

Overexpression of plasminogen activator inhibitor type-1 in HT-1080 fibrosarcoma cells promotes lung colonization and in vitro cell adhesion

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Summary Plasminogen activator inhibitor type 1 (PAI-1) is associated with tumour invasion, angiogenesis and metastatic spread. It is also a strong prognostic factor for relapse in a number of human cancers. This study was designed to investigate the effects of overexpression of PAI-1 on lung colonization by human HT-1080 fibrosarcoma cells. Full length PAI-1 cDNA was transfected in a non-aggressive HT-1080 clonal cell line (1–3C). Stable transfected clones were isolated of which one (3F52) secreted a 29-fold increase in PAI-1 protein levels ($29.1 \pm 6.5 \mu\text{g}/10^6$ cells/24 h) as compared with control mock transfected cells ($1.0 \pm 0.2 \mu\text{g}/10^6$ cells/24 h). 3F52 cells were significantly better able to form lung colonies after i.v. tail vein injection of athymic mice (mean number of colonies \pm SE 238 ± 105) as compared with control cells (8 ± 7). In addition, PAI-1 overexpressing cells were between 1.5 and 2.5 fold more adhesive to extracellular matrix membrane (ECM) proteins than mock transfected cells in an in vitro cell adhesion assay. These findings provide experimental support that PAI-1 facilitates tumour cell lodgement in vivo and adhesion of HT-1080 cells in vitro. © 2000 Harcourt Publishers Ltd

INTRODUCTION

Plasminogen activator inhibitor type 1 (PAI-1) is a specific inhibitor of two types of plasminogen activator (PA), tissue and urokinase plasminogen activators (tPA and uPA). TPA and uPA activate plasminogen to plasmin, a serine protease with broad substrate specificity that can result in matrix degradation as well as activation of metalloproteases and latent growth factors.^{1,2} In addition to its role as an inhibitor of PAs, PAI-1 has other non-proteolytic functions. It can block cell migration on vitronectin independently of its ability to inhibit proteolytic activity^{3,4} and exogenous PAI-1 can inhibit adhesion of some cell types by competing with cell surface urokinase plasminogen activator receptor (uPAR) for binding to vitronectin.⁵ In most, but not all experimental in vitro

and in vivo models, PAI-1 inhibits an invasive phenotype, tumour growth and angiogenesis.^{6–12} However, immunohistochemical and in situ hybridization analysis of PAI-1 shows that it is highly expressed in a variety of human cancers (for review, see ref. 1). In addition, levels of PAI-1 were shown to be considerably higher in metastases of colorectal tumours as compared with levels found in the primary carcinomas.¹³ Furthermore, PAI-1 is a very significant marker for relapse particularly in breast cancer.¹⁴ These clinical studies have shown that a coordinated expression of PAI-1, along with uPA and its receptor uPAR are synthesized by multiple cell types within the tumour milieu and that the expression of components of the plasminogen activation system varies between different types of cancer. It seems that a balance between plasmin generation and its inhibition by PAI-1 allows successful cell invasion and angiogenesis.¹⁵

Our group has previously described a correlation of PAI-1 expression in HT-1080 fibrosarcoma cells with lung colony formation after tail vein inoculation.¹⁶ In addition, in a preliminary study with PAI-1 transfected cells, a

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2–3-fold increase in PAI-1 also increased the number of *in vivo* lung colonies.¹⁷ Here, we report that fibrosarcoma cells transfected with PAI-1 cDNA that express a 29-fold increase of PAI-1 as compared with control cells form very significantly elevated numbers of lung nodules after tail vein injection of nude mice. *In vitro*, the ability of the PAI-1 overexpressing cells to induce angiogenesis was not affected as determined by a capillary tube formation assay but their adhesive capacity to ECM components was significantly increased as compared with control cells.

MATERIALS AND METHODS

Cell culture

The HT-1080 human fibrosarcoma cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 media (Sigma-Aldrich, Austria) supplemented with 10% heat-inactivated calf serum (Hyclone Laboratories Inc., Utah, USA), 2 mM L-glutamine, 100 U/mL penicillin, 250 ng/mL fungisone, and 100 µg/mL streptomycin (Bio Whittaker Inc., MD, USA). A clonal cell line (1–3C) that expressed low levels of PAI-1 was isolated from HT-1080 cells by limiting dilution.

