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Expression of Thrombospondin-1 in Human Pancreatic Adenocarcinomas: Role in Matrix Metalloproteinase-9 Production*

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Human pancreatic adenocarcinoma, an aggressive malignant disease, shows a strong desmoplastic reaction characterized by a remarkable proliferation of interstitial connective tissues. Thrombospondin-1 (TSP-1), a 450 kDa platelet and matrix glycoprotein, has been implicated in tumor invasion, angiogenesis and metastasis. TSP-1 and MMP-9 expression in pancreatic adenocarcinoma and control pancreas tissues was measured by immunohistochemistry. TSP-1 expression in pancreatic carcinoma cell lines, fibroblasts, and endothelial cells was measured by a competitive TSP-1 enzyme linked immunosorbent assay (ELISA). The effect of TSP-1 on MMP-9 production in pancreatic carcinoma cell lines was measured by zymography and Western blot analysis. Eighty five per cent (23/27) of cases of pancreatic adenocarcinoma showed increased TSP-1 staining in the desmoplastic stroma adjacent to tumor cells. No specific positive staining for TSP-1 was observed in the normal pancreatic tissues and the inflammatory areas. TSP-1 localized in tumor stroma surrounding the tumor cells expressing MMP-9. Using TSP-1 competitive ELISA, the secretion of TSP-1 by different pancreatic cancer cell lines into culture medium varied from 11.45 ± 14.08 to 275.82 ± 45.56 ng/10^6 cells/24 hours. The amounts of TSP-1 detected in both culture media and cell extracts from fibroblasts or endothelial cells were at least 2-3 fold higher than those from pancreatic cancer cells. TSP-1 augmented the production of matrix metalloproteinase-9, a matrix degrading enzyme, in pancreatic cancer cells *in vitro*. Stromally-derived TSP-1 up-regulates the production of MMP-9 by pancreatic adenocarcinoma. These data are consistent with the conclusion that TSP-1-rich stroma is involved in regulating matrix remodeling in tumor invasion. (Pathology Oncology Research Vol 7, No 4, 251–259, 2001)

Keywords: extracellular matrix; thrombospondin-1, matrix metalloproteinase; pancreatic cancer, tumor invasion, tissue inhibitor of metalloproteinase

Introduction

Pancreatic adenocarcinoma is one of the leading causes of cancer death in the industrialized societies. It is usually diagnosed when liver metastasis, vascular or perineural invasions are already present in most of the patients.[1] Although substantial progress has recently been made in our understanding of the molecular basis of pancreatic cancer, such as activating mutations of the K-*ras* oncogene,[2] microsatellite instability,[3] and abnormalities of several tumor suppresser genes including p53, p16/MTS-1, DPC and DCC,[4-6] the information about the factors involved in the invasion and metastasis of pancreatic cancer is so far limited.

The interactions between tumor cells and the surrounding stroma may play an important role in the progression of adenocarcinoma of the pancreas, which is characterized by a strong desmoplastic reaction.[7] The different components of the extracellular matrix are capable of modulating tumor cell growth, motility, differentiation, and gene expression.[8] One of the most notable extracellular macromolecules involved in tumor invasion and metastasis is thrombospondin-1 (TSP-1). TSP-1, a 450 kDa glycoprotein, has been repeatedly found to be present in increased levels in the stroma of invasive human breast carcinoma.[9,10] Accumulating evidence suggests that TSP-1 functions as a modulator in tumor invasion and angiogenesis.[11,12] Recent studies from our laboratory have shown that TSP-1 promotes breast tumor cell invasion both *in vitro* and *in vivo*.[13] In

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addition, we have discovered that TSP-1 can regulate the production of matrix metalloproteinase-9 (MMP-9 or gelatinase B) in endothelial cells[14] and the urokinase plasminogen activator (uPA)/plasminogen activator inhibitor (PAI-1) system in breast cancer cells.[15] These observations strongly suggest that TSP-1 may play a crucial role in proteolytic degradation of the extracellular matrix, an essential process during tumor invasion and metastasis.

Matrix metalloproteinases (MMPs) represent a family of zinc containing enzymes, which degrade extracellular matrix components, and are believed to play a central role in tumor cell invasion.[16] Currently, the MMP family includes three interstitial collagenases (MMP-1, 8, and 13), three stromelysins (MMP-3, 10, and 11), a small putative uterus metalloproteinase (PUMP-1, or MMP-7, or matrilysin), two type IV collagenases (gelatinases) in 72- and 92 kDa forms (MMP-2, and 9), a metalloelastase (MMP-12) and 3 newly described membrane-bound metalloproteinases (MMP-14, 15, and 16) . The activity of MMP is tightly regulated by gene expression as well as by proenzyme activation and the association with specific tissue inhibitors. MMP-9 is one of the two type IV collagenases of the MMP family and is capable of cleaving a wide range of extracellular matrix components, including denatured collagens (gelatins), native type I, III, IV , V, XI collagens, elastin, laminin A chain and myelin basic protein.[17,18] The expression of MMP-9 correlates well with metastasis in several tumor systems. For example, inhibition of MMP-9 expression by a ribozyme in rat sarcoma cells blocked their lung colonization. This result suggests a requirement for MMP-9 expression in metastasis in this tumor system.[19] MMP-9 gene expression *in vivo* localizes to both cancer cells and surrounding stromal cells in several cancers including pancreatic cancer suggesting the contribution of stromal cells in the regulation of proteolytic activity during tumor progression. Using a tumor-stromal cell co-culture model, several *in vitro* studies have demonstrated that the expression of MMP-9, but not other members of the MMP family, was induced in tumor cells such as squamous cell carcinoma cells by fibroblasts,[20] and *vice versa.*[21] However, the inducing factors have not been characterized. In this study, we show that the production of MMP-9 in human pancreatic cancer cells is augmented by the treatment of TSP-1, and TSP-1 is highly expressed in the desmoplastic stroma surrounding the tumor cells in human pancreatic cancer.

