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Increased Endothelial Cell Retraction and Tumor Cell Invasion by Soluble Factors Derived from Pancreatic Cancer Cells

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Background: Tumor cells induce endothelial cell retraction before invasion. In pancreatic cancer cells, the factors affecting endothelial cell retraction are not well-understood.

Methods: The activities of the endothelial cell retraction in conditioned media (CM) derived from three human pancreatic cancer cell lines, PSN-1, MiaPaca-2, and Capan-1, were measured for the amount of intercellular junctional transport of FITC dextran through an endothelial cell monolayer in a transwell cell culture system.

Results: The CM derived from the three pancreatic cancer cells induced endothelial cell retraction. The endothelial cell retraction activity in the CM from PSN-1 cells was significantly higher than those from MiaPaca-2 and Capan-1 cells. The CM from PSN-1 cells enhanced both the adhesion and the invasion of MiaPaca-2 and Capan-1 cells. The factors with endothelial cell retraction activity in the CM from PSN-1 cells were characterized as heat-stable, trypsin-sensitive glycoproteins ranging from 10,000 to 50,000 in molecular weight, and were found both in heparin-bound and unbound fractions.

Conclusions: PSN-1 cells produced and secreted at least two factors inducing the endothelial cell retraction. The factors could play an important role in the establishment of invasion and metastasis of PSN-1 cells.

Key Words: Invasion—Metastasis—Endothelial cell retraction—Pancreatic cancer.

Pancreatic cancer is the fourth or fifth leading cause of cancer death in the Western world and the incidence seems to be increasing (1,2). Although only surgical resection offers the patients an opportunity to live longer and to be possibly cured, only 15–20% of the patients with pancreatic cancer have a resectable tumor at the time of diagnosis. Despite the recent advances in the diagnostic and therapeutic modalities, the prognosis of the patients with adenocarcinoma of the pancreas is poor. Complete removal of macroscopically detectable cancer tissues does not prevent early tumor recurrence (3,4). Such recurrence probably arises from growth of occult cancer cells that had already invaded and metastasized out of the surgical region by the time of surgery (5–7). Therefore, understanding the biological basis of the aggressiveness, such as invasive and metastatic potential, in pancreatic cancer is important and could allow for better therapeutic approaches.

Tumor cell invasion and metastasis are a complex process involving several distinct, but essential steps. The critical steps are: escape from the primary tumor, dissemination through the circulation, lodgment in small vessels at distant sites, penetration through vessel walls, and growth in new sites as a secondary tumors (8–10). During this process, one of the most characteristic processes is the tumor cell migration through the endothelial cell layer (11). Although the integrity of the endothelium could play an important role as a barrier against tumor cell invasion (12,13), most studies, both in vivo and in vitro, have demonstrated that vascular endothelial cells retract before tumor cell intravasation and extravasation

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and that tumor cells invade through the interendothelial junctions (14–17). Furthermore, we previously demonstrated that retraction of mesothelial cells or endothelial cells was induced by the attachment of tumor cells or the addition of their conditioned medium (18,19). These studies suggest not only the involvement of endothelial or mesothelial cell retraction but also the importance of the interaction between tumor cell and endothelial or mesothelial cells in the establishment of tumor cell invasion and metastasis.

In this study, we have focused our attention on endothelial cell retraction during a tumor cell invasion and have further examined whether pancreatic cancer cell produces the factor(s) that can stimulate endothelial cell retraction in vitro.

MATERIALS AND METHODS

Cells and Cell Cultures

Human pancreatic cancer cell line PSN-1 (20) was a gift of Genetics Division, National Cancer Research Institute, Japan, and MiaPaca-2 and Capan-1 were obtained from the Japanese Cancer Research Resources Bank (Setagaya, Japan). The three pancreatic cancer cell lines were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% carbon dioxide. Calf pulmonary arterial endothelial (CPAE) cells were obtained from the Japanese Cancer Research Resources Bank. CPAE cells were cultured in a medium containing a 1:1 ratio of Dulbecco-modified minimal essential medium and Ham's nutrient mixture F12 (DME/F12; Sigma, St. Louis, MO, USA) supplemented with 20% FCS at 37°C in a humidified atmosphere with 5% carbon dioxide.

