Plasminogen activator inhibitor-1 and -3 Increase Cell Adhesion and Motility of MDA-MB-435 Breast Cancer Cells*

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Plasminogen activator inhibitor-1 (PAI-1), an inhibitor of urokinase plasminogen activator, is paradoxically associated with a poor prognosis in breast cancer. PAI-1 is linked to several processes in the metastatic cascade. However, the role of PAI-1 in metastatic processes, which may be independent of protease inhibitory activity, is not fully understood. We report herein that PAI-1, when added exogenously to or stably transfected in human MDA-MB-435 breast carcinoma cells, had disparate effects on adhesion to extracellular matrix proteins and motility in vitro. Specifically, exogenously added PAI-1 inhibited cell adhesion to vitronectin but not fibronectin, in agreement with the literature. By contrast, stably transfected PAI-1 stimulated adhesion to both proteins. Wild-type PAI-1 was required for this stimulation, because expression of a non-protease inhibitory T333R PAI-1 mutant failed to enhance adhesion. Compared with non-inhibitory PAI-1, wild-type PAI-1 also increased cell motility in chemotactic assays. Furthermore, stable transfection of a related serine protease inhibitor, plasminogen activator inhibitor-3 (PAI-3, or protein C inhibitor) gave results similar to wild-type PAI-1. The stimulatory activity of PAI-3 was not seen with a non-protease inhibitory P14 PAI-3 mutant (T341R). We show that a downstream effect of endogenous wild-type PAI-1 and PAI-3 overexpression, but not their non-inhibitory counterparts, was the altered expression of αv, α5, αv, αv, and β3 integrin subunits. Additionally, blocking antibodies to β3 integrin inhibited PAI-1-induced adhesion. Our data provide experimental support for the stimulatory and inhibitory effects of PAI-1 in metastasis and introduce PAI-3 as another serpin potentially important in malignant disease.

Metastasis is a multistep process that involves the coordinated events of proteolysis, adhesion, and migration. Plasminogen activator inhibitor-1 (PAI-11; SERPINE1) and plasminogen activator inhibitor-3 (PAI-3; also known as protein C inhibitor; SERPINS5) are related serine protease inhibitors (serpins) of the plasminogen activator system (1–5). PAI-1, the primary inhibitor of the plasminogen activator system, inactivates urokinase plasminogen activator (uPA), but it also has a role in cell adhesion and migration. Several studies have shown that PAI-1 expression in breast and other types of cancer is linked with a poor prognosis (6–8).

Identification of the biological function of PAI-1 in cancer is complicated by findings that PAI-1 can act independently of its protease inhibitory activity. PAI-1 inhibits in vitro adhesion of multiple cell lines to the extracellular matrix protein, vitronectin (VN) (9). PAI-1 binds VN (10), and this PAI-1-VN interaction blocks cell integrin (αvβ3 and αvβ3) adhesion to VN (11). The uPA receptor (uPAR) can also bind VN and has been identified as an integrin-independent cell surface VN receptor (9). Although PAI-1 and uPAR both share N-terminal binding sites on VN (10), PAI-1 has a higher affinity for VN, and consequently, competitively inhibits cell uPAR-VN binding (12). Deng et al. (9) proposed that increased PAI-1 could release cells bound to VN by uPAR and promote cell dissemination, possibly explaining the role of PAI-1 in a metastatic disease process.

In addition to the adhesive interactions, there is increasing evidence that PAI-1 can mediate cell migration (12–16). PAI-1 has been shown to either inhibit or stimulate cell migration on VN. Kjoller et al. (13) added exogenous PAI-1 to a modified Boyden chamber migration assay in which the filters were coated with VN and found that active PAI-1 bound to VN, blocked uPAR and integrin binding, and subsequently blocked migration of human epidermoid carcinoma Hep-2 cells. By contrast, Stahl and Mueller (15) found that exogenous PAI-1 stimulated melanoma cell migration on VN. It is unclear why exogenous PAI-1 had opposing effects in each of the studies, but the differences may be associated with differences in assay conditions, cell specificity, or protein conformation.

PAI-3 is synthesized in the liver and in numerous steroid-responsive organs. PAI-3 antigen has been detected in saliva, cerebral spinal fluid, amniotic fluid, tears, and semen (4, 17–19). In contrast to PAI-1, PAI-3 inhibits a broad array of proteases, including uPA, TPA, activated protein C, thrombin (free and bound to thrombomodulin), and acrosin (19–23). PAI-3, BSA, bovine serum albumin; CTX, chemotaxis; ECM, extracellular matrix; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FN, fibronectin; HTX, haptotaxis; LN, laminin; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; P14, non-inhibitory mutant for PAI-1 T333R and for PAI-3 T341R; PA, plasminogen activator; tPA, tissue-type plasminogen activator; uPA, urokinase plasminogen activator; serpin, serine protease inhibitor; uPAR, urokinase plasminogen activator receptor; VN, vitronectin; wt, wild-type; MEM, minimal essential medium; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
whose expression is increased in prostate cancer, also inhibits prostatic glandular kallikreins, suggesting that it may be linked to carcinogenesis in hormone-regulated tissues (17, 24). PAI-3 is found in many hormone-responsive tissues, is a uPA inhibitor, and can be localized to malignant breast tissue. The biological significance of a cancer expressing PAI-3 rather than other related serpins (PAI-1, PAI-2, or maspin) that have been implicated in various tumor cell biology processes is unknown (6, 7).

