Abstract. To clarify the potential involvement of plasmin(ogen) cascade proteins in the cell dissociation and subsequent invasion of pancreatic cancer cells, Western blot analysis, immunocytochemistry, immunohistochemistry, and in vitro invasion assay were performed in the cell lines or tissue of pancreatic cancer. The strong expression of plasmin(ogen), urokinase type plasminogen activator (uPA) and uPA receptor (uPAR), and apparently weak expression of the relevant proteins were found in the conditioned medium of dissociated (PC-1.0) and non-dissociated (PC-1) pancreatic cancer cells, respectively. Furthermore, uPA-treatment significantly induced the expression of plasmin(ogen) and uPAR in the conditioned medium of non-dissociated (PC-1) pancreatic cancer cells. Moreover, the expression of plasmin(ogen) and uPAR was stronger at the invasive front than at the center of human pancreatic cancer tissue. On the other hand, plasmin-treatment induced the expression of matrix metalloproteinase-2 (MMP-2), MMP-7 and MMP-9 in PC-1 cells. Simultaneously, plasmin-or uPA-treatments obviously induced the dissociation of cell colonies and in vitro invasiveness in PC-1 cells. The plasmin(ogen) cascade is closely involved in the invasion of pancreatic cancer cells and, especially in its early stage, cell dissociation. Targeting the plasmin(ogen) cascade may provide a new insight into molecular target therapy based on anti-invasion and anti-metastasis for pancreatic cancer.

Introduction

Exocrine pancreatic cancer is one of the most aggressive human tumors due to its high potential of local invasion and metastasis (1,2). Tumor invasion-metastasis has been known to be a complex multi-step process (3). However, thus far, the cellular and molecular mechanisms of the invasion-metastasis process of pancreatic cancer are not well elucidated. In our previous studies, two hamster pancreatic cancer cell lines with a different potential of invasion-metastasis (weakly invasive cell line, PC-1, and highly invasive cell line, PC-1.0) were established (4,5). In our recent investigations, activation of matrix metalloproteinase-7 (MMP-7) and the epidermal growth factor receptor (EGFR)-mediated mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signal transduction pathway have been found to be closely associated with the high potential of cell dissociation and subsequent invasion in pancreatic cancer cells (6-8). On the other hand, two families of secreted proteases, serine proteinases [essentially the plasmin(ogen) cascade] and matrix metalloproteinases (MMPs) are important for extracellular matrix (ECM) turnover. One of the most important physiological roles of plasmin is to activate MMP-7. There are two types of plasminogen activator, urokinase type plasminogen activator (uPA) and tissue type plasminogen activator (tPA). The activation of plasminogen to plasmin by tPA or uPA binding to its uPA receptor (uPAR) results in progressive degradation of extracellular matrix components and basement membrane and may also lead to the activation of metalloproteinases, latent growth factors, and proteolysis of membrane glycoproteins (9). All these processes may contribute to tumor invasion-metastasis. However, reports regarding the role of the plasmin(ogen) cascade in the early stage of the invasion-metastasis process (cell dissociation) of pancreatic cancer are few. Furthermore, the relationship between the plasmin(ogen) cascade and MMPs in the early stage of invasion-metastasis of pancreatic cancer is unclear. In this study, expression of the plasmin(ogen) cascade and MMPs in pancreatic cancer was examined to clarify the involvement of the plasmin(ogen) cascade and its relationship with MMPs in the cell dissociation and subsequent invasion of pancreatic cancer cells.

Materials and methods

Cell lines and cell culture. Hamster dissociated (PC-1.0) and non-dissociated (PC-1) pancreatic cancer cell lines were used. The PC-1 cell line was established from pancreatic ductal/ductular adenocarcinomas induced by BOP in a Syrian golden hamster (4). The PC-1.0 cell line was established from a subcutaneous tumor produced after the inoculation
of PC-1 cells (5). PC-1 cells grow as island-like colonies, whereas PC-1.0 cells exhibit a growth pattern of single cells.

