

HT-1080 FIBROSARCOMA CELL MATRIX DEGRADATION AND INVASION ARE INHIBITED BY THE MATRIX-ASSOCIATED SERINE PROTEASE INHIBITOR TFPI-2/33 kDa MSPI

C.N. RAO^{1*}, B. COOK¹, Yueying LIU¹, Krishna CHILUKURI¹, M. Sharon STACK², Donald C. FOSTER³, W. KISIEL⁴ and David T. WOODLEY¹

¹Department of Dermatology, Northwestern University School of Medicine, Northwestern University, Chicago, IL, USA

²Department of Obstetrics and Gynecology, Northwestern University School of Medicine, Northwestern University, Chicago, IL, USA

³Zymogenetics Inc., Seattle, WA, USA

⁴Department of Pathology, University of New Mexico School of Medicine, Albuquerque, NM, USA

The urokinase-urokinase receptor system plays a dominant role in the degradation and invasion of extracellular matrix (ECM) by tumor cells. In this system, urokinase bound to its cell receptor converts plasminogen to plasmin, a broad-spectrum serine protease that participates in the degradation and invasion of connective tissues by tumor cells. In this study, we evaluated whether these activities of plasmin are inhibited by a newly characterized human 32 kDa recombinant serine protease inhibitor called trypsin/tissue factor pathway inhibitor-2 (rTFPI-2). We found that rTFPI-2 dose-dependently inhibited fluid-phase plasmin as well as plasmin generated on the ECM and/or the cell surface of HT-1080 fibrosarcoma cells. The degradation of radiolabeled matrix as well as Matrigel invasion by these tumor cells is also inhibited by rTFPI-2 in a dose-dependent fashion. We have reported that rTFPI-2 is identical to 33 kDa extracellular matrix-associated serine protease inhibitor (33 kDa MSPI), whereas the 31 and 27 kDa MSPIs are under-glycosylated forms of the 33 kDa MSPI. We therefore evaluated the ability of MSPIs from the ECM of dermal fibroblasts to inhibit plasmin and found that the plasmin activity was effectively blocked by the MSPIs. We have also evaluated whether the HT-1080 cells synthesize and secrete the MSPIs and found that the synthesis and secretion of the MSPIs was undetectable in these cells. Collectively, our results suggest that rTFPI-2/33 kDa MSPI inhibits plasmin on the tumor cell and ECM surfaces as well as the degradation and invasion of matrix by HT-1080 fibrosarcoma cells. *Int. J. Cancer* 76:749–756, 1998.

© 1998 Wiley-Liss, Inc.

The extracellular matrix (ECM) of human skin cells and endothelial cells contains 3 Kunitz-type serine protease inhibitors with molecular sizes of 33-, 31-, and 27 kDa capable of inhibiting trypsin, chymotrypsin, plasmin (Pn) and pancreatic elastase (Rao *et al.*, 1995a,b,c; Lino *et al.*, 1998). Greater than 70% of these inhibitors synthesized and secreted by cultured skin cells are tightly associated with the ECM and have been designated matrix-associated serine protease inhibitors (MSPIs). The antiprotease function of the MSPIs is resistant to heat, acid and SDS treatments. The 3 MSPIs are biosynthetic products of a single gene product of molecular size 25 kDa due to differential glycosylation (Rao *et al.*, 1996). The 33 kDa MSPI (Rao *et al.*, 1996) was also found to be identical to a 32 kDa recombinant trypsin/tissue factor pathway inhibitor called rTFPI-2 from placenta (Sprecher *et al.*, 1994), which inhibits Pn, trypsin, plasma kallikrein, chymotrypsin, cathepsin G, factor XIa and factor VIIa-tissue factor complex. In contrast, neither MSPIs nor rTFPI-2 is active against thrombin, urokinase (uPA) and tissue-type plasminogen activator (tPA) (Petersen *et al.*, 1996). TFPI-2 was also found to be identical (Kisiel *et al.*, 1994) to a poorly characterized 30–38 kDa serine protease inhibitor from placenta called placental protein-5 or PP-5 (Butzow *et al.*, 1988). The genes for human and mouse PP-5/TFPI-2 are located on chromosomes 7q 22 and 6, respectively (Miyagi *et al.*, 1996a,b).

The importance of the uPA-uPA receptor (uPAR) system in tumor cell invasion and metastasis has been extensively documented (Faziola and Blasi, 1994; Nip *et al.*, 1995; Quattrone *et al.*, 1995). Characterization of antagonists to this receptor-ligand system has been the focus of numerous studies (Ossowski, 1988;

Cajot *et al.*, 1989; Crowley *et al.*, 1993; Stahl and Mueller, 1994; Kobayashi *et al.*, 1995; Min *et al.*, 1996). These studies documented that the ability of receptor-bound uPA to generate Pn from the zymogen plasminogen (Pg) is essential for the tumor cells to invade and metastasize tissues and that blockade of this activity leads to inhibition of *in vivo* tumor growth and metastasis. Alternately, high-affinity inhibitors of Pn can also be considered promising agents to regard to inhibition of tumor invasion and metastasis. Human plasma Pn inhibitors, namely, α_2 -antiplasmin and α_2 -macroglobulin, are ineffective in inhibiting Pn on the ECM and/or tumor cell surface (Knudsen *et al.*, 1985; Stephens *et al.*, 1989; Bizik *et al.*, 1990; Quax *et al.*, 1991; Reinartz *et al.*, 1993).

The purpose of our study was to evaluate whether the newly described rTFPI-2/MSPI inhibits 1) ECM- and/or tumor cell membrane-associated Pn activity; and 2) degradation and invasion of matrix by tumor cells. We report that rTFPI-2/MSPI is a dose-dependent inhibitor of these functions by highly invasive HT-1080 fibrosarcoma cells. Furthermore, the MSPIs from the ECM deposited by dermal fibroblasts (insoluble forms) also inhibit Pn. The MSPIs were not synthesized and secreted by the HT-1080 cells. Together these data suggest that the availability of functional MSPIs within the ECM may regulate the ability of tumor cells to invade and degrade the matrix *in vivo*.

MATERIAL AND METHODS

Material

Phorbol 12-myristate 13-acetate (PMA), and Pn substrate D-Val-Leu-Lys-para nitroanilide hydrochloride (S-2251) were purchased from Sigma (St. Louis, MO). RPMI-1640, glutamine, fetal bovine serum, and trypsin-EDTA were purchased from Northwestern University Cancer Center's Tissue Culture Facility. ECL reagent was a product of Amersham (Aylesbury, UK). Plasminogen (Pg) was a gift from Dr. J.S. Rao (Houston, TX). Lysine-Pg (lys-Pg) and high m.w. human recombinant uPA were gifts from Dr. B. Credo (Abbott Research Laboratories, Abbott Park, IL). High m.w. uPA from urine (80,000 U/mg protein) was purchased from Calbiochem (La Jolla, CA). ³⁵S-methionine was purchased from ICN (Irvine, CA). rTFPI-2 was purified from baby hamster kidney cell culture medium as described (Sprecher *et al.*, 1994). Different preparations used in our study contained the TFPI-2/33 kDa MSPI form as the predominant species (>95%) but also minor amounts of the 31 kDa

Grant sponsors: Northwestern University Memorial Hospital; Northwestern University Medical School; Grant sponsor: National Institutes of Health; Grant numbers: AR 41045, AR 33625, HL 35246, CA 58900.

