b). Exogenous TGF-beta1 led to a significant increase of the invasive potential of SW1990, CAPAN-2 and PANC-1 cells. Gabexate mesilate also blocked the TGF-beta1-induced invasiveness of SW1990, CAPAN-2 and PANC-1 cells (Figure 6c).

Table 2 summarizes the inhibitory effect of gabexate mesilate on liver metastasis. Using a splenic injection model in nude mice, we investigated whether *in vivo* liver metastasis of SW1990 and CAPAN-2 cells was enhanced by the addition of gabexate mesilate. Macroscopic and histological examination disclosed high potential liver metastasis in SW1990 cells (100%). Significant differences in liver metastatic potential, liver metastatic nodules, and liver

weight were observed between the control and the gabexate mesilate treatment group.



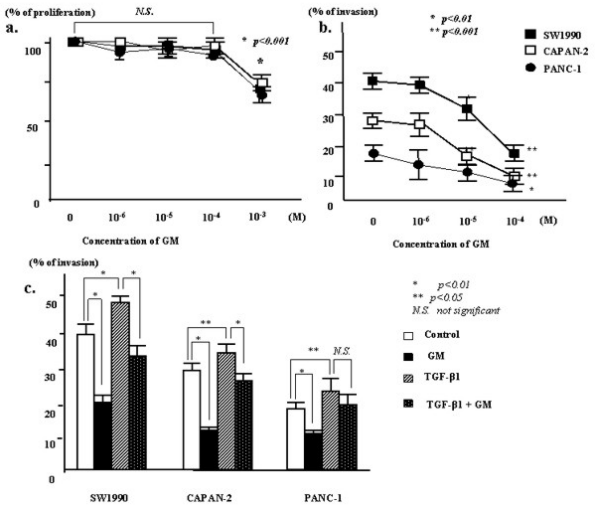


Figure 6. a. *In vitro* inhibitory effect of gabexate mesilate on human pancreatic cancer cell proliferation. We analyzed the effect of gabexate mesilate on cell proliferation after inhibition by using the cell proliferation ELISA system. **b.** *In vitro* inhibitory effect of gabexate mesilate on human pancreatic cancer cell invasion. The cells were cultured on cell culture inserts (Transwell®, Corning Inc., Corning, NY, USA) coated with Matrigel™ (BD Biosciences, San Jose, CA, USA) and treated with gabexate mesilate. **c.** The *in vitro* inhibitory effect of gabexate mesilate on TGF-beta1-induced invasiveness of human pancreatic cancer cells. The cells were cultured on cell culture inserts (Transwell®, Corning Inc., Corning, NY, USA) coated with Matrigel™ (BD Biosciences, San Jose, CA, USA) and treated with TGF-beta1, gabexate mesilate, or TGF-beta1 plus gabexate mesilate.

Conclusion

The process of pancreatic cancer cell metastasis has been shown to include multiple steps. In the progression of these steps, after detachment of cells from a primary tumor, tumor-cell invasion is necessary for infiltration into the blood vessels; invasion is also necessary for migration to a distant organ. There have been several reports that MMP-2, MMP-9, u-PA, and TAT play important roles in the progression of pancreatic cancer [5, 7, 13]. We suggested

Figure 5. a. The inhibitory effect of total TAT and TGF-beta1-induced TAT production by gabexate mesilate in vitro. Total TAT levels in control, gabexate mesilate treatment, TGF-beta1 treatment, or TGF-beta1 plus gabexate mesilate treatment culture supernatants with 1 μg/mL enterokinase were measured by the substrate assay. **b.** Inhibitory effect of the levels of u-PA by gabexate mesilate in vitro. The u-PA concentrations measured by the human u-PA ELISA kit include single-chain, two-chain, and both receptor and PAI-1-bound u-PA in the control, gabexate mesilate treatment, TGF-beta1 treatment, or TGF-beta1 plus gabexate mesilate treatment culture supernatants. **c.** Inhibitory effect of the production of MMP-2 by gabexate mesilate in vitro. The control, gabexate mesilate treatment, TGF-beta1 treatment, or TGF-beta1 plus gabexate mesilate treatment culture supernatants were measured for total production of MMP-2 by using the MMP-2 activity kit.