The cells were incubated in RPMI-1640 (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO₂ to 95% air. The cells were serum-starved overnight before experiments.

Tissue samples. All of the tissue samples were obtained during surgery at the Department of Surgery II, Kumamoto University Hospital from October 1989 to July 2001. Specimens were from 37 pancreatic cancers. The median age of the patients with pancreatic cancer was 63.5 years (range, 35-78 years). These patients included 14 males and 23 females. Histologically, these consisted of 13 well-differentiated, 20 moderately differentiated, and 4 poorly differentiated adenocarcinomas. All of the tissue samples were histologically examined, and the pathological diagnoses were confirmed.

Antibodies. Murine against plasminogen/plasmin antibody (American Diagnostica, CT), rabbit anti-uPA antibody (Innovative Research, Southfield, MI), rabbit anti-uPAR antibody (American Diagnostica), and goat anti-MMP-2, -MMP-7, and -MMP-9 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used in this study. FITC labeled fluorescence secondary antibodies (Santa Cruz Biotechnology) were also used.

Preparation of cell lysate and condensation of conditioned medium. The PC-1.0 and PC-1 cells were grown in 90-mm dishes containing 10 ml of RPMI-1640 plus 10% fetal bovine serum. After growing to subconfluence, the medium was replaced with serum-free medium and incubated for 36 h. The PC-1 cells were treated with or without 1 μg/ml uPA (Innovative Research). The cell lysate and condensation of conditioned medium were prepared according to a previous study (6).

Western blot analysis. Western blotting was performed as described previously (6). In brief, samples of equivalent total protein (20 μg) were run in 7.5% polyacrylamide slab gels. Human plasminogen, plasmin (Innovative Research), uPA and uPAR positive control (American Diagnostica) were used as positive control.

Immunofluorescent staining and fluorescence intensity (FI) analysis. PC-1.0 and PC-1 cells were planted on the chamber...
slides and incubated before the experiment. The PC-1 cells were treated with or without 5 μg/ml plasmin for 36 h.

After incubation, immunofluorescent staining was performed as described previously (7). For examination of plasmin(ogen) expression on the plasma membrane, the cells were treated without Triton X-100. The control slides were prepared as follows: a) sections were processed without a primary antibody; b) normal goat or mouse serum and non-specific goat or mouse IgG were used instead of a primary antibody.

Finally, 6 cells in the image were chosen randomly to measure the fluorescence intensity (FI) with the software Fluoview 500 (version 4.3, Olympus, Japan). The averages were used for FI analysis of the expression of plasmin(ogen), MMP-2, MMP-7, and MMP-9.

\section*{In vitro invasion assay}

The \textit{in vitro} invasion assay was performed using Invasion Chambers (Becton Dickinson Labware, Bedford, MA) as described previously (6). The PC-1.0 and PC-1 cells were incubated for 12 h at 37˚C and PC-1 cells were pretreated with or without 5 μg/ml plasmin or 1 μg/ml uPA.

\section*{Immunohistochemical analysis}

Immunohistochemical staining was performed using the avidin-biotin-peroxidase complex technique (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) as described previously (7). The control slides were prepared as follows: a) sections were processed without a primary antibody; b) normal mouse or rabbit serum and non-specific mouse or rabbit IgG were used instead of a primary antibody.

\section*{Statistical analysis}

The average FI of the expressions of plasmin(ogen), MMP-2, MMP-7, and MMP-9 in different experimental groups, as well as the numbers of pancreatic cancer cells counted in the invasion assay were examined by unpaired Student’s t-test using the StatView computerized program (SAS Institute Inc., Cary, NC). A probability value <0.05 was considered significant.