*Correspondence to: Department of Dermatology, Northwestern University Medical School, Tarry Bldg., Room 4-711 303-East Chicago Avenue, Chicago, IL-60611-3008, USA. Fax: (312) 908-1984.
E-mail: n-chilukuri@nwu.edu

Received 10 November 1997; Revised 4 February 1998

species (<5%). Antibodies against rTFPI-2 were generated in rabbits (Sprecher *et al.*, 1994), and the IgG fraction was purified by protein A-column chromatography.

Cell culture

HT-1080 fibrosarcoma cells were obtained from the ATCC (Rockville, MD). The SV-40-transformed human skin fibroblast (t12FB) cell line was originally supplied by Dr. C.L. Goolsby (Chicago, IL) (Goolsby *et al.*, 1991). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 50 µg/ml penicillin and 50 µg/ml streptomycin.

Pn inhibition assays

The inhibition of Pn by rTFPI-2/33 kDa MSPI was investigated in a variety of settings in which Pn was generated from Pg by recombinant uPA in fluid phase and uPA was added onto an ECM surface derived from t12FB, or uPA was bound to cell membranes of HT-1080 fibrosarcoma cells. In the fluid-phase assay, 100 IU recombinant uPA, 0.4 µM Pg and 0.4 mM S-2251 were incubated at 23°C for 2 hr with (50–200 nM) or without rTFPI-2/33 kDa MSPI. Assays were performed in 200 µl of a buffer containing 15 mM Tris-HCl, pH 7.40, 1 mM CaCl₂, 1 mM MgCl₂, 0.15 M NaCl, 1% DMSO. Free nitrophenolate anion (NA), which is formed from S-2251 by Pn, was quantitated by monitoring the absorbance at 405 nm in a Beckman (Fullerton, CA) DU spectrophotometer. The absorbance value at 405 nm in the absence of the inhibitor was considered to be 100%. The absorbance values from S-2251 with Pg alone, and uPA alone were considered background (<0.5%) and were subtracted.

The ability of rTFPI-2/33 kDa MSPI to inhibit Pn on an ECM surface produced by t12FB was also determined. t12FB were cultured to confluence in 24-well tissue culture plates and ECM prepared as previously described (Rao *et al.*, 1995a,b,c). Endogenous Pg, uPA and tPA were removed from the ECM by extraction with 100 mM 6-aminohexanoic acid (6-AH) in 15 mM Tris-HCl, pH 7.40 for 1 hr at 23°C. The MSPIs from the ECM are not removed by 6-AH extraction (data not shown). Therefore, the MSPIs from the t12FB ECM were removed by extraction with 50 mM glycine-HCl, pH 2.5, for 30 min at 23°C as described (Rao *et al.*, 1995b,c, 1996). We found that this treatment removes >90% of the MSPIs from the t12FB ECM (data not shown). It has been reported that these treatments do not remove matrix proteins such as von Willebrand factor and fibronectin from the ECM (Mimuro *et al.*, 1987). The ECM following 6-AH and 50 mM glycine-HCl, pH 2.5, extractions was then washed 4 times with 15 mM Tris-HCl, pH 7.40 and used as a surface to test whether exogenous rTFPI-2/33 kDa MSPI inhibits Pn, as described above for the fluid-phase assay, except that the incubation was maintained for 90 min.

Experiments were also designed to determine whether rTFPI-2/33 kDa MSPI inhibits Pn on the surface of HT-1080 fibrosarcoma cells. Briefly, HT-1080 cells were grown to confluence, detached with 15 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA and washed with RPMI-1640 0.1% BSA. Endogenous receptor-bound uPA was removed by incubation of the cells with 50 mM glycine-HCl, pH 3.0, 0.15 M NaCl for 10 min. After acid incubation, the cells were washed 3 times with RPMI-1640 0.1% BSA and incubated with recombinant uPA (10,000 IU for 40–50 × 10⁶ cells in 5 ml of RPMI-1640 0.1% BSA) for 30 min with end-over-end rotation. Thus the uPAR were recharged with fresh uPA. Unbound uPA was removed by washing the cells 3 times with 30 ml of Hanks' balanced salt solution without phenol red (HBSS), 0.1% BSA solution; the tumor cells were then used in Pn inhibition assays. Cells (1.5 × 10⁶) were incubated by end-over-end rotation for 90 min at 23°C in 500 µl of a solution containing HBSS, 0.1% BSA, 0.3 µM Pg and 0.4 mM S-2251 and without or with 50–200 nM of rTFPI-2/33 kDa MSPI. Pg was omitted in control reactions to obtain non-specific amidolytic activity by cells. Free NA was measured at 405 nm after a 90 min incubation period. The absorbance value in the absence of recombinant inhibitor was considered 100%. To ensure that the production of Pn by uPA-

charged HT-1080 cells is due to the exogenous uPA, those cells treated with acid buffer for removing endogenous uPA but not charged with exogenous uPA were used as controls in the Pn assays.

ECM degradation assays

Radiolabeled t12FB ECM was used as the substrate for degradation by HT-1080 fibrosarcoma cells. To prepare radiolabeled ECM, t12FB were plated in 24-well plates in RPMI-1640 that contained 10% FCS and 2–3 µCi of ³⁵S-methionine/ml. Radioactive methionine was added daily for 3 days or until the cells grew to confluency. The cultures were washed several times with PBS and the ECM prepared as described by Rao *et al.* (1996). The radiolabeled ECM plates were stored at 4°C until analysis. Prior to use, the plates were washed twice with 15 mM Tris-HCl pH 7.40 for 3 min and then extracted to remove Pg, uPA and tPA, as described above. The ECM was then washed 4 times with 15 mM Tris-HCl, pH 7.4, and incubated with HT-1080 fibrosarcoma cells (2 × 10⁶) in 400 µl of RPMI-1640 containing 0.1% BSA and 2 µg Pg. rTFPI-2/33 kDa MSPI was included in the reaction mixtures in increasing concentrations from 0 to 200 nM. Degradation of ECM by tumor cells was allowed for 2 hr at 23°C with gentle shaking. In some cases, Pg was omitted from the reaction mixtures to obtain non-specific (Pg-independent) degradation of radiolabeled ECM by tumor cells. After incubation, the samples were mixed with 10 ml of Ready Safe liquid scintillation cocktail (Beckman) and the radioactivity counted in a scintillation counter (Beckman model 6000 LS). The radioactivity released from the ECM in the absence of recombinant inhibitor was considered 100%. Non-specific degradation of ECM by tumor cells (<3%) was subtracted from all values.