Preparation of Conditioned Media

The conditioned media (CM) were prepared from the cultures of pancreatic cancer cells in a serum-free RPMI 1640 medium at 37°C for 48 h. Briefly, 1×10^6 tumor cells were seeded on 100-mm plastic culture dishes and cultured for 3 to 4 days at 37°C in RPMI 1640 medium supplemented with 10% FCS. After the cells were washed three times with phosphate-buffered saline (PBS) to remove the serum components, they were incubated with 10 ml serum-free RPMI 1640 for 48 h. The culture medium was collected and centrifuged at 3,000 rpm for 10 min. The collected supernatant was dialyzed against DME/F12 medium for 24 h at 4°C and concentrated on a Diaflo ultrafiltration membrane (Amicon, Beverly, MA, USA). The concentrated CM was dialyzed against PBS for 24 h at 4°C. The protein amounts of the concentrated CM were, determined by Coomassie blue plus protein assay regent (Pierce, Rockford, IL, USA). After the concentrated CM was adjusted to 1 mg/ml by adding PBS, it was subjected to assays and biochemical characterizations. In some experiments, tumor cells were cultured with cycloheximide, benzyl-*N*-acetyl-a-*D*-galactosaminide (Bzl-GalNac), or swansonine for 48 h. After dialysis against DME/F12 medium for 24 h at 4°C, the collected media were processed and used for assays.

Endothelial Cell Retraction Assay

The extent of endothelial cell retraction was measured as the amount of fluorescein isothiocyanate (FITC) labeled dextran (average molecular weight, 70,000) (Sigma) that passed across an endothelial cell monolayer as previously described (21,22) with some modifications (Fig. 1). Briefly, the CPAE cells were cultured to form a monolayer for 3 days on the polycarbonate membranes with a 0.4-μm pore size coated with the reconstituted basement membrane Matrigel (Becton Dickinson, Bedford, MA, USA) of each upper chamber of the transwell chamber (Costar, Cambridge, MA, USA). After the removal of the culture medium, 250 μl of the fresh culture medium containing 1 mg/ml of FITC dextran with and without CM was added into the each upper transwell chamber. The lower transwell chamber was filled with 1

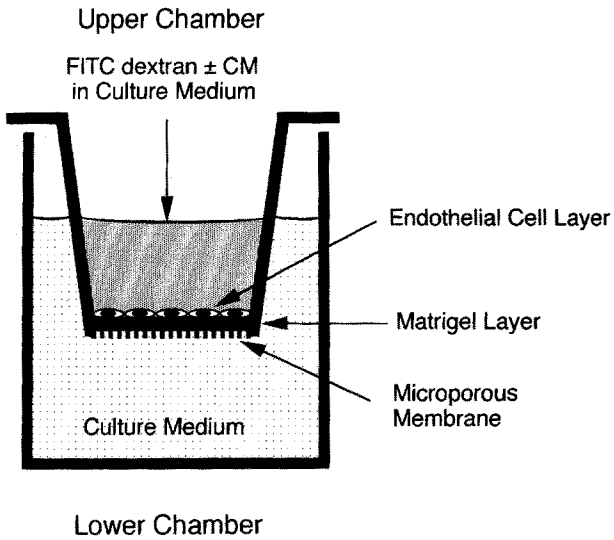

FIG. 1. A schematic representation of the transwell chamber assay for measuring the endothelial cell retraction activity of the CM derived from tumor cells. If CM are capable of retracting endothelial cells and exposing interendothelial spaces, the amount of diffusion of FITC dextran from the upper to the lower chamber should be substantially increased.

ml of the same medium without FITC dextran and the transwells were cultured at 37°C for a given period. Following culture, the amount of FITC dextran in the lower chamber was quantified by Amicon Scan Control SPF-500 (Amicon) under excitation and emission wavelengths of 490 nm and 530 nm, respectively. The concentration of FITC dextran in the lower chamber was calculated from the relative fluorescence intensity against 1 mg/ml FITC dextran in culture medium. Each assay was performed in triplicate. The endothelial cell retraction activity was represented by the mean concentration of FITC dextran in the lower chamber.