Stable transfection of sense and antisense PAI-1 cDNA

An EcoR I-Bgl II fragment of full length human PAI-1 cDNA (1426 bp) was inserted in a sense orientation into an expression plasmid BCMGS-Neo kindly provided by the Institute of Molecular Pathology, Vienna, Austria. An antisense construct was prepared by insertion of a Sal I-EcoR I fragment (1005 bp) of PAI-1 cDNA in an opposite orientation into the expression plasmid. Exponentially growing 1–3C fibrosarcoma cells were plated in six well dishes at a density of 5×10^5 cells/well and grown overnight to 50% confluence. Cells were transfected with either sense, antisense or control plasmids with Lipofectamine (GibcoBrl, Life Technologies, Austria) according to the manufactures instructions. Stable transfected clones were isolated by neomycin resistance and continually grown in medium containing 100 µg/mL of geneticin (Sigma-Aldrich, Austria). PAI-1 protein and activity levels were measured in the conditioned media of cells by ELISAs (Technoclone Inc., Austria).

Lung colonization assay

Four to six week old balb/c athymic mice were obtained from Vienna University Research Institute for Experimental Animals and acclimatized for one week before use. Transfected cell lines were washed twice in serum containing media without geneticin antibiotic and

resuspended in PBS at 3×10^6 cells/mL. 0.1 mL was slowly injected into the tail vein. Twenty-one days later mice were sacrificed, lungs were stained with 15% india ink (Pelikan, Germany) by intratracheal injection, harvested and fixed in Fekete's solution (70% ethanol, 10% formaldehyde and 5% glacial acetic acid). The total number of unstained colonies on the lung surface was counted under a dissection microscope.

Primary tumour formation

Transfected cell lines were washed twice in serum containing media without geneticin and resuspended in PBS at 1×10^7 cells/mL. 0.1 mL was inoculated subcutaneously into 4–6-week-old balb/c athymic mice. Twenty-one days later mice were sacrificed, palpable tumours excised and weighed.

Capillary tube formation assay

The effect of PAI-1 on angiogenesis was determined by a co-culture of transfected cell lines with human umbilical vein endothelial cells (HUVEC) by using transwell plates (Costar Europe, the Netherlands). In the lower chamber, tumour cells were plated in duplicate and allowed to grow to confluence overnight. Confluent HUVECs (Technoclone Inc., Austria) at passage 4 that were previously incubated for 24 h in M199 medium (Sigma-Aldrich, Austria) containing 5% supplemented calf serum, but without additional growth supplements were added to transwell inserts coated with 1 mg of matrigel (Becton Dickinson, MA, USA). Resulting capillary tubes were photographed using phase-contrast microscopy (six randomly selected fields) and analysed using NIH-Image software. The total length of tubes and capillary branching points were calculated.

Cell adhesion assay

Ninety-six well plates were coated with 5 µg/well of either matrigel, human vitronectin, human fibronectin (Technoclone, Inc., Austria), or rat collagen type I (Upstate Biotechnology Inc, NY, USA) in HBSS (BioWhittaker Inc., MD, USA) for 2 h at 37°C. Non-specific sites were blocked by addition of 100 µL 3% BSA (Sigma-Aldrich, Austria) in HBSS for 30 min at 37°C. Tumour cells were washed twice with RPMI containing 0.1% BSA and adjusted to 2×10^5 cells/mL. Ninety-six well plates were washed twice with HBSS and 100 µL cell suspension (2×10^4 cells) was added to the wells. Cells were incubated for 15, 30 or 60 min at 37°C and 5% CO₂. After incubation, non-attached cells were aspirated and wells were washed twice with RPMI containing 0.1% BSA. Attached cells were quantified by addition of 50 µL