Matrigel invasion and cell attachment assays

Invasion assays were performed as described previously (Albini *et al.*, 1987). Briefly, polycarbonate filters (8 µm pore size) of the invasion chambers were coated with Matrigel (200 µg/filter, Collaborative Research, Bedford, MA). Recombinant inhibitor was added to the upper wells (0–75 nM final concentrations) in 100 µl of a medium containing RPMI-1640, 0.1% BSA and 2 µg lys-Pg. Cultured HT-1080 fibrosarcoma cells were detached and suspended in Tris-saline, 1 mM EDTA, pH 7.40, buffer and washed twice with RPMI-1640, 0.1% BSA solution; 400 µl tumor cell (100,000 cells) suspension in RPMI-1640, 0.1% BSA, 0.01% gentamicin solution was placed in the upper compartment chamber. The lower compartment chamber contained serum-free conditioned medium (CM) from HT-1080 cells as a chemo-attractant. After incubation for 20 hr at 37°C, filters were removed and stained with Diff-Quick (Baxter, McGaw Park, IL), as suggested by the manufacturer. Invasive cells adhering to the lower surface of the filter were quantitated using a light microscope (×400). Cells from 18 non-overlapping fields from triplicate filters were counted, and the average was taken for analysis. Attachment of tumor cells to Matrigel (5–20 µg/well) was measured as described previously (Pierschbacher and Ruoslahti, 1984).

Pn inhibition assays by MSPIs from t12FB ECM

In assays determining whether the MSPIs from the ECM of t12FB (insoluble MSPIs) inhibit Pn, 0.25–0.3 × 10⁶ cells were cultured in 24-well plates and treated with PMA (50 ng/ml) overnight to induce the synthesis and secretion of MSPIs into ECM (Rao *et al.*, 1995a,b,c, 1996). The MSPIs from the ECM of control and PMA-treated t12FB were quantitated by immunoblotting with antiTFPI-2 antibody (Rao *et al.*, 1996). Pg and Pg activators were removed from the ECM of control and PMA-treated t12FB by extraction with 6-AH as described above in Pn inhibition assays. These matrices were then used as surfaces to generate Pn from 1 µg of Pg by reacting with 50 IU of recombinant uPA. S-2251 was included in the reaction mixtures and incubated for 2 hr at 23°C for determining Pn activity. In some assays 10 µg of antiTFPI-2 IgG or control IgG were included in the reaction mixtures to determine

their effect on Pn activity on ECM of PMA-treated t12FB. The data were evaluated for statistical significance by StatWorks program.

Synthesis and secretion of MSPIs by HT-1080 cells

Cells were cultured in RPMI-1640 containing 10% FBS, 50 µg/ml penicillin and 50 µg/ml streptomycin. Cells were grown to 80–90% confluence in 100-mm tissue culture dishes and the medium replaced with serum-free medium or the serum-free medium supplemented with PMA, at a concentration of 50 ng/ml. After culturing the cells for 24 hr serum-free conditioned medium (CM), cell-lysate (CL) and ECM fractions from control and PMA-treated cells were collected (Rao *et al.*, 1995a,c, 1996). The CM and CL fractions were analyzed for MSPIs by trypsin-affinity chromatography followed by Western blotting. Briefly, trypsin (2 mg/ml) was coupled to Reactigel agarose beads following the manufacturer's instructions (Pierce, Rockford, IL). The CM and CL were incubated with 100 µl of trypsin-Reactigel beads that were previously equilibrated with 15 mM Tris-HCl, pH 7.40 (equilibration buffer). The incubation was continued for 1 hr at room temperature with end-over-end rotation. The supernatant was removed and the trypsin-Reactigel beads were washed 4 times with equilibration buffer. Trypsin-bound proteins were extracted into 100 µl of SDS-PAGE sample buffer without β-mercaptoethanol, and MSPIs were detected (in 25 µl) by Western blotting with antiTFPI-2 antibody (Rao *et al.*, 1996). The ECM was extracted with 1.50 ml of the SDS-PAGE sample buffer, and a 25 µl aliquot of the extract was also assayed for MSPIs. As positive controls, t12FB cultures were treated with PMA, and the CM, CL and ECM fractions were simultaneously and similarly processed for the quantitation of MSPIs.

Western blotting

Proteins were boiled for 3 min, separated by SDS-PAGE using 12% polyacrylamide gels and electroblotted onto nitrocellulose membranes (Towbin *et al.*, 1979). After electroblotting, the membranes were blocked with 4% non-fat dry milk in 10 mM Tris-HCl, 150 mM NaCl, pH 7.40, containing 0.1% Tween-20 (TTBS) for 2 hr at 23°C. Then the membranes were incubated for 2 hr at 23°C or overnight at 4°C with normal rabbit serum or antiTFPI-2 antibody, diluted 1:2,000 in TTBS containing 1% BSA. After several washes, the membranes were incubated for 1 hr with a peroxidase-conjugated secondary antibody, diluted 1:3,000 in TTBS, 1% BSA solution. The immunoreactive proteins were identified using the ECL reagent system, following the manufacturer's instructions. The proteins were quantitated by scanning the bands using an imaging densitometer (BioRad model GS 670, Hercules, CA).

RESULTS

rTFPI-2/33 kDa MSPI inhibits fluid-phase Pn as well as Pn associated with the ECM or the cell surface of HT-1080 fibrosarcoma cells

It has been previously reported that rTFPI-2 does not inhibit uPA or tPA but inhibits Pn with an inhibition constant of 3 nM (Petersen *et al.*, 1996). Here, we have designed experiments to examine whether Pn is inhibited by the inhibitor as it is being produced from Pg by uPA in fluid phase, by uPA added onto a t12FB ECM-surface or by the uPA bound to the cell surface of HT-1080 fibrosarcoma cells.

Nanomolar concentrations of rTFPI-2/33 kDa MSPI inhibited 70–95% of the amidolytic activity of fluid-phase Pn produced from Pg with uPA (Fig. 1). Similarly, the inhibitor inhibited 60–90% of the amidolytic activity of Pn when the enzyme was produced on an ECM surface derived from t12FB (Fig. 2) and/or the cell surface of HT-1080 fibrosarcoma cells (Fig. 3a). In the latter experiment, the exogenous uPA is responsible for the production of Pn, as indicated in the control experiment shown in Figure 3b, which confirms that the cells not charged with uPA generated very little Pn (<10%). Together, these results suggest that the fluid-phase Pn as well as Pn

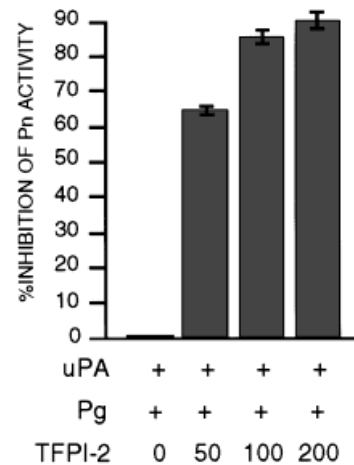


FIGURE 1 – Inhibition of fluid-phase Pn by rTFPI-2/33 kDa MSPI. rTFPI-2/33 kDa MSPI (0, 50, 100 and 200 nM) was incubated with recombinant uPA (100 IU) and S-2251 (0.4 mM final concentration) for 2 hr at 23°C, and the absorbance at 405 nm was determined. The absorbance value without the inhibitor is considered 100%. uPA alone and Pg alone were also incubated with S-2251 to determine the background absorbance. The values represent the average of triplicate determinations, which differed from 0 to 2%.