Materials and Methods

Antibodies

Goat anti-human TSP-1 used in immunohistochemistry and rabbit anti-human TSP-1 used in ELISA were characterized elsewhere.[9,22] Mouse monoclonal anti-human TSP-1 IgG (P10) for neutralizing experiments was purchased from Chemicon (Temecula, CA). The other antibodies used in immunohistochemistry and Western blot analysis were obtained from the following sources: sheep anti-human MMP-9 IgG from the Binding Site Co. (Birmingham, England); monoclonal anti-human MMP-9 IgG from Oncogene Research Products, Calbiochem (Cambridge, MA); polyclonal rabbit anti-human TIMP-1 from Triple Points (Forest Grove, OR); horseradish peroxidase-conjugated second antibodies from Amersham (Arlington Heights, IL).

TSP-1 purification

TSP-1 was purified from Ca^{2+} ionophore A23187-activated platelets as previously described.[23] All TSP-1 was further purified to remove bound TGF-β1 according to the procedure of Murphy-Ullrich *et al.*[24] TGF-β1 levels were monitored by a TGF-β1 ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). TSP-1 preparations used for cell culture were routinely checked for endotoxin content by the E-TOXATE kit (Sigma, Chemical Co, St. Louis,MO), and no detectable levels of endotoxin were found.

Tissue specimens and immunohistochemistry

Total of 27 cases of pancreatic adenocarcinoma with internal controls of normal pancreas obtained from the Graduate Hospital (Philadelphia, PA) were studied. Specimens were formalin-fixed, paraffin embedded, and cut as 5 μm serial sections. Sections were deparaffinized and rehydrated by sequential incubation in graded xylene-ethanol solutions. Endogenous peroxidase activity was quenched by treatment with 1.5% H_2O_2 for 15 min. Nonspecific binding in the tissue sections was blocked by normal horse serum. The primary antibodies, either polyclonal affinity purified goat anti-human TSP-1 IgG or sheep anti-human MMP-9 IgG, were incubated overnight at 4°C. The antibodies have been previously characterized and used for immunohistochemistry studies.[9,14] Non-immune IgG was used as negative controls. Immunohistochemical staining was performed using a biotin-streptavidin immunoperoxidase method (ABC Elite, Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB) as chromogen. Sections for TSP-1 staining were treated with 0.1% trypsin, 0.1% $CaCl_2$ in 20 mM Tris-HCl buffer, pH 7.6, for 10 min at room temperature for antigen retrieval before incubation with the antibody against TSP-1. All sections were counter-stained with hematoxylin. The intensity of TSP-1 and MMP-9 staining was graded on a scale of 0 to 3+, with 0 representing no detectable and 3+ representing the strongest staining.

Cell culture and treatments

Following cell lines were obtained from the American Type Culture Collection (Rockville, MD): human pancreatic adenocarcinoma cell lines, AsPC-1, BxPC-3, Colo357,

Panc-1, Panc89 and T3M4; bovine aortic endothelial (BPAE) cells; and human foreskin fibroblasts. AsPC-1 and BxPC-3 were maintained in RPMI-1640. BPAE cells were maintained in Ham's F12K, and other cell lines were cultured in DMEM. All culture media were supplemented with 10% fetal bovine serum, 50 U/ml of penicillin, 50 µg/ml of streptomycin and 50 µg/ml of gentamicin sulfate, and the cultures were kept in 5% CO_2 at 37°C. For studies of metalloproteinase activity in response to TSP-1, cells were cultured in 96-well cell culture plates in regular media until 90% confluent. Cells were washed with and incubated in serum-free media containing insulin-transferrin-sodium (ITS) (Sigma). Different concentrations of TSP-1 and other reagents were added. After 48 h incubation, unless specified otherwise, the conditioned media were collected, clarified by centrifugation, and assayed by zymography and Western blotting. The amount of conditioned media used in these assays was corrected for cell numbers in each well. Cell viability after treatments was monitored by the trypan blue exclusion assay.