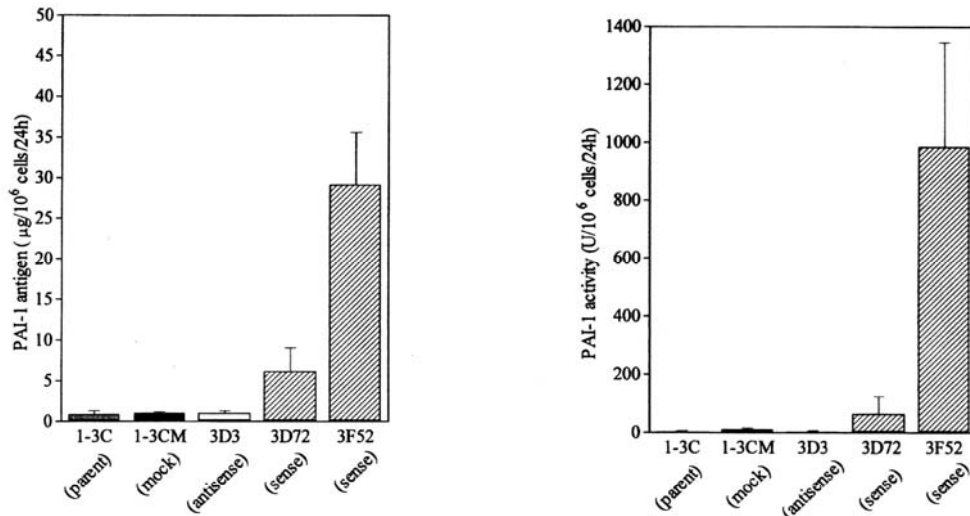


Fig. 1 Expression of PAI-1 antigen and activity following transfection of 1-3C cells. Levels of total PAI-1 antigen (left panel) and PAI-1 activity (right panel) were determined in conditioned media by ELISA for the 1-3C parent cell line (grey bars), mock transfected cells (black bars) and in clonal cell lines transfected with antisense PAI-1 cDNA (open bars) or sense PAI-1 cDNA (hatched bars). Values represent the mean \pm SD of between 3-10 independent determinations.

of 0.5% crystal violet (Sigma-Aldrich, Austria) in 25% methanol. After 10 min, excess stain was removed by washing three times with distilled water. Plates were allowed to dry overnight and crystal violet pigment was dissolved in 100 μ L 0.1 M sodium citrate (Sigma-Aldrich, Austria) in 50% ethanol for 20 min. Cell adhesion was quantified by determination of absorbance at 540 nm and 405 nm reference in an ELISA plate reader.

RESULTS

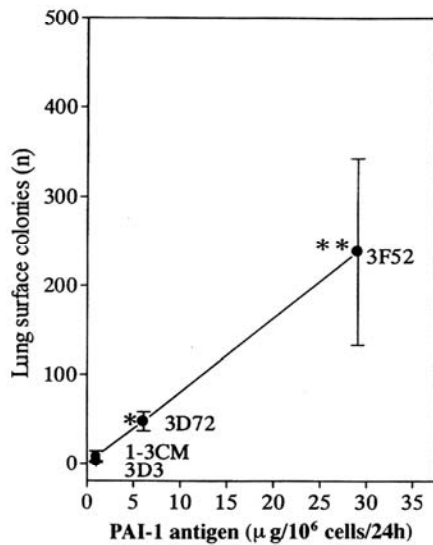
Figure 1 illustrates the expression of total PAI-1 protein and active PAI-1 levels following stable transfection of 1-3C fibrosarcoma cells with either sense or antisense PAI-1 cDNA. As a control, cells were also transfected with the expression plasmid. No difference in PAI-1 secretion was observed following transfection with the vehicle alone (1-3CM) as compared with the parent cells (1-3C). Two clones were selected after transfection with sense PAI-1 cDNA. One expressed a 6-fold increase in total PAI-1 antigen (3D72). A second (3F52) secreted over a 29-fold increase in total PAI-1 antigen levels as compared with the control 1-3CM cells. Following transfection with antisense PAI-1 cDNA an additional clone was isolated (3D3). No significant reduction in total PAI-1 protein was observed, but active PAI-1 levels were 67% reduced as compared with mock transfected 1-3CM control cells.