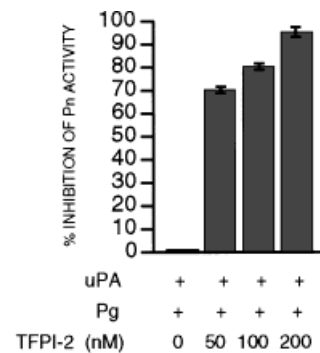


FIGURE 2 – Inhibition of Pn on t12FB ECM surface by rTFPI-2/33 kDa MSPI. ECM was extracted with 6-AH to remove endogenous uPA, tPA and Pg as described under Material and Methods. rTFPI-2/33 kDa MSPI (0, 50, 100 and 200 nM) was incubated with recombinant uPA (400 IU), Pg (0.4 µM) and S-2251 (0.4 mM) at 23°C for 90 min. At the end of incubation, absorbance at 405 nm was determined. Pg alone or uPA alone were also incubated with S-2251 for determining the background absorbance. The values represent the average of 4 determinations, which differed from 0 to 3%.

produced on the t12FB ECM surface and the tumor cell surface is rapidly inhibited by rTFPI-2/33 kDa MSPI.

rTFPI-2/33 kDa MSPI inhibits Pn-mediated degradation of ECM by HT-1080 fibrosarcoma cells

To determine whether the ECM degradation by HT-1080 fibrosarcoma cells is blocked, experiments were performed with tumor cells plated on radiolabeled ECM synthesized by t12FB. When 2×10^6 HT-1080 cells were plated onto wells containing the radiolabeled ECM, the tumor cells released 60–70% of the radioactivity (compared with the radioactivity solubilized with 5 µg of trypsin as 100%) from ECM in 2 hr in the presence of Pg. In comparison, only 2–5% of radioactivity was solubilized from the ECM without Pg (data not shown and Jones and DeClerck, 1980). In the presence of rTFPI-2/33 kDa MSPI (25–300 nM), the release of ECM radioactivity by the tumor cells was inhibited in a dose-dependent manner (30–80%, Fig. 4).

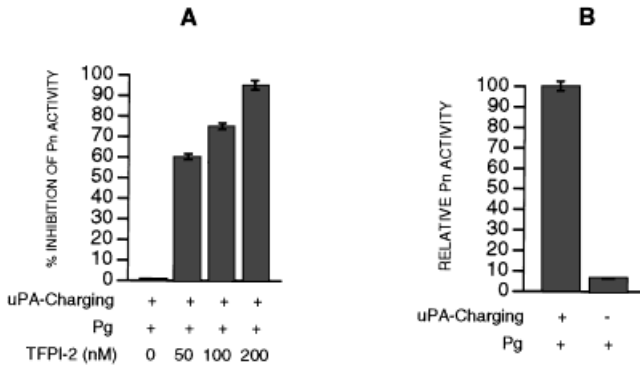


FIGURE 3 – (a) Inhibition of Pn on the cell surface of HT-1080 fibrosarcoma cells by rTFPI-2/33 kDa MSPI. HT-1080 fibrosarcoma cells (1.5×10^6) were treated with acid to remove the receptor-bound endogenous uPA and incubated with fresh recombinant uPA, as described in Material and Methods. The cells were incubated with rTFPI-2/33 kDa MSPI (0, 50, 100 and 200 nM), Pg (0.3 μ M) and S-2251 (0.4 mM) at 23°C for 90 min with end-over-end rotation. At the end of incubation, absorbance at 405 nm was determined. Background absorbance was determined by incubating the cells without Pg. This value was subtracted from all values. Values represent the average of four determinations, which differed from 0 to 3%. (b) Exogenous uPA is responsible for the production of Pn. HT-1080 cells were treated with acid to remove the endogenous, cell surface uPA and incubated with Pg and S-2251 as described above for (a). Absorbance at 405 nm was determined and compared with the absorbance value by those cells charged with recombinant uPA. Each value represents the average of quadruplicate determinations, which differed from 0 to 3%. Compared with the uPA-charged cells, the cells not charged with uPA generated very little (<10%) Pn.

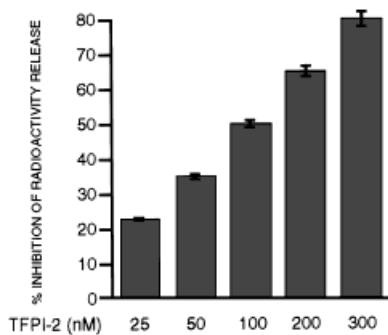


FIGURE 4 – rTFPI-2/33 kDa MSPI inhibits radiolabeled ECM degradation by HT-1080 fibrosarcoma cells. Tumor cells (2×10^6) were plated onto radiolabeled ECM and the degradation of ECM in the presence (25–200 nM) or absence of the inhibitor was determined as described in Material and Methods. Values represent the average of 4 determinations, which differed by <5%.

rTFPI-2/33 kDa MSPI inhibits Matrigel invasion by HT-1080 fibrosarcoma cells

Experiments were also performed to determine whether rTFPI-2/33 kDa MSPI could prevent tumor cells from invading through a reconstituted basement membrane (Matrigel). Tumor cell invasion of Matrigel in the absence of recombinant inhibitor was considered 100% (Fig. 5a). As shown in Figure 5b–d, inhibitions of 35%, 51% and 75% of the invasion of tumor cells through Matrigel were observed in the presence of 3 nM, 15 nM and 75 nM of the recombinant inhibitor, respectively. In contrast, 25–200 nM concentrations of the inhibitor had no effect on the attachment of the HT-1080 cells to Matrigel or on the growth rate of these cells (data not shown). These results suggest that rTFPI-2/33 kDa MSPI blocks Matrigel invasion by HT-1080 fibrosarcoma cells without

influencing the attachment ability of these cells to Matrigel or their growth.

33, 31 and 27 kDa MSPIs from PMA-treated t12FB ECM (insoluble MSPIs) inhibit Pn

We previously reported that PMA stimulated the synthesis and secretion of MSPIs into t12FB ECM (Rao *et al.*, 1995b,c, 1996). Quantitating the MSPIs from cell lysate, conditioned medium, and ECM fractions of control or PMA-treated t12FB revealed that 75–80% were ECM associated, and 20–25% were cell associated. None or very little (0–2%) were found in the conditioned medium. Here, we examined whether the MSPIs from the t12FB ECM are capable of inhibiting Pn. For this purpose, ECMs were prepared from control and PMA-treated t12FB, as described (Rao *et al.*, 1996). As shown in Figure 6a, compared with the ECM from control t12FB (4 representative samples), the ECM from PMA-treated t12FB contains 10- to 12-fold more of the MSPIs. By using an immunoblot with rTFPI-2/33 kDa MSPI standards (0.5–10 ng), the ECM derived from $0.25\text{--}0.3 \times 10^6$ PMA-treated t12FB contains 4–5 ng of MSPIs compared with 0.4–0.5 ng from the ECM deposited by the same number of control t12FB. To demonstrate that these ECM-bound MSPIs are functional, the ECMs were first cleared of endogenous Pg, uPA and tPA with 6-AH and then used as surfaces to generate Pn from Pg by uPA, as described in the methods section. As shown in Figure 6b, the amidolytic activity of Pn on the ECM from PMA-treated t12FB (average of 4 samples) was significantly lower than the Pn activity on ECM from untreated t12FB. To demonstrate that this decrease of Pn activity is due to the MSPIs within the ECM, 10 μ g of antiTFPI-2 IgG or control IgG was added to the reaction mixtures. As shown in Figure 6c, anti-TFPI-2 antibody but not normal rabbit IgG recovered the Pn activity to the levels observed on the ECM from control t12FB ECM. Following immunoblotting procedures that can detect 20 ng of PAI-1 and protease nexin-1, the ECM from both control and PMA-treated t12FB did not reveal the presence of these two inhibitors (data not shown). Collectively, these results suggest that the MSPIs from the t12FB ECM are functional.