Enzyme-linked immunosorbent assay (ELISA)

Quantitation of TSP-1 secreted into the culture medium by cells was performed using a competitive ELISA. Cells were plated in 6-well culture plates at $1.5x10^5$ cells per well in growth medium and incubated for 1-3 days until subconfluent. The cells were then washed 3 times and incubated in serum-free medium for additional 24 hours. The culture medium was then collected, clarified by centrifugation and stored at –70°C. The cells were then harvested with trypsin and the cell number was counted. In some experiments, the cellular fraction was obtained by incubating the cells with lysis buffer (PBS containing 0.2% Triton X-100, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 mM leupeptin, 1 mM EDTA). Samples were assayed for TSP-1 by a competitive ELISA using a rabbit monospecific anti-TSP-1 antibody as previously described.[22,25] The standard curves were generated using purified TSP-1 (2-100 ng/ml), which was diluted to the same extent as the samples. The serum-free culture medium and lysis buffer served as negative controls.

Table 1. **Normal, benign, and neoplastic pancreatic tissues immunostained for TSP-1**

Tissue	*No.*	*TSP-1 Staining*	
		Epithelial Cell	*Stroma*
Normal pancreas *	11	–	+/–
Chronic pancreatitis *	2	–	+/–
Ductal adenocarcinomas	27	+	+/+++
		3 cases	23 (85%) cases

**Normal pancreas and chronic pancreatitis specimens are from internal controls.*

Zymograghy

Conditioned medium was separated by 10% SDS-PAGE in which the gels contained 1 mg/ml gelatin under non-reducing conditions as previously described.[26] After electrophoresis, the gels were washed with 2.5% Triton X-100 to remove SDS and incubated overnight at 37°C in 50 mM Tris buffer containing 150 mM NaCl, 10 mM $CaCl_2$, and 0.2% NaN_3, pH 7.5. The gels were stained with 0.05% Coomassie Brilliant Blue R-250 in acetic acid: isopropanol: water (1:3:6, v/v/v) and destained with 5% acetic acid. Bands of gelatinase activity appeared as transparent areas against a blue background. Gels were then photographed and the gelatinase activities were determined by quantitative densitometry.

Immunoblotting

Conditioned media were concentrated (4-10 fold) using a microconcentrator (Gelman Sciences Inc, Ann Arbor, MI). Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford). Samples of equal amount were fractionated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Micron Separations Inc, Westborough, MA). Nonspecific sites on the membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at 4°C overnight. The immunoblots were then incubated with primary antibodies (anti-MMP-9 pAb: 3 µg/ml; anti-MMP-9 mAb: 2 µg/ml; and anti-TIMP-1 pAb: 0.2 µg/ml) for 3 hours at room temperature. After washing, the immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. The bound antibodies were detected using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL).

Results

Expression of TSP-1 and MMP-9 in human pancreatic adenocarcinomas

Of the 27 cases of pancreatic adenocarcinomas studied for TSP-1 expression, 23 cases (85%) showed increased TSP-1 staining in the desmoplastic stroma juxtaposed to tumor cells and/or at the basement membrane associated with the malignant ductal epithelium *(Table 1 and Figure 1).* In most cases, tumor epithelial cells had little or no staining for TSP-1. Only in 3 poorly differentiated pancreatic adenocarcinoma sections, tumor cells showed weak TSP-1 staining. The TSP-1 positive staining was heterogeneously distributed with preference to the peripheral areas of the tumor nests. In some tissues, there was a gradient stromal distribution of TSP-1 around the tumor: ie. the closer to the tumor, the higher the TSP-1 levels in the stroma. In addition, the abundance of TSP-