\section*{Results}

Different expressions of plasmin(ogen) cascade proteins in dissociated (PC-1.0) and non-dissociated (PC-1) pancreatic cancer cells

The plasmin(ogen) expression in PC-1.0 and PC-1 cells. The results of Western blotting showed that the cellular expression of plasmin(ogen) in PC-1.0 was stronger than that in PC-1 cells. No apparent induction of cellular expression of plasmin(ogen) in PC-1 cells was observed after uPA-treatment (Fig. 1A). However, the cytoplasm localized expression of plasminogen was weak in both PC-1.0 (FI=7.5±1.8, Fig. 1B-C) and PC-1 cells (FI=8.0±1.1, P>0.05, Fig. 1B-D). The FI of plasmin(ogen) expression is shown in Fig. 1B-E.

In contrast to the cellular expression, the expression of plasminogen and plasmin in the conditioned medium detected by Western blotting was significantly different in PC-1.0 and PC-1 cells. A large amount of both plasminogen and plasmin proteins were detected in the conditioned medium of PC-1.0 cells, but were not detectable in that of PC-1 cells. However, uPA-treatment significantly induced the expression of plasminogen and plasmin protein in the conditioned medium of PC-1 cells.

The uPA expression in PC-1.0 and PC-1 cells. Western blotting analysis showed that the intracellular pro-uPA protein was weak, with no marked difference between PC-1.0 and PC-1 cells (Fig. 2A). On the contrary, more uPA protein was found in the conditioned medium of PC-1.0 cells than in that of PC-1 cells (Fig. 2B).
Figure 3. Expression of plasminogen cascade in human pancreatic cancer tissue. In non-malignant tissue, as shown by the black arrow, the expression of plasminogen was faint (A). The expression of uPAR (C) and uPA (E) was not detectable. On the other hand, overexpression of plasminogen (B), uPAR (D), and uPA (F) was observed in malignant tissue. Furthermore, the expression of plasminogen and uPAR was stronger at the invasive front (black arrow) than at the center (white arrow) of pancreatic cancer tissue.

Figure 4. Effects of plasminogen cascade proteins on the cell morphology, intracellular MMP expression, as well as in vitro invasiveness of pancreatic cancer cells. A), morphological changes induced by plasmin or uPA-treatment in non-dissociated cells (PC-1). The dissociated cells (PC-1.0) were growing as single cells (A), whereas non-dissociated cells (PC-1) were growing as island-like cell colonies (B). The cell colonies of PC-1 cells were obviously dissociated and the cells elongated and formed pseudopodia by plasmin-treatment (C) and uPA-treatment (D). Papanicolaou staining, original magnification x400. B), enhanced expression of MMP-2, MMP-7, and MMP-9 by plasmin-treatment in non-dissociated cells (PC-1). Constitutive expression of MMP-2 (A), MMP-7 (B), and MMP-9 (C) was observed in PC-1.0 cells. In contrast, the expression of MMP-2 (D), MMP-7 (E), and MMP-9 (F) in PC-1 cells was faint. However, the expression of MMP-2 (G), MMP-7 (H), and MMP-9 (I) in PC-1 cells was induced by plasmin-treatment. Immunofluorescent staining, original magnification x400. The FI of MMP-2, MMP-7, and MMP-9 expression is shown in (J). Black bars, PC-1.0 cells; white bars, PC-1 cells; grey bars, plasmin-treated PC-1 cells; S, significant. C), enhanced in vitro invasiveness of non-dissociated cells (PC-1) by plasmin or uPA-treatment. PC-1.0 cells present an obviously higher invasive ability than PC-1 cells. The plasmin-treatment or uPA-treatment markedly induced the invasiveness of PC-1 cells. I, PC-1.0 cells; II, PC-1 cells; III, plasmin-treated PC-1 cells; IV, uPA-treated PC-1 cells; S, significant.
The uPAR expression in PC-1.0 and PC-1 cells. The results of Western blotting showed no apparent differences in cellular uPAR expression between PC-1.0 and PC-1 cells. Moreover, the cellular uPAR expression in PC-1 cells was not significantly induced by uPA-treatment (Fig. 2C).