Adhesion Assay

The confluent CPAE cell monolayer was incubated with and without CM for 5 h at 37°C prior to the assay. Cultured tumor cells, detached from culture dishes by treatment with a mixture of 0.02% EDTA and 0.05% trypsin, were suspended in RPMI 1640 medium containing 10% FCS at a concentration of 1×10^7 cell/ml. The suspended tumor cells were labeled by incubating with a fluorescence probe, 3′-*O*-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxylfluorescin diacetoxymethylester (BCECF-AM) at 37°C for 30 min and then rinsed to remove excess BCECF-AM. Labeled tumor cells at a concentration of 2×10^5 cells/ml were placed on the CPAE cell monolayer in 12-well tissue culture plate and incubated for 1 h at 37°C. After nonadherent cells were removed by aspiration and a gentle rinse with PBS, the monolayer was lysed in 1% Triton X-100 at room temperature. The fluorescence intensity of the lysate was measured under excitation and emission wavelengths of 490 nm and 530 nm, respectively.

In Vitro Transcellular Invasion Assay

The invasive ability of tumor cells into the endothelial cell monolayer was measured as the transcellular migration activity of the tumor cells by the method previously described (18,19) with some modifications. Briefly, CPAE cells were grown to confluence on a 35-mm plastic culture dish coated by Matrigel. After rinsing once with the culture medium, the CPAE cell monolayer was incubated with and without the CM for 5 h at 37°C. After the CPAE cell monolayer was rinsed with the culture medium, tumor cells (5×10^4 cells/dish) were seeded onto it and incubated for 16 h at 37°C. After nonadherent cells were removed by aspiration, the remaining cell layer was fixed in 10% formalin in PBS. The number of penetrated single tumor cells and tumor cell colonies formed under the CPAE cell monolayer in 96 different visual fields (1.13 mm^2 each) was counted under a phase-contrast microscope. Activity of in vitro transcellular invasion was expressed as the penetrated single tumor cells and colonies per square centimeter.

Scanning Electron Microscopy

CPAE cell monolayers were carefully washed with PBS at 37°C by aspiration and fixed by 1.5% glutaraldehyde in PBS for 10 min at 37°C. After postfixation in 1% osmium tetroxide/1 m*M* $CaCl_2$/0.1 *M* sodium phosphate buffer (pH 7.2) for 0.5 h at room temperature, the monolayers were dehydrated through a graded series of ethanol and transferred to Freon 113. After they were coated with 50–100Å of gold/palladium, the sample was observed in a SM-LBG40 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Characterization of CM Derived from PSN-1 Cells

The CM was sequentially fractionated with ultrafiltration membranes XM300 (molecular weight cutoff, 300,000; Amicon), YM100 (molecular weight cutoff, 100,000; Amicon), and XM50 (molecular weight cutoff, 50,000; Amicon). These fractions were finally concentrated by Centriprep-10 (molecular weight cutoff, 10,000; Amicon). We measured the activity of the endothelial cell retraction in each fraction.

The fractionated CM that retained the most endothelial cell retraction activity was applied to a heparin–Sepharose column (Pharmacia, Piscataway, NJ, USA) equilibrated in PBS. The bound material was eluted with a linear gradient of 0–2 *M* NaCl. Fractions containing unbound and bound material were tested for their ability to induce endothelial cell retraction.

RESULTS

To confirm whether an endothelial cell monolayer functions as a barrier for the diffusion of FITC dextran through the membrane, we first measured the amount of FITC dextran in the lower chamber which passed through a transwell membrane without or covered with an endothelial cell monolayer. The amount of FITC dextran passing through the membrane covered with an endothelial cell monolayer was significantly lower than that without the monolayer (Fig. 2). Despite the endothelial cell monolayer covering the membrane, addition of the CM derived from the three pancreatic cells at a concentration of 50 μg/ml in the upper transwell chambers caused increases in the concentration of FITC dextran in the lower chambers in a time-dependent manner (see Fig. 2). The effect was also dose dependent and

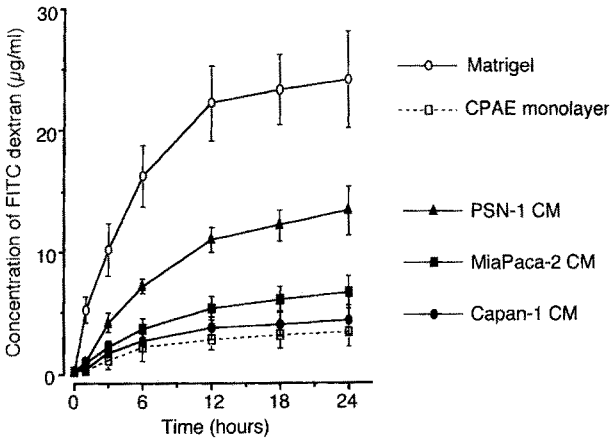

FIG. 2. Time course study of the endothelial cell retraction. Matrigel-coated polycarbonate membrane only is indicated by open circles, solid lines. CPAE cell monolayer on a Matrigel-coated polycarbonate membrane is indicated by open squares, dotted lines. Addition of CM (50 μg/ml) derived from PSN-1 (closed triangles) cells, MiaPaca-2 cells (closed squares), and Capan-1 cells (closed circles) on CPAE cells monolayer is indicated. Values are mean ± SD from triplicate experiments.

reversible (data not shown). The endothelial cell retraction activity of the CM derived from PSN-1 cells was significantly higher than that derive from MiaPaca-2 and Capan-1 (see Fig. 2).