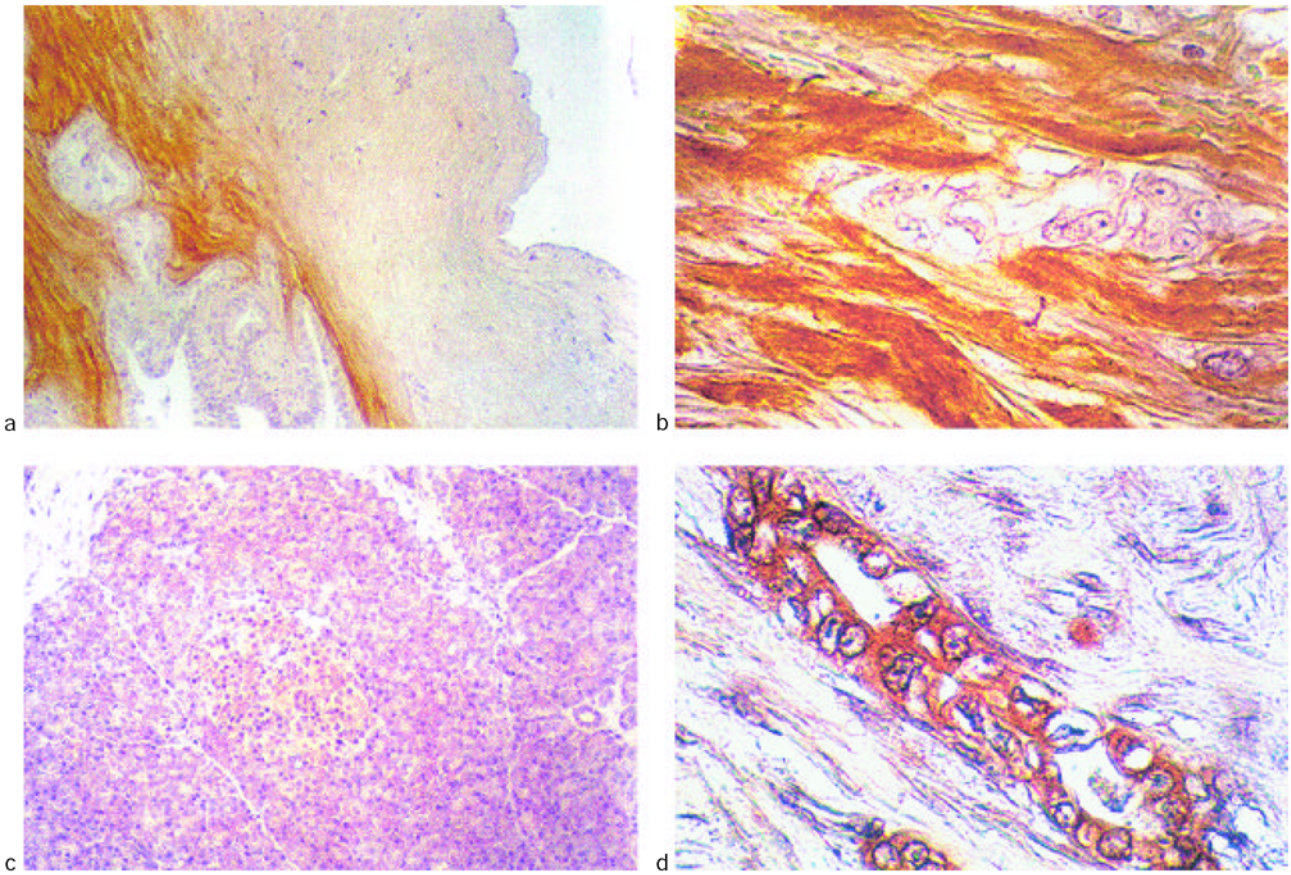

Figure 1. *Representative immunohistochemical staining of TSP-1* ***(a, b, c)*** *and MMP-9* ***(d)*** *in pancreatic ductal carcinoma* ***(a, b, d)*** *and normal pancreas tissues* ***(c)*** *is shown. Sections were stained by the abc immunoperoxidase method (brown stain) and counterstained with hematoxylin (Blue nuclei stain). Magnification:* ***a*** *and* ***c****, X 120;* ***b*** *and* ***d****, X 500.*

1 in the tumor tissue trended to be positively associated with the extent of the desmoplastic reaction of the tumor. No specific positive staining for TSP-1 was observed in the normal pancreatic tissues and the inflammatory areas. Our results suggest that high expression of TSP-1 in human pancreas is associated with neoplastic transformation. The presence of TSP-1 at the border of tumor cells and normal stroma further suggests an important role of TSP-1 in the tumor-stromal interaction in human pancreatic cancer.

Recent studies have suggested that the interaction between tumor cells and surrounding stroma plays an important role in modulation of tumor pericellular proteolysis by up-regulating MMP-9, one of the major metalloproteinases participating in tumor invasion.[20,27,28] Our observation[14] that TSP-1 can regulate MMP-9 production in endothelial cells promted us to hypothesize that high levels of TSP-1 in the stroma of the pancreatic cancer tissues may regulate the expression of MMP-9 in pancreatic tumor cells. Immunohistochemically, we investigated 13 pancreatic adenocarcinoma specimens for MMP-9 expression using a polyclonal antibody which recognizes both the proenzyme and the active forms of MMP-9 (***Figure 1***). These specimens were serially sectioned and also immunostained for TSP-1. Normal pancreas or chronic pancreatitis were used as an internal control. We detected moderate to strong intracytoplasmic staining for MMP-9 in tumor epithelial cells in 85% of the cases. In normal pancreas, MMP-9 staining was occasionally positive in islet cells and ductal cells. MMP-9 staining was also detected focally in disrupted acinar cells, islet cells and in inflammatory cells in chronic pancreatitis lesions (***Table 2***). Staining of stroma was negative in normal pancreas and inflammatory tissues and weak in tumor specimens. When the immunostain of MMP-9 was compared to that of TSP-1 in the pancreatic adenocarcinomas, we found that the areas where TSP-1 was strongly positive in the stroma also showed strong staining for MMP-9 in adjacent carcinoma cells (***Figure 1b and d***). However in some areas, positive MMP-9 staining was also detected in tumor cells, whereas TSP-1 was negative in the surrounding stroma. These observations suggest that TSP-1 can be one of the factors that regulate the expression of MMP-9 in pancreatic adenocarcinomas.