HT-1080 cells do not synthesize and secrete MSPIs

To determine the synthesis and secretion of MSPIs by HT-1080 cells, we subjected the CM, CL and ECM fractions from control and the PMA-treated cells to Western blotting with anti-TFPI-2 IgG (Fig. 7) or normal rabbit IgG (data not shown). We have used trypsin-Reactigel beads to isolate MSPIs from the CM and CL of HT-1080 cells and a control cell line, t12FB, which synthesizes and secretes MSPIs (Rao *et al.*, 1996). In sharp contrast to the heparin-Sepharose beads used in our earlier study (Rao *et al.*, 1996), trypsin-Reactigel beads did not bind non-specific proteins, as demonstrated using CM and CL from PMA-treated t12FB (Fig. 7). However, the trypsin-bound MSPIs are recovered as smaller (by 1–2 kDa) molecular size inhibitors (Rao *et al.*, 1995a). The MSPIs were undetectable in the CM, CL and ECM from PMA-treated or control HT-1080 cells while they were readily detectable in the CM, CL and ECM of PMA-treated t12FB (Fig. 7). A control inhibitor, namely, plasminogen activator inhibitor-1 (PAI-1), was detected in both the CM and ECM of control and PMA-treated HT-1080 cells, suggesting that the isolation of these samples is complete (data not shown). These results suggest that the MSPIs are not synthesized and secreted by HT-1080 cells.

DISCUSSION

Proteolytic degradation of ECM is considered to be an essential step for malignant cells to invade and metastasize to distant tissues (reviewed in Danø *et al.*, 1985; Mignatti and Rifkin, 1995). Protease inhibitors capable of protecting the ECM from degradation by malignant cells could therefore have potential utility as therapeutic agents for blocking the formation of metastasis. Here, we have demonstrated that a newly described human serine protease inhibitor designated rTFPI-2 or 33 kDa MSPI is a potent

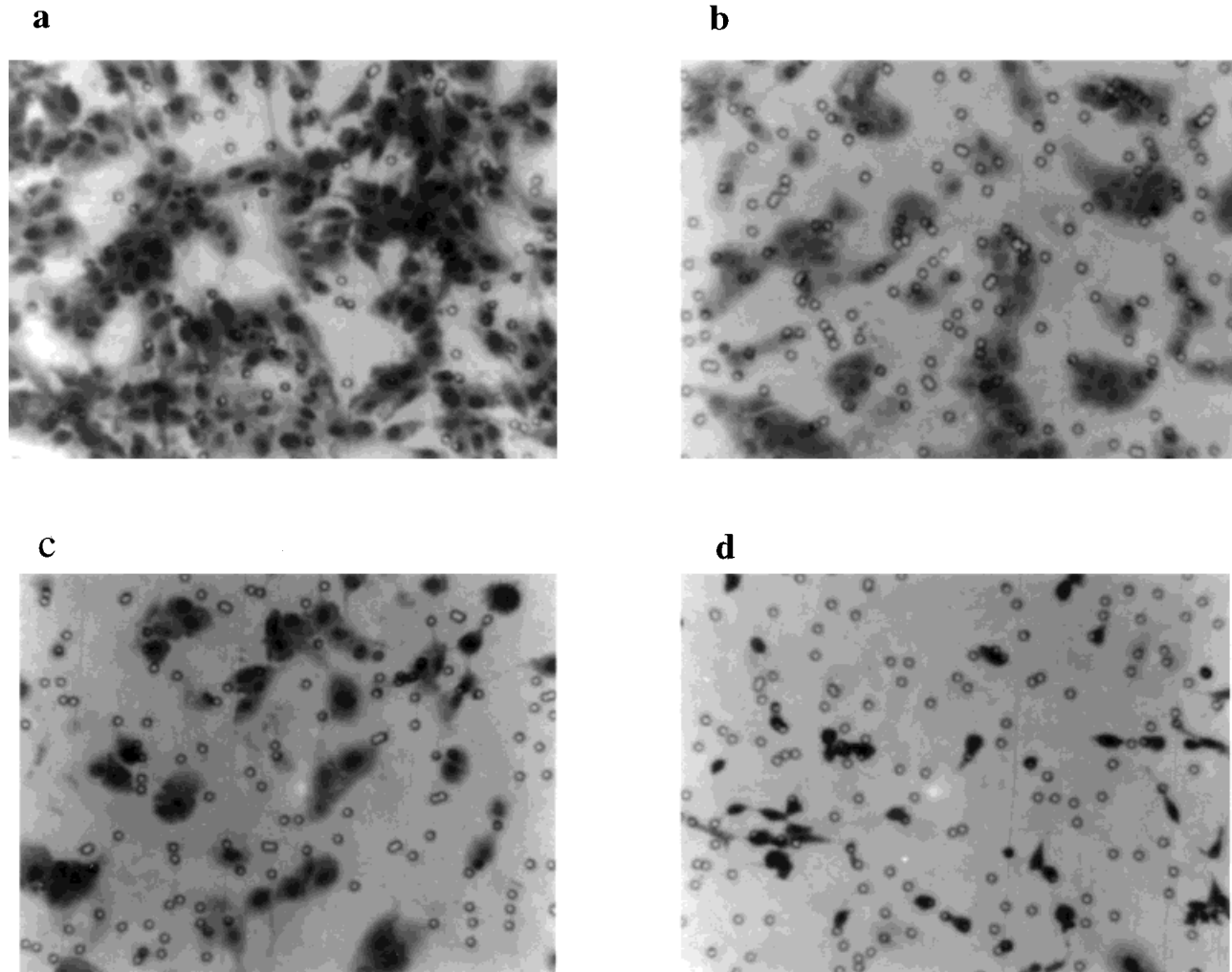


FIGURE 5 – rTFPI-2/33 kDa MSPI inhibits the invasion of Matrigel by HT-1080 fibrosarcoma cells. Tumor cells (0.1×10^6) were seeded onto polycarbonate membranes coated with Matrigel. Photomicrographs ($400\times$) of the tumor cells that invaded the Matrigel and collected on the underside of the filter are shown. (a) Without the inhibitor. (b–d) In the presence of 3, 15 and 75 nM of the inhibitor, respectively. Invasion of tumor cells through Matrigel was quantitated as described in Material and Methods. The values represent the average of cells counted in 18 non-overlapping fields from triplicate filters. The experiments were repeated 3 times with identical results.

inhibitor of ECM degradation and invasion by highly invasive HT-1080 fibrosarcoma cells. In addition to the inhibition of fluid-phase Pn, rTFPI-2/33 kDa MSPI also inhibits Pn generated on the tumor cell-, and/or ECM- surfaces in a dose-dependent fashion. We have also shown that insoluble MSPIs from the ECM are capable of inhibiting Pn. Based on these observations, we hypothesize that rTFPI-2/33 kDa MSPI play an important role in protecting the matrix from degradation and invasion by metastatic tumor cells (Fig. 8). This is further strengthened by our earlier observations showing that majority of MSPIs synthesized and secreted by cultured cells are associated with the ECM (Rao *et al.*, 1996). Acid or high salt is required to separate the MSPIs from ECM, suggesting a tight association between the MSPIs and ECM. Together, our data suggest that MSPIs are available at the cell/matrix interface and effectively block the metastatic tumor cells from degrading and invading the surrounding connective tissues.