Scanning electron microscopy confirmed that the treatment of the CM induced the endothelial cell retraction, resulting in slight thickening of the endothelial cells, irregularity of the cell borders, and increased interendothelial spaces and exposed regions of underlying extracellular matrix (Fig. 3).

To evaluate the contribution of increased endothelial cell retraction on tumor cell invasion through the endothelial cell monolayer, we examined the invasive abilities of tumor cells, which were measured as number of tumor cells and colonies under the endothelial cell monolayer (18,19). The invasion ability of PSN-1 cells was the strongest among the three pancreatic cancer cells, just as they showed the strongest endothelial cell retraction activity in their CM. Furthermore, the CM derived from PSN-1 cells increased the numbers of MiaPaca-2 and Capan-1 cells adhering to and invading the endothelial monolayers (Fig. 4). However, although CMs derived from MiaPaca-2 and Capan-1 cells also increased the invasion of PSN-1 cells, the extent of the increased invasion was lower than that seen with the CM derived from PSN-1 cells.

We next performed a preliminary characterization of the factor(s) inducing endothelial cell retraction. Addition of the CM did not change the growth rates either of the CPAE or pancreatic cancer cells. The activity in the CM was retained after freezing and thawing, or heating at 80°C for 20 min. Treatment of the CM derived from PSN-1 cells with trypsin (500 μg/ml) at 37°C for 1 h decreased the endothelial cell retraction activity. Furthermore, pretreatment of PSN-1 cells by cycloheximide, which is known to block protein synthesis, reduced the activity in the CM to less than 50% of the untreated control. Pretreatment of PSN-1 cells with Bzl-GalNac, an *O*-linked carbohydrate chain processing inhibitor (23), also reduced the endothelial cell retraction activity in the CM, whereas swansonine, an *N*-linked carbohydrate chain processing inhibitor (24), did not (Fig. 5), thus suggesting that the factor(s) inducing endothelial cell retraction are likely to be *O*-linked glycoproteins.

In order to define the molecular weight of the factors with endothelial cell retraction stimulatory activity, the CM derived from PSN-1 cells was sequentially fractionated with ultrafiltration membranes. More than 80% of the activity was retained in the fraction of the molecular weight range from 10,000 to 50,000 (see Fig. 5). Furthermore, since it has recently been described that endo-

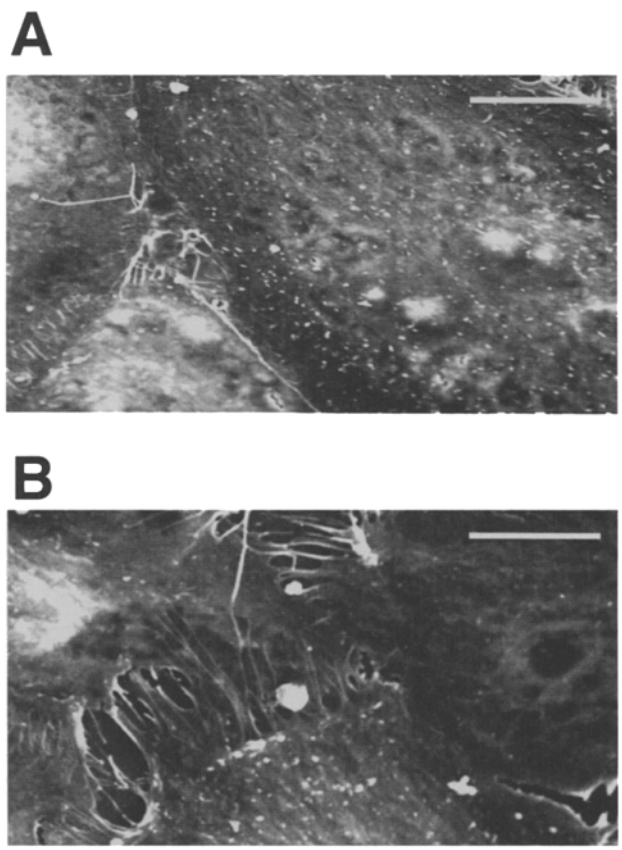

FIG. 3. Scanning electron microscopy of the endothelial cell retraction. CPAE cell monolayer was incubated without (A) or with (B) the CM (50 μg/ml) derived from PSN-1 cells for 5 h. Bars indicate 10 μm.