Table 2. **Normal, benign, and neoplastic pancreatic tissues immunostained for MMP-9**

Tissue	*No.*	*MMP-9 Staining*	
		Epithelial Cells	*Stroma*
Normal pancreas*	11		–
Chronic pancreatitis*	2	focal islet, duct cells +/++ acinar, islet & inflammatory cells	–
Ductal adenocarcinomas	13	++/+++ (11/13) ductal tumor cells	+

**Normal pancreas and chronic pancreatitis specimens are from internal controls.*

Table 1 and 2. *Specimens were formalin-fixed, paraffin embedded, and cut as 5 mm serial sections. TSP-1 protein expression in these specimens was investigated by immunohistochemical staining using a polyclonal affinity purified goat anti-human TSP-1 IgG. MMP-9 expression was studied using a polyclonal sheep anti-human MMP-9 IgG. Non-immune goat IgG and sheep IgG were used as negative controls. Immunohistochemical staining was performed by the immuno-peroxidase technique (ABC system). For TSP-1 staining, sections were treated to retrieve antigen with 0.1% trypsin in 0.1% $CaCl_2$, 20 mM Tris (pH 7.8) for 10 min at room temperature before straining. The intensity of staining was graded on a scale of 0 to 3+, with 0 representing no detectable and 3+ representing the strongest staining.*

TSP-1 production in pancreatic cancer and normal cell lines

To further investigate the production of TSP-1 in pancreatic cancer, we measured the secretion of TSP-1 in the culture medium and the cellular fraction of TSP-1 in five pancreatic adenocarcinoma cell lines by ELISA. One normal fibroblast cell line and one endothelial cell line, which are representative cells in the tumor stroma, were included in the study. As shown in ***Figure 2a***, the amounts of TSP-1 detected in both culture media and cell extracts from fibroblast or BPAE cells were much higher than those from pancreatic cancer cells. The secretion of TSP-1 by different pancreatic cancer cell lines into culture medium varied from 11.45±14.08 to 275.82±45.56 ng/10^6 cells/24 hours whereas there were almost undetectable amounts of TSP-1 in pancreatic cancer cell extracts. In addition, the treatment of TGF-β1 increased TSP-1 secretion in AsPC-1 cells by approximate 64% (***Figure 2b***). These results are consistent with our above immunohistochemical study and previous observations made in other tumor systems. [29] In summary, we found that carcinoma cells can produce TSP-1 and that this synthesis can be regulated by cytokines whereas the

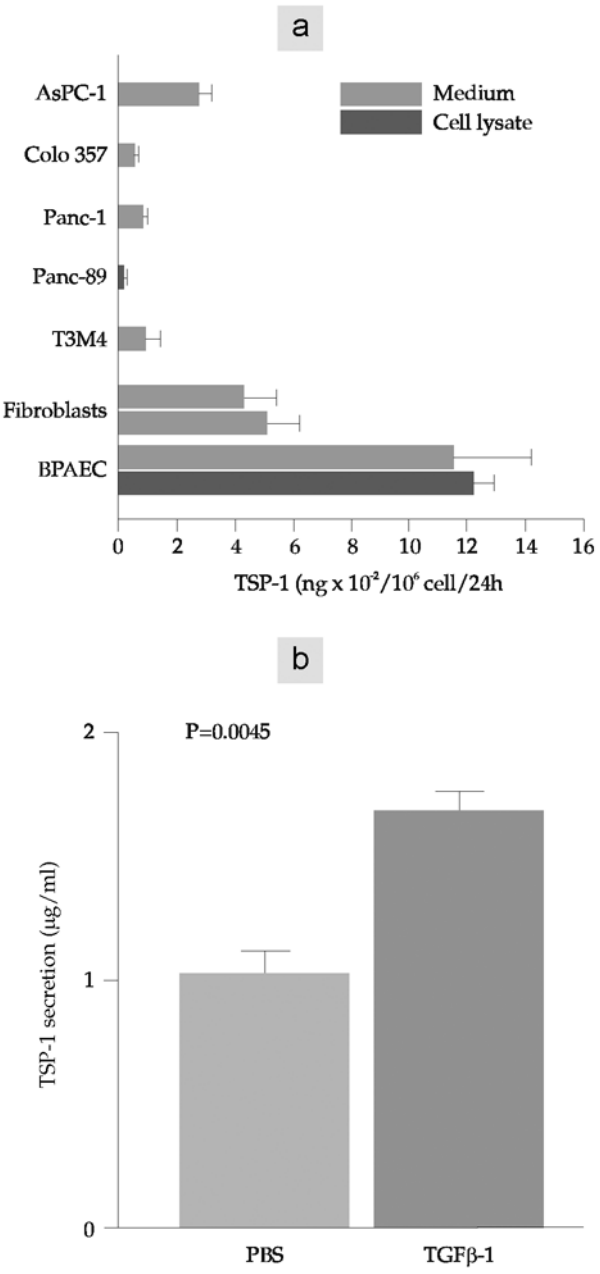

Figure 2. ***a)*** *Quantification of TSP-1 production in pancreatic cancer and normal cell lines by ELISA.* ***b)*** *Effect of TGF-β on TSP-1 production in AsPC-1 cells. Cells were plated in 6-well culture plates at 1.5x10^5 cells per well in growth medium and incubated for 1-3 days until sub-confluent. The cells were then washed 3 times and incubated in serum-free medium with or without TGF-β (1 ng/ml) for an additional 24 hours. The culture medium was then harvested. The cellular fraction was obtained by incubating the washed cell with lysis buffer (PBS containing 0.2% Triton X-100, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 µM leupeptin, 1 mM EDTA). Quantitation of TSP-1 secreted into the culture medium and the cellular fraction was performed using a competitive ELISA using a rabbit anti-TSP-1 antibody. Purified human platelet TSP-1 (2-100 ng/well) was used as a standard.*