Metastatic tumor cells utilize a variety of proteases including serine proteases, MMPs and cysteine proteases to degrade and invade connective tissues during invasion and metastasis. rTFPI-2/33 kDa MSPI is a broad-spectrum serine protease inhibitor with specificity toward Pn, trypsin, chymotrypsin, plasma kallikrein,

cathepsin G, factor VIIa-tissue factor and factor XIa (Petersen *et al.*, 1996). These activities for TFPI-2/MSPI explain the mechanisms by which the inhibitor inhibits matrix degradation and Matrigel invasion by tumor cells.

The ability of rTFPI-2/33 kDa MSPI to inhibit cell surface Pn of HT-1080 fibrosarcoma cells and their invasion of Matrigel may be of potential clinical significance in view of a study involving PAI-1. It has been found that wide variations in host PAI-1 expression, from complete absence to marked overexpression, failed to influence the metastatic potential of B16-F10 melanoma cells in a murine model (Eitzman *et al.*, 1996). Thus, PAI-1/plasminogen activator (uPA and tPA) balance may not significantly influence the tumorigenicity or metastatic potential of the murine melanoma cells. In contrast to PAI-1, which must compete with Pg for binding to the receptor-bound uPA, rTFPI-2/33 kDa MSPI inhibits Pn, which is directly responsible for matrix degradation and invasion. Interestingly, MSPIs were undetectable in the CM, CL and ECM of HT-1080 cells, suggesting that the MSPIs are not synthesized by these tumor cells. Currently we are testing whether the mRNA for MSPIs is also not expressed in HT-1080 cells. Nonetheless, our results clearly suggest that the extracellular Pn/MSPIs ratio for

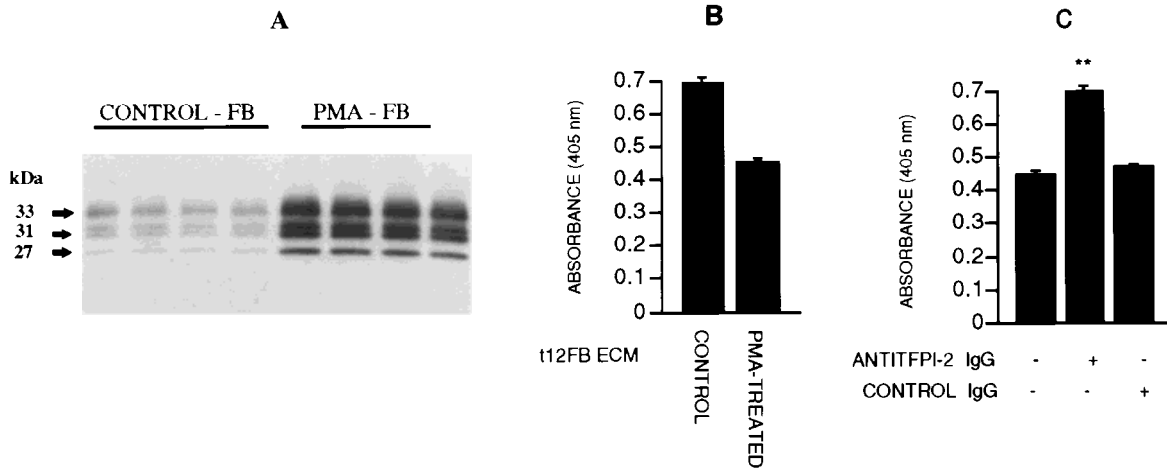


FIGURE 6 – 33, 31 and 27 kDa MSPIs from t12FB ECM inhibit Pn. (a) The MSPIs from the ECM of control and PMA-treated t12FB were quantitated by immunoblotting with antiTFPI-2 antibody, as described in Material and Methods. Compared with the ECM from control t12FB, the ECM from PMA-treated cells contains 10- to 12-fold more of the 3 MSPIs. (b) ECM from control and PMA-treated t12FB were subjected to treatment with 6-AH for removing Pg, uPA and tPA, and then Pn activity was assayed as described in Material and Methods. The MSPIs were intact after 6-AH treatment. Values represent the mean of 4 determinations, which differed from 0 to 2%. Pn activity was significantly inhibited on the ECM from PMA-treated t12FB. (c) To determine whether the reduced Pn activity on t12FB ECM from PMA-treated t12FB was due to the 33, 31 and 27 kDa MSPIs, 10 µg of anti-TFPI-2 IgG or normal rabbit IgG were included during the Pn assays. Values represent the mean of 4 determinations. The anti-TFPI-2 antibody but not the control IgG relieved the Pn inhibition on ECM from PMA-treated t12FB, suggesting that the MSPIs within the ECM are functional. The significance of the data was evaluated using the StatWorks program. The experiment was repeated 3 times with identical results.

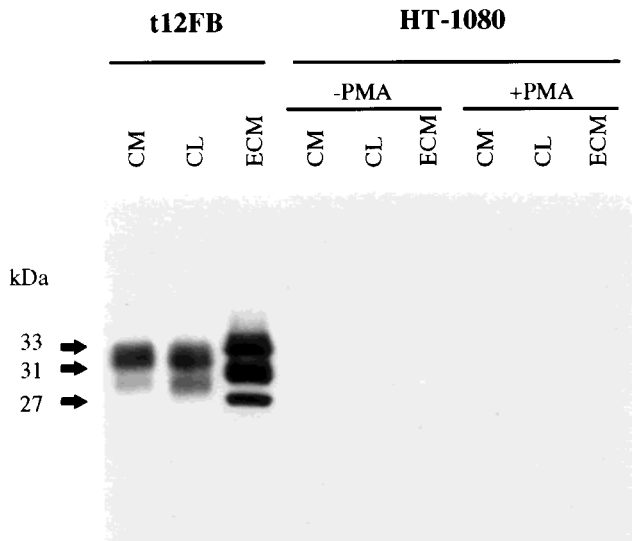


FIGURE 7 – MSPIs are not detected in the serum-free conditioned medium (CM), cell-lysate (CL) and extracellular matrix (ECM) of HT-1080 cells. CM, CL and ECM samples from control and PMA-treated HT-1080 cells and PMA-treated t12FB were subjected to SDS-PAGE using 12% acrylamide gels. The proteins were electroblotted onto nitrocellulose membranes and subjected to Western blotting with anti-TFPI-2 IgG. The MSPIs are detected in the 3 samples from PMA-treated t12FB but not in the samples from control or PMA-treated HT-1080 cells. The 33, 31 and 27 kDa MSPIs are marked by arrowheads. Normal rabbit IgG do not react with the MSPIs (not shown).

HT-1080 cells is 1:0 or is heavily in favor of the enzyme. This may explain in part the highly invasive behavior of HT-1080 cells.