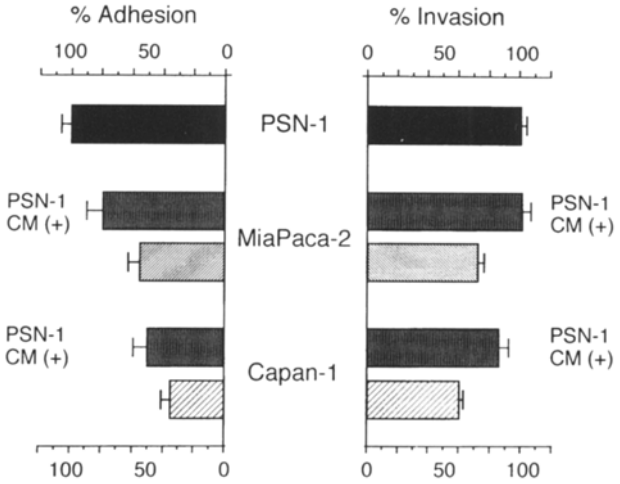

FIG. 4. The effect of CM (50 μg/ml) from PSN-1 on adhesion and invasion of MiaPaca-2 and Capan-1 cells to the CPAE cell monolayer. The CPAE cell monolayers were incubated in the absence and presence of CM from PSN-1 for 5 h prior to the adhesion and invasion experiments. The number of cells adhering to the CPAE cell monolayer after 1 h of incubation and the number of penetrated cells/colonies under the CPAE cell monolayer after 16 h of incubation were expressed as percentage compared with those of PSN-1.

thelial cell injury and basement membrane damage by proteases produced by tumor cells lead to endothelial cell retraction and invasion (25,26), we investigated whether proteases affect the endothelial cell retraction activity in

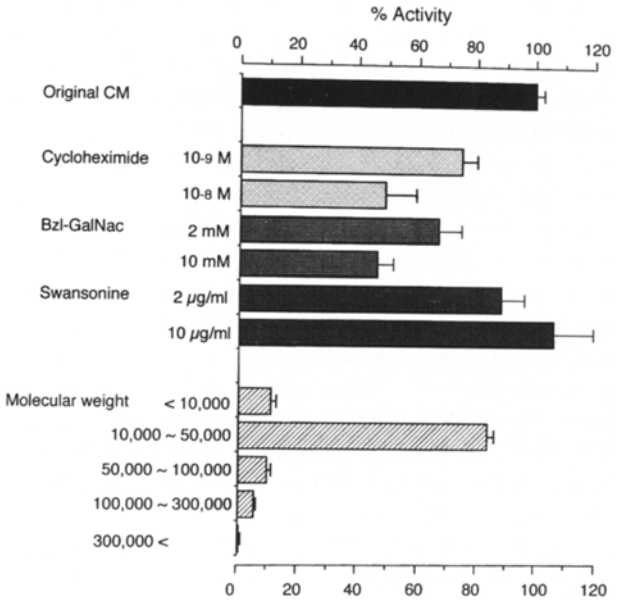

FIG. 5. The effect of protein synthesis and processing inhibitors on the endothelial cell retraction-stimulatory activity in the CM derived from PSN-1, and molecular weight size distribution of the endothelial cell retraction-stimulatory activity. Treated and fractionated CM at the equivalent dilution was subjected to assay. The endothelial cell retraction activity was expressed as zero in the absence of CM and as 100% in the presence of the original CM.