stromal cells in the tumors are likely the major contributors of TSP-1. The stromally produced TSP-1 likely accounts for the high levels of TSP-1 in carcinomas.

Regulation of MMP-9 and TIMP-1 expression in pancreatic cancer cells by TSP-1

To further test the hypothesis that stromal TSP-1 is involved in the regulation of matrix degradation by pancreatic cancer cells, we investigated the production of MMP-9 and its natural inhibitor, TIMP-1, in two pancreatic adenocarcinoma cell lines, AsPC-1 and BxPC-3, in response to exogenous TSP-1 treatment. We first analyzed the conditioned media harvested from AsPC-1 and BxPC-3 cell cultures treated with TSP-1 for 48 hours using gelatin zymography. As compared with the two major gelatinases, 72 kDa gelatinase A (MMP-2) and 92 kDa gelatinase B (MMP-9), produced by HT1080 cells stimulated by PMA, the 72 kDa gelatinase A was the major enzyme produced by AsPC-1 cell while the 92 kDa gelatinase B was the only one detected in BxPC-3 cells under basal conditions. The treatment of TSP-1 (40 μg/ml) increased the production of 92 kDa gelatinase B by 2-4 fold in both AsPC-1 and BxPC-3 cell lines, but only slightly increased the production of 72 kDa gelatinase A (1.2 fold) in AsPC-1 cells (***Figure 3a***). The stimulation of MMP-9 production by TSP-1 in BxPC-3 cells was dose-dependent and saturable (***Figure 3b***). The stimulatory effect of TSP-1 on MMP-9 production was specific since a monoclonal anti-TSP-1 antibody (p10) abolished the stimulatory effect of TSP-1 on MMP-9 production (data not shown).

The identity of the 92 kDa gelatinase detected in gelatin zymography was further confirmed by Western blot analysis using two different specific antibodies. As shown in ***Figure 4,*** the monoclonal anti-MMP-9 antibody detected greater amounts of 92 kDa gelatinase B in both TSP-1 and PMA treated BxPC-3 cell cultures as compared to the buffer control. In addition, the polyclonal anti-MMP-9 antibody recognized a 92 kDa gelatinase as well as its active form generated by the treatment of samples with 4-aminophenymercuric acetate (APMA), a known specific activator of MMP ***in vitro***.

Since TIMP-1 forms a complex with MMP-9 and regulates its function, and its expression is regulated coordinately with the expression of MMP-9 in several tumor cell lines,[30,31] we also tested the effect of TSP-1 on TIMP-1 expression in AsPC-1 and BxPC-3 cells by Western blot analysis. As shown in ***Figure 5***, the conditioned media from both AsPC-1 and BxPC-3 cell cultures treated with TSP-1 or PMA contained significantly greater amount of 29 kDa TIMP-1 as compared to buffer controls. We also consistently observed an unidentified band migrating around 70 kDa in the conditioned media from HT1080 cells treated with PMA. A variation of signal intensity between the 29 kDa TIMP-1 band and the 70 kDa unknown band in the same sample was noticed in different experiments, suggesting the existence of a TIMP-1 complex with unidentified proteins.

Discussion

We found that TSP-1, a platelet and matrix protein, was expressed at higher levels in human pancreatic adenocarcinomas as compared to normal pancreatic and chronic pan-