Regulation of Pn activity on the surface of tumor cells has been shown to influence the invasive and metastatic behavior of these cells (Ossowski, 1988; Cajot *et al.*, 1989; Crowley *et al.*, 1993;

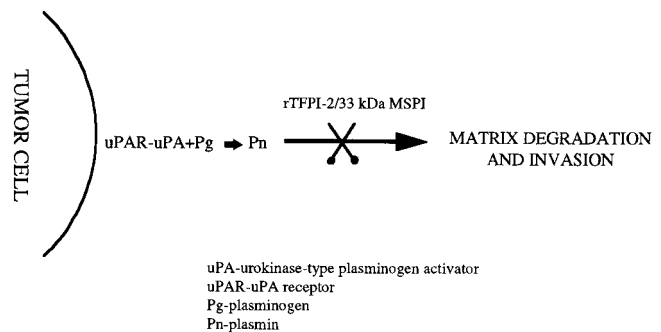


FIGURE 8 – Schematic representation of the site of action of rTFPI-2/33 kDa MSPI in matrix degradation and invasion by tumor cells.

Stahl and Mueller, 1994; Min *et al.*, 1996). However, Pn associated with the ECM or the membranes of cultured cells is resistant to inhibition by known physiologic serum serine protease inhibitors (Knudsen *et al.*, 1987; Stephens *et al.*, 1989; Bizik *et al.*, 1990; Quax *et al.*, 1991; Reinartz *et al.*, 1993). Based on these observations, it has been suggested that metastatic tumor cells generate “unregulated” Pn activity, which potentiates metastatic behavior (Kwaan, 1992; Kramer *et al.*, 1994). Many tumor cells, particularly the HT-1080 fibrosarcoma cell line utilized in this study, employ the uPA/uPAR system to activate Pg, resulting in Pn-mediated ECM degradation and invasion as well as proMMP-1 and -3 activation (reviewed in Danø *et al.*, 1985; Mignatti and Rifkin, 1995). As shown in our study, fluid-phase Pn, the Pn generated on the ECM and/or the tumor cell surface was inhibited by rTFPI-2/33 kDa MSPI in a dose-dependent manner. Furthermore, nanomolar concentrations of rTFPI-2/MSPI inhibited HT-1080 cells from degrading and invading the matrix. The ECM-bound MSPIs are functional too. This unique combination of efficacies suggests that the newly characterized rTFPI-2/MSPI may be of therapeutic importance in a variety of human cancers.

Sabapathy *et al.* (1997) have investigated the requirement of the PAs/Pn system in the formation of polyoma middle T antigen-, and End. (transformed endothelial) cell-induced vascular tumors in newborn mice. While the PAs/Pn system is not required for the development of polyoma middle T antigen-inducible tumors, it played a role in the End. cell-induced tumor formation. The absence of both PAs led to a loss in the invasiveness and proliferation of End. cells. Currently, the level of MSPIs expression in the wild-type and mutant End. cells is unknown. In *in vitro* fibrin gel models, addition of broad-spectrum serine protease inhibitors, namely, aprotinin and soybean trypsin inhibitors, inhibited the development of tumor cysts by End. cells, suggesting a role for serine protease inhibitors in vascular tumor formation (Montesano *et al.*, 1990). From these results, it appears that the newly identified TFPI-2/MSPIs may influence the development of End. cell-induced vascular tumors.

In conclusion, our present data demonstrate that rTFPI-2/MSPI is an effective inhibitor of Pn, regardless of whether the enzyme is in the fluid phase or is cell or matrix associated. This results in effective inhibition of matrix degradation and HT-1080 cell invasion. Together, our data suggest that the availability of TFPI-2/MSPI may serve as a control mechanism for regulation of matrix degradation and tumor invasion *in vivo*.

ACKNOWLEDGMENTS

This work was supported in part by grants from Northwestern University Memorial Hospital and Northwestern University Medical School (CNR) and National Institutes of Health grants AR 41045 and AR 33625 (DTW), HL 35246 (WK) and CA 58900 (MSS).