The PAI-1 expressing cell lines were analysed for their ability to colonize the lung after tail vein injection. A statistically significant positive correlation was observed between PAI-1 secreted by the transfected cell lines and

the number of pulmonary colonies determined (Fig. 2). Both PAI-1 sense transfected cell lines, 3D72 and 3F52 produced significantly higher numbers of lung colonies ($P < 0.02$ and $P < 0.005$ respectively) than mock transfected cells. The antisense PAI-1 cell line, 3D3 resulted in fewer colonies than the control cells, but this was not statistically significant. The transfected cell lines were also analysed for their ability to produce solid tumours in nude mice. No palpable tumours were detected following injection of the mock transfected cells or the overexpressing PAI-1 cells in nude mice (data not shown).

A recent report has shown that host PAI-1 may be necessary for angiogenesis.¹² The ability of the PAI-1 transfected cell lines to influence angiogenesis was therefore tested in a co-culture assay with human umbilical vein endothelial cells. A network of capillary tubes was observed following co-culture with mock transfected control cells. No differences in total tube length or capillary branching points were observed following co-culture of HUVEC with either sense or antisense PAI-1 transfected cells (data not shown).

Apart from the well-defined role of PAI-1 as an inhibitor of tPA and uPA, it has also been shown to influence adhesion on vitronectin. PAI-1 can block adhesion of cells that form cellular attachments via uPAR - vitronectin interactions by virtue of PAI-1's increased affinity for vitronectin.⁵ We assayed whether the adhesive properties of fibrosarcoma cells that continuously secrete high PAI-1 were altered in an in vitro cell adhesion assay (Fig. 3). Ninety percent confluent cultures were harvested and washed to remove exogenous PAI-1 before addition



to ECM proteins. Following 30 min incubation, adhesion of 3D72 cells was increased by 1.5-fold to matrigel, collagen type 1, fibronectin and vitronectin coated wells as compared with mock transfected cells. Adhesion of 3F52 cells was also 1.5 fold increased to matrigel and collagen type 1 and 2.5 and 2-fold increased to fibronectin and vitronectin coated wells respectively as compared with mock transfected cells. Additionally, the increased adhesive phenotype of 3F52 cells was also observed at shorter incubation times (15 min) indicating that the increase in adhesion of the cells was not likely due to a protective effect on the ECM proteins by PAI-1 deposition by the cells (data not shown).

DISCUSSION

Most transfection studies with PAI-1 have usually resulted in inhibition of tumour cell invasion, tumour growth and spread most likely due to inhibition of the uPA dependent plasminogen activation cascade.⁷⁻⁹ However, few of these studies have analysed the effects of PAI-1 on the later stages of metastasis formation with an assay specific for tumour cell lodgement as used in these experiments. This model is generally known as an assay of experimental metastasis formation. In this study the effects of elevated PAI-1 expression in a non-aggressive clonal cell line of human fibrosarcoma were examined. We have shown that cells that secrete high levels of PAI-1 form significantly increased numbers of experimental lung metastases as compared with control transfected cells. Clinically the PAI-1 content of metastases has been shown to be elevated as compared with the corresponding primary tumour in colorectal cancer¹³ and

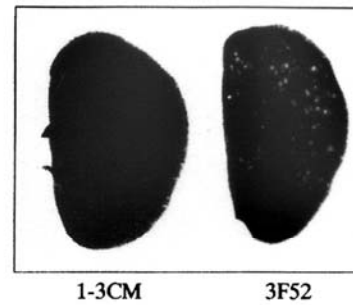


Fig. 2 Influence of PAI-1 on lung colony formation. Mean total PAI-1 antigen values of 1-3 C sense, antisense and mock transfected cells were plotted against the mean number \pm SE of pulmonary nodules determined after tail vein inoculation of nude mice (five to six mice per clone). * $P < 0.02$, ** $P < 0.005$ by Mann-Whitney-U test as compared with mock transfected 1-3CM cells (left panel). Visualization of lung colonies for mock transfected control cells (1-3CM) and high PAI-1 sense transfected cells (3F52) (right panel).