However, soluble uPAR (suPAR) protein was detected in the conditioned medium of PC-1.0 cells, whereas nearly no suPAR was detectable in the conditioned medium of PC-1 cells. In addition, the suPAR protein in the conditioned medium of PC-1 cells was significantly increased after uPA-treatment (Fig. 2D).

The expression of plasmin(ogen) cascade proteins in human pancreatic cancer tissue. In non-malignant tissue, faint immunostaining of plasmin(ogen) protein (Fig. 3A) and no immunostaining of uPAR (Fig. 3C) and uPA (Fig. 3E) proteins were observed in non-malignant pancreatic tissue. In pancreatic cancer tissue, the increased expression of plasmin(ogen) (Fig. 3B), uPAR (Fig. 3D) and uPA (Fig. 3F) was observed, both at the center (white arrow) and at the invasive front (black arrow). Furthermore, the expression of plasmin(ogen) and uPA at the invasive front was significantly stronger than that at the center of the same pancreatic cancer tissue (Fig. 3B and D, respectively).

Effects of plasmin(ogen) cascade proteins on the cell morphology, intracellular MMP expression, as well as in vitro invasiveness of pancreatic cancer cells

Induction of the dissociation of cell colonies in non-dissociated pancreatic cancer cells, PC-1, by plasmin or uPA-treatment.

The dissociated pancreatic cancer cells (PC-1.0) grew as single cells (Fig. 4A-A) whereas the non-dissociated pancreatic cancer cells (PC-1) grew as island-like cell colonies (Fig. 4A-C). Interestingly, the treatment with 5 μg/ml plasmin or 1 μg/ml uPA for 36 h obviously induced the dissociation of cell colonies of PC-1 cells (Fig. 4A-C and A-D).


In dissociated cells (PC-1.0), constitutive intracellular expression of MMP-2 (FI=15.6±4.3, Fig. 4B-A), MMP-7 (FI=19.2±5.2, Fig. 4B-B), and MMP-9 (FI=22.6±5.6, Fig. 4B-C) proteins was observed. However, the intracellular expression of MMP-2 (FI=3.5±1.7, P<0.05, Fig. 4B-D), MMP-7 (FI=11.5±2.9, P<0.05, Fig. 4B-E), and MMP-9 (FI=3.4±1.9, P<0.05, Fig. 4B-F) proteins in PC-1 cells was weak. In contrast, the intracellular expression of MMP-2 (FI=32.0±6.7, P<0.05, Fig. 4B-G), MMP-7 (FI=23.7±5.4, P<0.05, Fig. 4B-H), and MMP-9 (FI=18.6±4.9, P<0.05, Fig. 4B-I) was significantly induced in PC-1 cells by plasmin-treatment. The FI of MMP-2, MMP-7, and MMP-9 expression is shown in Fig. 4B-J.


As shown in Fig. 4C, dissociated cells (PC-1.0) exhibited a strong invasive capability (invasive cell number = 31.3±4.2). Contrarily, non-dissociated pancreatic cancer cells (PC-1) showed a weak invasive capability (invasive cell number = 10.7±3.1, P<0.01), but the invasive capability was significantly enhanced by 12-h plasmin-treatment (invasive cell number = 60.3±11.5, P<0.01) or uPA-treatment (invasive cell number = 32.7±7.4, P<0.01).