REFERENCES

- ALBINI, A., IWAMOTO, H., KLEINMAN, H.K., MARTIN, G.R., AARONSON, S.A., KOZLOWSKI, J.M. and MCEWAN, R.N., A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.*, **47**, 3239–3245 (1987).
- BIZIK, J., LIZINOVA, A., STEPHENS, R.W., GROFOVA, M. and VAHERI, A., Plasminogen activation by tPA on the surface of human melanoma cells in the presence of α 2-macroglobulin secretion. *Cell Regul.*, **1**, 895–905 (1990).
- BUTZOW, R., HUHTALA, M.L., BOHN, H., VIRTANEN, I. and SEPPALA, M., Purification and characterization of placental protein-5. *Biochem. biophys. Res. Comm.*, **150**, 483–490 (1988).
- CAJOT, J.F., SCHLEUNING, W.D., MEDCALF, R.L., BAMAT, J., TESTUZ, L. and SORDAT, B., Mouse L cells expressing human prourokinase-type plasminogen activator: effects on extracellular matrix degradation and invasion. *J. Cell Biol.*, **109**, 915–925 (1989).
- CROWLEY, C.W., COHEN, R.L., LUCAS, B.K., LIU, G., SHUMAN, M.A. and LEVINSON, A.D., Prevention of metastasis by inhibition of the urokinase receptor. *Proc. nat. Acad. Sci. (Wash.)*, **90**, 5021–5025 (1993).
- DANØ, K., ANDREASEN, P.A., GRØNDALH-JOHNSON, J., KRISTENSEN, P., NIELSEN, L.S. and SKRIVER, L., Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.*, **44**, 139–239 (1985).
- EITZMAN, D.T., KRAUSS, J.C., SHEN, T. and GINSBURG, D., Lack of plasminogen activator inhibitor-1 effect in a transgenic mouse model of metastatic melanoma. *Blood*, **87**, 4718–4722 (1996).
- FAZIOLI, F. and BLASI, F., Urokinase-type plasminogen activator and its receptor: new targets for anti-metastatic therapy? *Trends Pharm. Sci.*, **15**, 25–29 (1994).
- GOOLSBY, C.L., WILEY, J.E., STEINER, M., BARTHOLDI, M.F., SCOTT-CRAMM, L. and KRAEMER, P.M., Karyotype evolution in a simian virus 40-transformed tumorigenic human cell line. *Cancer Genet. Cytogenet.*, **49**, 231–248 (1991).
- JONES, P.A. and DECLERCK, Y.A., Destruction of extracellular matrices containing glycoproteins, elastin, and collagen by metastatic human tumor cells. *Cancer Res.*, **40**, 3222–3227 (1980).
- KISIEL, W., SPRECHER, C.A. and FOSTER, C.A., Evidence that a second human tissue factor pathway inhibitor-2 (TFPI-2) and human placental protein-5 are equivalent (letter). *Blood*, **84**, 4384–4385 (1994).
- KNUDSEN, B.S., SILVERSTEIN, R.L., LEUNG, L.L.K., HARPEL, P.C. and NACHMAN, R.L., Binding of plasminogen to extracellular matrix. *J. Biol. Chem.*, **261**, 10765–10771 (1985).
- KOBAYASHI, H., GOTOH, J., HIRASHIMA, Y., FUJIE, M., SUGINO, D. and TERAO, T., Inhibitory effect of a conjugate between human urokinase and urinary trypsin inhibitor on tumor cell invasion *in vitro*. *J. Biol. Chem.*, **270**, 8361–8366 (1995).
- KRAMER, M.D., REINARTZ, J., BRUNNER, G. and SCHIRRMACHER, V., Plasmin in pericellular proteolysis and cellular invasion. *Invasion Metastasis*, **14**, 210–222 (1994).
- KWAAN, H.C., The plasminogen-plasmin system in malignancy. *Cancer Metastasis Rev.*, **11**, 291–311 (1992).
- LINO, M., FOSTER, D.C. and KISIEL, W., Quantification and characterization of human endothelial cell-derived tissue factor pathway inhibitor-2. *Arterioscler. Thromb. Vasc. Biol.*, **18**, 40–46 (1998).
- MIGNATTI, P. and RIFKIN, D.B., Biology and biochemistry of proteinases in tumor invasion. *Physiol. Rev.*, **73**, 161–195 (1995).
- MIMURO, J., SCHLEEF, R.R. and LOSKUTOFF, D.J., Extracellular matrix of cultured bovine aortic endothelial cells contains functionally active type 1 plasminogen activator inhibitor. *Blood*, **70**, 721–728 (1987).
- MIN, Y.H., DOYLE, V.L., VITT, C.R., ZANDONELLA, C.L., STRATTON-THOMAS, J.R., SHUMAN, M.A. and ROSENBERG, M., Urokinase receptor antagonists inhibit angiogenesis and primary tumor growth in syngenic mice. *Cancer Res.*, **56**, 2428–2433 (1996).
- MIYAGI, Y., YASUMITSU, H., KOSHIKAWA, N., MATSUDA, Y., ITOH, H., HORI, T.-A., AOKI, I., MISUGI, K. and MIYAZAKI, K., Cloning of the cDNA encoding mouse PP5/TFPI-2 and mapping of the gene to chromosome 6. *DNA Cell Biol.*, **15**, 975–954, (1996a).
- MIYAGI, Y., YASUMITSU, H., MIYATA, S., KKAWA, N., HIRAHARA, F., AOKI, I., MISUGI, K. and MIYAZAKI, K., Assignment of the human PP5/TFPI-2 gene to 7Q 22 by FISH and PCR-based human mapping panel analysis. *Genomics*, **35**, 267–268 (1996b).
- MONTESANO, R., PEPPER, M.S., MOHLE-STEINLEIN, U., RISAU, W., WAGNER, E.F. and ORCI, L., Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing middle T oncogene. *Cell*, **32**, 319–328 (1990).
- NIP, J., RABBANI, S.A., SHIBATA, H.R. and BRODT, P., Coordinated expression of the vitronectin receptor and the urokinase type-plasminogen activator receptor in metastatic melanoma cells. *J. Clin. Invest.*, **95**, 2096–2103 (1995).
- OSSOWSKI, L., *In vivo* invasion of modified chorioallantoic membrane by tumor cells: the role of cell surface bound urokinase. *J. Cell Biol.*, **107**, 2437–2445 (1988).
- PETERSEN, L.C., SPRECHER, C.A., FOSTER, D.C., BLUMBERG, H., HAMAMOTO, T. and KISIEL, W., Inhibitory properties of a novel human Kunitz-type protease inhibitor homologous to tissue factor pathway inhibitor. *Biochemistry*, **35**, 266–272 (1996).
- PIERSCHBACHER, M.D. and RUOSLAHTI, E., Variants of the cell recognition site of fibronectin that retain attachment promoting activity. *Proc. nat. Acad. Sci. (Wash.)*, **81**, 5985–5988 (1984).
- QUATTRONE, A., FABBI, G., ANICHINI, E., PUCCI, M., ZAMPERINI, A., CAPACCIOLI, S. and DEL ROSSO, M., Reversion of the invasive phenotype of transformed human fibroblasts by anti-messenger oligonucleotide inhibition of urokinase receptor gene expression. *Cancer Res.*, **55**, 90–95 (1995).
- QUAX, P.H.A., VAN MUIJEN, G.N.P., WEENING-VERHOFF, E.J.D., LUND, L.R., DANØ, K., RUITER, D.J. and VERHEIJEN, J.H., Metastatic behavior of human melanoma cells in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor and urokinase-mediated degradation. *J. Cell Biol.*, **115**, 191–199 (1991).
- RAO, C.N., GOMEZ, D.E., WOODLEY, D.T. and THORGEIRSSON, U.P., Partial characterization of novel serine protease inhibitors from human umbilical vein endothelial cells. *Arch. Biochem. Biophys.*, **319**, 55–62 (1995a).
- RAO, C.N., LIU, Y.Y., PEAVEY, C.L. and WOODLEY, D.T., Novel extracellular matrix-associated serine protease inhibitors from human skin cells. *Arch. Biochem. Biophys.*, **317**, 311–314 (1995b).
- RAO, C.N., PEAVEY, C.L., LIU, Y.Y., LAPIERE, J.C. and WOODLEY, D.T., Partial characterization of matrix-associated serine protease inhibitors from human skin cells. *J. Invest. Dermatol.*, **104**, 379–383 (1995c).
- RAO, C.N., PRASAD REDDY, L., LIU, Y.Y., O'TOOLE, E.A.O., REEDER, D.J., FOSTER, D.C., KISIEL, W. and WOODLEY, D.T., Extracellular matrix-associated serine protease inhibitors (Mr 33,000, 31,000, and 27,000) are

- single-gene products with differential glycosylation: cDNA cloning of the 33-kDa inhibitor reveals its identity to tissue factor pathway inhibitor-2. *Arch. Biochem. Biophys.*, **335**, 82–92 (1996).
- REINARTZ, J., BATRLA, R., BOUKAMP, P., FUSENIG, N. and KRAMER, M.D., Binding and activation of plasminogen at the surface of human keratinocytes. *Exp. Cell Res.*, **208**, 197–208 (1993).
- SABAPATHY, T.K., PEPPER, M.S., KIEFER, F., MOHLE-STEINLEIN, U., TACCHINI-COTTIER, F., FETKA, I., BREIER, G., RISAU, W., CARMELIET, P., MONTESANO, R. and WAGNER, E.F., Polyoma middle T-induced vascular tumor formation: the role of the plasminogen activator/plasmin system. *J. Cell Biol.*, **137**, 953–963 (1997).
- SPRECHER, C.A., KISIEL, W., MATHEWES, S. and FOSTER, D.C., Molecular cloning, expression and partial characterization of a novel human tissue factor pathway inhibitor. *Proc. nat. Acad. Sci. (Wash.)*, **91**, 3353–3357 (1994).
- STAHL, A. and MUELLER, B.M., Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells *in vitro*. *Cancer Res.*, **54**, 3066–3071 (1994).
- STEPHENS, R.W., PÖLLANEN, J., TAPIOVAARA, H., LEUNG, K.C., SIM, P.S., SALONEN, E.M., RÖNNE, E., BEHRENDT, N., DANØ, K. and VAHERI A., Activation of pro-urokinase and plasminogen on human sarcoma cells: a proteolytic system with surface-bound reactants. *J. Cell Biol.*, **108**, 1987–1995 (1989).
- TOWBIN, H., STAEBELIN, T. and GORDON, J., Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. nat. Acad. Sci. (Wash.)*, **76**, 4350–4354 (1979).