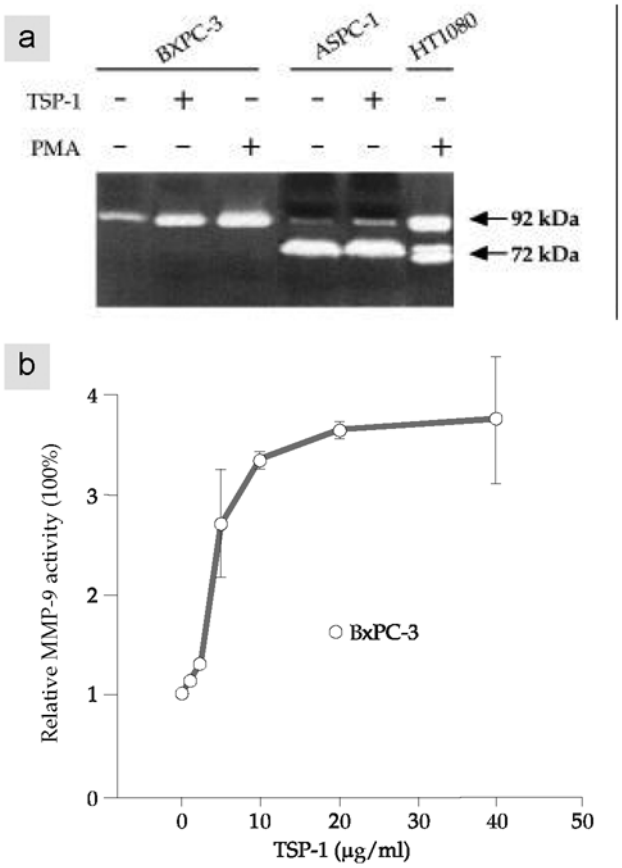

Figure 3. *Effect of TSP-1 on MMP-9 production in pancreatic cancer cells.* ***a)*** *Zymographic analysis of conditioned media from cultured BxPC-3 and AsPC-1 cells treated with TSP-1 or PMA. Cells were cultured in 96-well tissue culture plates under serum-free conditions and treated with TSP-1 (40 μg/ml) or PMA (50 ng/ml) for 48 h. The conditioned media were collected and analyzed by gelatin zymography. The conditioned medium from HT1080 cells treated with PMA served as a positive control. MMP-9 (92 kDa gelatinase B) and MMP-2 (72 kDa gelatinase A) are marked by arrows.* ***b)*** *Semi-quantitation of the effect of TSP-1 on MMP-9 production in BxPC-3 cells. The conditioned medium from BxPC-3 cells treated with increasing concentrations of TSP-1 (0-40 μg/ml) was analyzed by zymography. Relative 92 kDa gelatinase activities on zymography were determined by quantitative densitometry. The gelatinase activity in conditioned medium from cultures without TSP-1 treatment was assigned a value of 1.0. Data were expressed as mean ± SD of three experiments.*

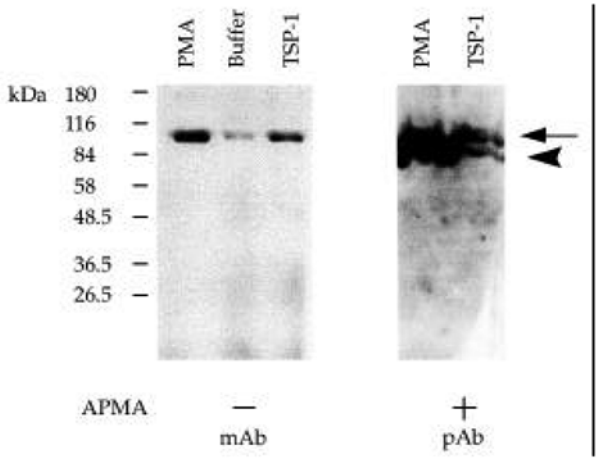

Figure 4. *Western blotting analysis of MMP-9 in the conditioned medium from BxPC-3 cells treated with TSP-1 or PMA. Aliquots of conditioned media were generated from cultures of BxPC-3 cells treated with or without TSP-1 or PMA for 48 h, concentrated 4-fold, and analyzed by immunoblotting using a monoclonal mouse anti-human MMP-9 IgG (left panel) and a polyclonal sheep anti-human MMP-9 IgG (right panel). Proenzyme form of MMP-9 (92 kDa) is indicated by an arrow, and the activated MMP-9 (83 kDa) following APMA (4-aminophenylmercuric acetate) exposure is indicated by an arrowhead.*

creatitis tissues. The TSP-1 staining patterns observed in this study are similar to previously described TSP-1 staining in invasive breast ductal carcinomas[9,32] suggesting a common mechanism involved in the regulation of TSP-1 expression in these desmoplastic-rich tumors. Consistent with the observation ***in vivo*** that excessive TSP-1 deposits were present predominantly in the desmoplastic stroma adjacent to pancreatic tumor tissues, we found that fibroblasts and endothelial cells synthesize much more TSP-1 than pancreatic cancer cells ***in vitro***. These data strongly suggest a stromal source of TSP-1, the expression of which may be induced by tumor derived factors. However, pancreatic tumor cells may also contribute to TSP-1 synthesis and deposition in the tumor-stromal border, since our present data showed that pancreatic cancer cells can secrete TSP-1 ***in vitro***, though in relatively lower amounts, and that TSP-1 secretion can be regulated by TGF-β1, which shows enhanced expression in pancreatic cancer.[33] In fact, TSP-1 was immunohistochemically detected in tumor cells ***in vivo*** in 3 poorly differentiated pancreatic cancer specimens. The regulatory mechanisms that control TSP-1 biosynthesis in tumor tissues are not clearly understood at present. Since the TSP-1 gene contains a serum response element and its expression is induced by multiple growth factors ***in vitro***, it is highly likely that the excessive TSP-1 in tumor stroma is a result of a growth factor rich environment around the tumor tissue. The results from several studies showing that TSP-1 expression was down-regulated ***in vitro*** by introducing oncoproteins such as Myc[34] may explain the low presence of TSP-1 in tumor or transformed cells. However, further investigation is required to elucidate the autocrine and/or paracrine mechanisms involved in TSP-1 expression ***in vivo*** by tumor cells and/or stromal cells.