Table 2. Effect of gabexate mesilate (GM) on liver metastasis in nude mice.

Cell lines	Treatment	Liver metastasis in nude mice [a]	Number of liver metastases	Liver weight (g)
SW1990	Control group	4/4 (100%)	32±5.6	2.9±0.9
	GM treatment group	2/4 (50%)	0.75±0.96	1.5±0.2
			P<0.01	*P<0.01*
CAPAN-2	Control group	1/4 (25%)	1	1.4±0.2
	GM treatment group	0/4 (0%)	0	1.5±0.1
				P NS
PANC-1	Control group	0/4 (0%)	0	1.7±0.4

[a] Human pancreatic cancer cells in the form of a suspension having a final concentration of $7.5x10^6$ cells/mL in PBS were injected into the spleen of six-week-old female Balb/c nude mice under ether anesthesia. After injection, the spleen was extracted. 0.05 mg of GM was injected intra-peritoneally every day for 2 weeks. The mice were sacrificed under anesthesia after 4 weeks to measure the liver weight and the number of metastatic tumors in the liver. Data show mean±SD.

that the expression of TAT and u-PA were correlated with invasion and metastasis more than MMPs in pancreatic cancer.

Gabexate mesilate also inhibited the liver metastasis of SW1990 and CAPAM-2 cells in nude mice. The mechanism of the inhibition of liver metastasis was more complex in the *in vivo* model. We found that gabexate mesilate reduced the production of TGF-beta1 and VEGF and inhibited the activation of u-PA and TAT. Angiogenesis is important for tumor growth and metastasis. The formation of tumor microvessels is stimulated by angiogenic factors such as VEGF. VEGF is a 45-kDa glycoprotein which is mitogenic for endothelial cells. In the present study, we found that the production of VEGF in the culture supernatant of SW1990 cells was higher than that in the other two cell lines, and that only SW1990 originally revealed liver metastatic potential by *in vivo* splenic injection. Previous studies have shown that the overexpression of VEGF is correlated with the progression and hematogenous metastasis of pancreatic cancer [35, 37, 38]. Moreover, we had reported that TGF-beta1 up-regulated the production of VEGF in the pancreatic cancer cell lines [35]. Based on these results, we suggested that gabexate mesilate inhibited liver metastasis of pancreatic cancer cell lines in nude mice by inhibition of invasion and the production of growth factors such as TGF-beta1 and VEGF.

Our findings suggest that gabexate mesilate could inhibit the invasiveness, proliferation, and liver metastatic potential of pancreatic cancer cell lines by three mechanisms: 1) antagonizing the activities of TAT and u-PA; 2) decreasing the activation of PAR-2 and 3) suppressing the production of TGF-beta1 and VEGF. Gabexate mesilate has been used for the treatment of hyperenzymemia and acute pancreatitis in Japan, the Republic of Korea, Italy, and Taiwan. There has been no serious side effect reported, even when medication involved a high dosage of gabexate mesilate. These findings indicate that gabexate mesilate is potentially useful in the treatment of invasion, proliferation and metastasis of pancreatic cancer. Therefore, we believe it could be a useful therapeutic modality for anti-metastatic and anti-angiogenic treatment of pancreatic cancer.

Keywords Matrix Metalloproteinase 2; Pancreatic Neoplasms; Transforming Growth Factor beta; Trypsinogen; Urinary Plasminogen Activator; Vascular Endothelial Growth Factors

Abbreviations MT: membrane type; PAT: pancreatic acinar trypsinogen; TAT: tumor-associated trypsinogen; u-PA: urokinase-type plasminogen activator

Conflict of interest The authors have no potential conflicts of interest

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