TABLE 1. *Effect of protease inhibitors on endothelial cell retraction*

Protease inhibitor	Dose	% Activity[a]
TIMP	1 μg/ml	106.3 ± 7.4
Aprothinin	10^{-7} *M*	98.3 ± 6.7
	10^{-6} *M*	104.3 ± 5.9
Leupeptin	10^{-5} *M*	89.8 ± 6.5
	10^{-4} *M*	118.4 ± 5.0
E-64[b]	10^{-5} *M*	115.8 ± 15.0
	10^{-4} *M*	89.1 ± 8.9
Gabexate mesilate	10^{-6} *M*	92.6 ± 13.1
	10^{-5} *M*	98.3 ± 6.9
Pepstatin	10^{-5} *M*	115.8 ± 2.0

[a] Endothelial cell retraction activity is expressed as mean percentage ± SD compared with the activity of the CM only.
[b] *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane.

the CM. The endothelial cell retraction in the fraction of the molecular weight range 10,000–50,000, however, was not inhibited by the six protease inhibitors examined (Table 1).

To further purify the CM, the fractionated CM in the molecular weight range 10,000–50,000 was applied to a heparin–Sepharose column. The collected unbound fractions and the bound fractions eluted with a linear gradient of 0 to 2 *M* NaCl were tested in an endothelial cell retraction assay. We observed two peaks with activity, one corresponding to heparin-unbound fractions and the other corresponding to heparin-bound fractions eluted by about 0.8 *M* NaCl (Fig. 6A). When the aliquot of the collected fractions in the heparin-bound peak were analyzed by SDS-PAGE, we found a single silver-stained components of molecular weight ~28,000 and additional faint component (see Fig. 6B). More than ten compo-

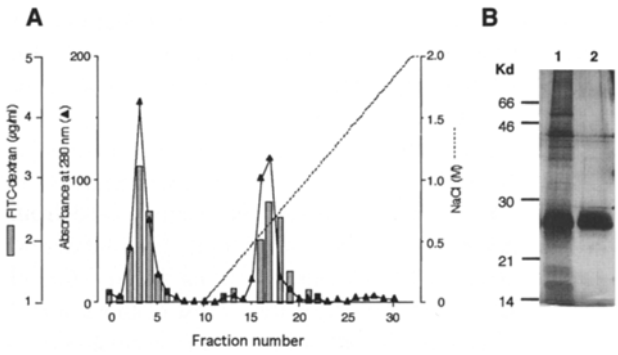

FIG. 6. Partial purification of the CM derived from PSN-1 cells. (**A**) Heparin–Sepharose column chromatography of the fraction in the molecular weight range 10,000–50,000 after ultrafractionation, indicating endothelial cell retraction activity (*bars*), absorbance at 280 nm (*closed triangles and solid line*), and NaCl concentration (*dotted line*). (**B**) SDS–polyacrylamide gel electrophoresis and silver staining of the fraction in the molecular weight range 10,000–50,000 (lane 1) and the active pool from fractions 17 and 18 in the heparin–Sepharose column chromatography (lane 2).

nents were, however, found in the heparin-unbound fractions (data not shown).

DISCUSSION

In this study we addressed the hypothesis that human pancreatic cancer cells produce and secrete factors that stimulate endothelial cell retraction, which could contribute to the establishment of tumor cell and host cell microenvironments that enhanced tumor cell invasion and metastasis in vivo. The present findings clearly demonstrated that this was the case in vitro. First, an endothelial cell monolayer functioned as a barrier against the diffusion of macromolecules such as dextran. This barrier function was disrupted to varying degrees by the endothelial cell retraction induced by the addition of CM derived from three pancreatic cancer cells. Second, among the three pancreatic cancer cells, the CM derived from PSN-1 cells had the strongest influence on endothelial cell retraction. Third, the CM of the PSN-1 cells increased the adhesion and invasion of the two remaining pancreatic cancer cells, suggesting that the increased endothelial cell retraction was closely correlated with the enhancement of the adhesion of the tumor cells to the endothelial cell monolayer and also with their invasion. Since it has been already proposed that metastatic tumor cells adhere preferentially to the extracellular matrix underlying endothelial cells (15,27), increased interendothelial junction spaces induced by endothelial cell retraction could result in an increased exposure of the extracellular matrix. This would ultimately enhance adhesion and invasion of the tumor cells. These findings also suggested that since the CM influence neither growth nor motility of the pancreatic cancer cells, the CM derived from PSN-1 cells should affect the endothelial cells in either a paracrine or endocrine manner and establish a preferential microenvironment for tumor cell invasion and metastasis.