The role of TSP-1 in tumor progression is multifunctional. TSP-1 has been shown to regulate tumor growth, tumor cell adhesion and invasion, hematogenous spread as well as tumor angiogenesis (for review).[11,12,35] The understanding how TSP-1, as an extracellular matrix protein, exerts these activities is the focus of current studies. We made an observation that TSP-1 can modulate endothelial cell tube formation and invasion by up-regulating MMP-9, an important matrix metalloproteinase involved in angiogenesis and cancer metastasis.[14] In the present study, we expanded our observations to the pancreatic cancer system to test the hypothesis that TSP-1 is involved in pancreatic cancer invasion and metastasis by regulating extracellular matrix degradation. Using zymography and immunoblotting, we found that TSP-1 increased MMP-9 production by 2 to 3 fold in two pancreatic cancer cell lines, AsPC-1 and BxPC-3, whereas no or negligible effects were observed on MMP2 production (***Figure 3***). The effect of TSP-1 was more prominent in BxPC-3, a cell line established from the metastatic site of a pancreatic cancer patient. TSP-1 seems to regulate the expression of MMP-9 at the post-transcriptional level since we also detected the increase of MMP-9 mRNA following TSP-1 treatment as measured by Northern blot analysis. These data suggest that TSP-1 may participate in pancreatic cancer invasion and metastasis by up-regulating MMP-9. Our immunostaining result showing that MMP-9 positive pancreatic cancer cells were surrounded by TSP-1 rich stroma further supports this view. In addition, we also demonstrated that TSP-1 and PMA increased TIMP-1 production in pancreatic cells as well (***Figure 5***). The coordinating expression of MMP-9 and TIMP-1 has

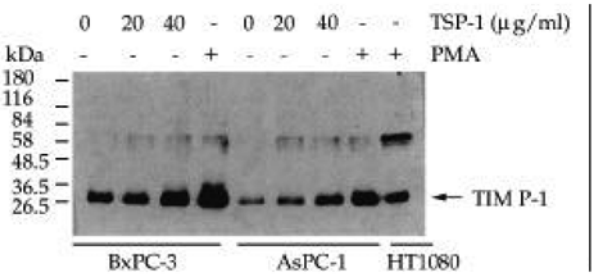

Figure 5. *Western blotting analysis of TIMP-1 in the conditioned media from BxPC-3 and AsPC-1 cells treated with TSP-1 or PMA. Cells were cultured in 24-well tissue culture plates under serum-free conditions and treated with indicated concentrations of TSP-1 or PMA (100 ng/ml) for 48 h. The conditioned media were collected, concentrated 4-fold, and analyzed by immunoblotting using a polyclonal rabbit anti-human TIMP-1 IgG. The conditioned medium from HT1080 cells treated with PMA served as a positive control. TIMP-1 (28.5 kDa) is shown by an arrow.*

been found in tumor cells and endothelial cells in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cytokines such as interleukin-1 (IL-1) and tumor necrosis factors-(TNF-α).[31] These results suggest that TSP-1 may share the same signaling pathways, such as protein kinase C (PKC) pathway, that mediate the stimulation of MMP-9 and TIMP-1 expression by phorbol esters, IL-1 or TNF. Since the inhibition of MMP activity by TIMP is reversible,[36] co-regulation of TIMP-1 expression with MMP-9 may provide a protection from excessive proteolysis, which may interfere with the biological processes mediated by MMP-9. Since TIMP-1 is capable of stimulating the growth of a variety of cells including those derived from tumors such as human breast adenocarcinoma, leukemia, the elevated TIMP-1 production by TSP-1 may function as a growth-promoting factor in pancreatic cancer, which is independent of its activity of inhibition of MMPs. Despite limited information on the role of matrix metalloproteinase in pancreatic cancer invasion and metastasis, the significance of MMP-9 in these processes is becoming recognized. In a study using ***in situ*** hybridization and Northern blot analysis, Gress ***et al***[87] reported that elevated levels of MMP-9 mRNA, as well as the transcripts of MMP-2, TIMP-1 and TIMP-1 but not those of MMP-1 and MMP-3, were detected in the majority of pancreatic cancer tissue samples as compared to normal controls. Consistent with our MMP-9 protein localization result, MMP-9 mRNA was also mainly, but not exclusively found in tumor cells. In support of a role of MMP-9 in pancreatic cancer invasion, Hirata ***et al***[88] demonstrated that the expression and activity of MMP-9 in pancreatic cancer cells was suppressed by loxiglumide, a cholecytskinin receptor antagonist, and that was correlated with the decrease of pancreatic cancer cell invasion. Taken together, our data suggest that TSP-1, which is present in the stroma surrounding the pancreatic tumor cell, is likely to be involved in modulating pancreatic cancer cell invasion by up-regulation of MMP-9 and TIMP-1.

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