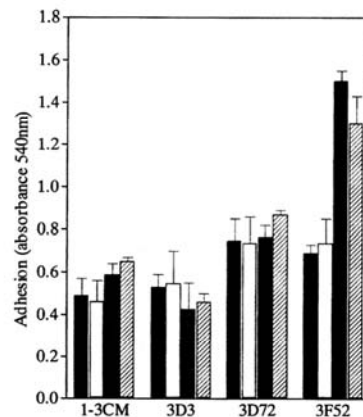


Fig. 3 Effect of PAI-1 on tumour cell adhesion in vitro. The control mock transfected, antisense and sense PAI-1 transfected cell lines were allowed to adhere to matrigel (dark grey bars), collagen type 1 (open bars), fibronectin (light grey bars) or vitronectin (hatched bars) for $t = 30$ min. Values plotted represent mean absorbance \pm SD from one representative experiment. Similar results were obtained in three independent experiments.

ovarian cancer.¹⁸ That PAI-1 can positively increase tumour cell lodgement may be one explanation for these observations and help to explain why PAI-1 is a very strong prognostic risk factor for relapse in a variety of cancers.

There are a number of mechanisms by which high PAI-1 expression might increase tumour cell lodgement in vivo. Fibrin formation has been implicated in promoting metastasis dissemination as has been shown for cells expressing high levels of tissue factor, the initiator of blood coagulation.¹⁹ Similarly, fibrin dissolution would be inhibited in the presence of elevated PAI-1 levels.

Alternatively, PAI-1 can influence angiogenesis. Although some studies have shown PAI-1 to block capillary tube formation and angiogenesis *in vitro* and *in vivo*,^{8,10} host PAI-1 was recently shown to be necessary for tumour angiogenesis.¹² In a co-culture system *in vitro* we found no effect of tumour cell secreted PAI-1 on capillary tube formation by this fibrosarcoma clonal cell line (1-3C). In contrast when a second HT-1080 clonal cell line (26-6) was transfected with sense PAI-1, capillary tube formation was inhibited (data not shown). The 26-6 cell line was also able to form primary tumours in nude mice, as is generally the case for the highly tumorigenic HT-1080 cell line. These differential effects of PAI-1 in two clonal cell lines of HT-1080 may depend on differing angiogenic stimuli secreted by the two clonal cell lines and the utilization of PAI-1 dependent and independent angiogenic pathways. We indeed have found higher constitutive levels of both PAI-1 and vascular endothelial growth factor (VEGF) in the 26-6 cell line. In case of the 1-3C cells, given that no differences were observed on primary tumour growth or on capillary tube formation with cells secreting elevated PAI-1, the increase in pulmonary metastases observed was not likely due to alterations in tumour growth or angiogenesis. We do acknowledge that additional clonal variations among our transfected cells may have given rise to the results described in this report. However, we have tried to minimize confounding factors by transfecting a clonal cell line of HT-1080 and not the heterogenous HT-1080 cells themselves that are known to exhibit cellular variation of uPA, tPA and PAI-1.²⁰

In addition to regulating plasminogen activation, PAI-1 can also influence cellular adhesion and migration. Due to its high affinity for vitronectin, PAI-1 blocks binding of vitronectin to at least two of its receptors, uPAR and $\alpha_v\beta_3$ integrin. Through this mechanism PAI-1 can inhibit cell adhesion to and migration on vitronectin.³⁻⁵ To our knowledge this is the first study to show that high PAI-1 expressing cells are more adhesive as compared to their lower PAI-1 counterparts. Fibrosarcoma cells use integrins for cellular attachment. UPAR may directly interact with integrins and alter their affinity for matrix proteins and subsequent adhesion.²¹ Specifically, the β_1 and β_3 sub-units associate with uPAR on HT-1080 cells.²² Interestingly, previous studies have shown that antisense uPAR transfected glioblastoma cells were more adhesive to ECM proteins²³ and resulted in differential expression of integrin sub-units.²⁴ PAI-1 can alter uPAR levels by internalization of uPAR/uPA/PAI-1 complexes and this may be one explanation for the differences in adhesion of the sense PAI-1 transfected cells. In summary, the data presented in this study provide evidence that PAI-1 can influence the later stages of metastasis formation by promoting lung colonization and that one possible

explanation may be due to alterations in the adhesive phenotype of high PAI-1 expressing cells.

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