A variety of factors that stimulate endothelial cell retraction in vivo and in vitro have already been documented (28,29). These include vasoactive substances (e.g., histamine, bradykinin, serotonin), lipid mediators (e.g., leukotriene C_4, platelet-activating factor), thrombin, cytokines, and growth factors. Most of these stimulants have been implicated in increasing vascular permeability and facilitating leukocyte transmigration through the endothelial cell monolayers during inflammation. Although tumor cell extravasation and leukocyte emigration share many phenotypic similarities on the basis of cell–cell interactions, the tumor cell-derived factors responsible for endothelial cell retraction still remain obscure. In this study, we provided evidence that human pancreatic cancer cells PSN-1 produce and secrete factors that stimulate endothelial cell retraction. The factors were heat-stable, ranged in molecular weight from 10,000 to 50,000, and consist of at least two different molecules: heparin-bound and unbound. The endothelial cell retraction activity in the CM was reduced by pretreatment of PSN-1 cells with either a protein synthesis inhibitor, cycloheximide, or an *O*-linked carbohydrate chain processing inhibitor, Bzl-GalNac, but was not inhibited by the pretreatment with swansonine, an *N*-linked carbohydrate chain processing inhibitor. Therefore, the factors are more likely to be glycoproteins in which *O*-linked glycosylation may play an important role in the activity. Furthermore, although proteases produced by tumor cells lead to endothelial cell retraction and invasion (25,26), the present findings suggested that the factors with endothelial cell retraction-stimulating activity were not likely to possess the protease-like activity.

There are only a few reports about the factor derived from tumor cells that induces endothelial cell retraction. Honn et al. (30) reported that tumor cell-derived 12(*S*)-hydroxyeicosatetraenoic acid [12(*S*)-HETE] induced endothelial cell retraction. They, however, did not show any direct correlation of the endothelial cell retraction activity with tumor cell invasion. Unlike the endothelial cell retraction-stimulating factors proposed in this study, 12(*S*)-HETE is a lipoxygenase metabolite of arachidolic acid. It is synthesized in response to the adhesion of tumor cells on endothelial cells and is not detectable in the culture medium (30,31). Although further investigations will be necessary to clarify the relationship, if any, between these two molecules that participate in endothelial cell retraction, the factors in this study clearly differ from 12(*S*)-HETE. Furthermore, a few growth factors and cytokines, including vascular endothelial growth factor/vascular permeability factor (VEGF/VPE) (32,33), tumor necrosis factor (TNF) (34), and interleukin-6 (IL-6) (22), have been reported to increase endothelial cell permeability, probably due to physical endothelial cell retraction. However, unlike VEGF/VPF, the factors derived from PSN-1 cells did not have any growth-stimulatory activity for endothelial cells. No immunoreactive VEGF/VPE and TNF were detected either in the heparin-bound or unbound fractions in the CM, as assessed by immunoblot assay, and antibodies against VEGF/VPE, TNF, and IL-6 did not block the endothelial cell retraction activity of the factors (data not shown). Although we found a faint band in the heparin-unbound fractions by immunoblot assay with an anti-IL-6 antibody, the same level of purified IL-6 as that in the CM was too low to induce the endothelial cell retraction (unpublished data). Thus, the present findings suggest that

the factors with endothelial cell retraction-stimulating activity derived from PSN-1 cells are not identical with VEGF/VPE, TNF, and IL-6.

Although the factors shown in this study have not been identified yet, because invasive pancreatic cancer cells such as PSN-1 cells produce and secrete the factors inducing endothelial cell retraction, compounds that inhibit the function of the factors may have clinical applications as antiinvasive and antimetastatic agents. Alternatively, since determination of the activity to induce endothelial cell retraction could indicate the aggressiveness of the tumor cells, measurement of the factors in a patient's blood, if possible, may serve as prognostic determinants in patient selection. Since the value of surgery in all patients who are thought to have potentially resectable pancreatic cancer has been questioned, and the need to identify and to appropriately treat patients in whom the cancer's biologic behavior offers a substantial chance for long-term survival after resection has been emphasized (3,35), such determinants may have a significant impact on prognostic evaluation of patients and hence on a better therapeutic approach in terms of treatment cost and quality of life.

In conclusion, the results of this in vitro study indicate that human pancreatic PSN-1 cells produce and secrete the factors with endothelial cell retraction-stimulatory activity and that the factors could play important role in the establishment of tumor invasion and metastasis. Further efforts to identify the molecules and mechanisms involved in endothelial cell retraction would bring better understanding of the biological basis of the aggressiveness in pancreatic cancer and could allow oncologists better therapeutic approaches.

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