Discussion

Pericellular proteolysis plays a crucial role in tumor cell invasion (10). Plasmin acts both directly and indirectly (through activation of certain MMPs) to degrade proteins of the ECM and basement membrane, thereby facilitating ECM degradation, tumor proliferation, invasion and metastasis (11,12). In the current study, plasmin(ogen) was distinctly expressed in pancreatic cancer cells which have apparently different dissociation status and invasion abilities. A strong plasmin(ogen) expression was detected at the plasma membrane and in the conditioned medium of dissociated cells, which were growing as single cells and had a high invasion ability. On the contrary, the plasmin(ogen) expression was not detectable either at the plasma membrane or in the conditioned medium of non-dissociated cells, which were growing as island-like cell colonies and had a weak invasion ability. Moreover, plasmin-treatment significantly induced the cell dissociation and in vitro invasion ability of non-dissociated cells. In addition, the plasmin(ogen) protein was overexpressed in human pancreatic cancer tissue and a stronger plasmin(ogen) expression was observed at the invasive front than at the center of pancreatic cancer tissue. Collectively, the current results demonstrate that plasmin(ogen) is closely involved in the invasion-metastasis process of pancreatic cancer cells, especially in the process of cell dissociation, which is its first important step. The differently expressed plasmin(ogen) at the plasma membrane and in the conditioned medium rather than in the cytoplasm of pancreatic cancer cells may imply that the interaction of plasmin(ogen) with other molecules is essential for the activation and functioning of plasmin(ogen).

On the other hand, the basement membrane is composed mainly of type IV collagen, laminin, heparin sulfate proteoglycans, and entactin (13). Gelatinase A (MMP-2) and gelatinase B (MMP-9), and their balance in cancer cells is thought to be related to the invasiveness of the cells (14). In addition, MMP-7 positivity was reported to be significantly correlated with the extent of tumor invasion, lymph node and distant metastasis in pancreatic carcinoma (15). In the current study, the simultaneous induction of cell dissociation and expression of MMP-2, MMP-7, and MMP-9 by plasmin-treatment were observed in non-dissociated cells, PC-1. These results indicate that interactions between the plasmin(ogen) cascade and MMPs may cooperate in achieving extracellular matrix degradation. Furthermore, several active MMPs can activate other MMPs, thus representing a positive-feedback mechanism (16,17). As a result, cell dissociation and an enhanced invasiveness of pancreatic cancer cells are induced.

uPA, another important molecule in the plasmin(ogen) cascade, was also found to be differently expressed in the conditioned medium of dissociated and non-dissociated pancreatic cancer cells. uPA-treatment induced the plasmin(ogen) expression, cell dissociation, as well as invasiveness of non-dissociated cells. However, tPA, another plasminogen activator, showed no difference in expression
between the cytoplasm or conditioned medium of dissociated and non-dissociated cells in the preliminary experiment (data not shown). Although tPA can also convert plasminogen to plasmin, it is mainly reported to be involved in fibrinolysis (18). These results suggest that it may be uPA which serves as a plasminogen activator in the cell dissociation and subsequent invasion-metastasis process of pancreatic cancer cells.

Compared with cellular localized uPAR, the uPAR present in body fluids (soluble uPAR, suPAR) was reported to be a more important prognostic marker (19,20). Although no obvious difference in uPAR expression was observed between the cytoplasm of dissociated and non-dissociated cells, more suPAR protein was found in the conditioned medium of dissociated cells than in the conditioned medium of non-dissociated cells in this study. Furthermore, the suPAR in the conditioned medium of non-dissociated cells was markedly increased by uPA-treatment. The uPAR protein was also found to be overexpressed in human pancreatic cancer tissue and the expression at the invasive front was stronger than that at the center of the pancreatic cancer tissue. Hence, all these data may indicate that uPAR is also one of the important molecules involved in the invasion-metastasis process of pancreatic cancer. However, because no difference in expression of uPA was found between the invasive front and the center of pancreatic cancer tissue, the different expression patterns of uPAR and uPA in pancreatic cancer tissue imply that, besides binding to uPA, uPAR may play an independent role in the invasion-metastasis of pancreatic cancer.

In summary, the plasmin(ogen) cascade is closely involved in the cell dissociation and subsequent invasion of pancreatic cancer cells. Production, secretion, and activation of plasminogen, rather than its activators uPA and tPA, possibly serves as a rate-limited step in the cell dissociation and subsequent invasion of pancreatic cancer. Targeting the invasive properties of pancreatic cancer, such as the plasminogen(ogen) cascade, may provide a new therapeutic strategy for the anti-tumor therapy of pancreatic cancer.

References