We hypothesized that the role of PAI-1 in cell adhesion and motility would be independent of its ability to inhibit serine proteases. Because PAI-3 also inhibits uPA but does not bind VN, it was used to compare and contrast to the interactions of PAI-1. We established stably transfected MDA-MB-435 breast cancer cells expressing wild-type and non-inhibitory mutants of PAI-1 and PAI-3 and characterized their biological properties. In contrast to our initial hypothesis, what we report here is that changes in adhesion, integrin expression, and cell migration of MDA-MB-435 cells was dependent on expression of wild-type PAI-1 and PAI-3 and was not observed from their non-inhibitory serpin counterparts. These results suggest that expression of PAI-1 and PAI-3 may render a tumor cell better able to invade and may partly explain the association of PAI-1 with metastatic disease.

EXPERIMENTAL PROCEDURES

Cells—MDA-MB-435 and MDA-MB-231 breast tumor cells and the hepatocellular carcinoma cell line Hep G2 were obtained from the University of North Carolina Tissue Culture Facility. Cells were maintained as monolayer culture in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% sodium pyruvate in a humidified chamber with 5% CO2 at 37 °C. MDA-MB-435 cells were transfected with pcDNA1 vectors (Invitrogen) containing wt-PAI-1 (1), P14-PAI-1 (T510R), wt-PAI-3 (55), or P14-PAI-3 (T314R) using Effectene (Qiagen) according to the manufacturer’s recommendations. Stably transfected clones were selected for resistance to the neomycin analogue, G418 (Invitrogen).

Reverse Transcription PCR—Total RNA was isolated from the MDA-MB-435 cells and HepG2 cells using RNeasy (Qiagen). Two micrograms of RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) per the manufacturer’s recommendations. cDNA was amplified with each cycle consisting of a 1-min denaturation step at 94 °C, a 1-min annealing step at a primer-specific temperature (given in parentheses below for each primer set), and a 1-min elongation step at 72 °C. A pre-amplification denaturation at 94 °C for 5 min and a post-amplification elongation at 72 °C for 5 min were also included. The primer sequences were: PAI-1 (61 °C), sense 5′-AACAGACGGCGACGCTGT-3′, antisense 5′-CTGAACAGTGCAGTGCATCTACC-3′; PAI-3 (55 °C), sense 5′-AGGCAAGGAGAACAGCTGACTGC-3′, antisense 5′-CTGTTAAGAATCGCTTCTGAGG-3′; p53 (55 °C), sense 5′-GCACGCGGCTGAGGAAGAAGCAG-3′, antisense 5′-CGGCACTCCTCCATCCGACAAGGAACTGCTC-3′; p53 (55 °C), sense 5′-GACATTCCACCATGCTCCCTGCG-3′; p53 (50 °C), sense 5′-GGACAACTGTCTCGGTCCAGG-3′; p53 (50 °C), sense 5′-TTTCCCAGCTGGCTGCTGCTG-3′; p53 (50 °C), sense 5′-TATCAGGCGTGGCAGCGCAG-3′; p53 (50 °C), sense 5′-GCACCTGGGCTGAGGAGAAAGCAG-3′; p53 (50 °C), sense 5′-GCATTGGCGGTCAAGGAACTGCTC-3′; p53 (50 °C), sense 5′-GGTCCTCCTGGCAGCTGCTC-3′; p53 (50 °C), sense 5′-GGTCGTGGGGCTGGGAGCAG-3′; p53 (50 °C), sense 5′-GGAGGAGTCCGTTGTGGACTGC-3′; annexin II, sense 5′-CTTGGGCAGAAGAAGCTGCTG-3′; annexin II, sense 5′-CTTGGGCAGAAGAAGCTGCTG-3′; annexin II, sense 5′-CTTGGGCAGAAGAAGCTGCTG-3′; annexin II, sense 5′-CTTGGGCAGAAGAAGCTGCTG-3′. 

Immunohistochemistry—Cells were plated at a density of 20,000/12 mm² on sterile glass coverslips and allowed to adhere in normal growth medium containing 10% FBS for 4 h, washed 1× with phosphate-buffered saline (PBS), and incubated overnight in serum-free MEM. Cells were fixed in 30% ethanol with 2% paraformaldehyde for 30 min at room temperature and permeabilized with a 0.5% Triton X-100 buffer containing 300 mM sucrose, 20 mM Hepes (pH 7.4), 50 mM NaCl, and 3 mM MgCl₂ for 5 min on ice. Cells were blocked in 10% normal serum from the same species as the secondary antibody for 30 min. Staining was performed at room temperature for 1 h with the following primary antibodies: rabbit monoclonal anti-human PAI-3 (Molecular Innovations, Southfield, MI) and mouse monoclonal anti-human PAI-3 (G4–2, prepared in our laboratory). Biotinylated secondary antibodies (Vector Laboratories) were used in conjunction with the Vectastain ABC Kit for detection. Nuclei were counterstained with hematoxylin.

Enzyme-linked Immunosorbent Assay—ELISAs were used to determine the concentration of PAI-1 or PAI-3 protein secreted by the transfected cells. A PAI-1 Immulise kit (BioPool International) and PAI-3 Paired Antibody Sandwich ELISA kit (Affinity Biologicals) were used according to the manufacturer’s instructions.

Adhesion Assay—Forty-eight well tissue culture plates were coated with VN (BD Bioscience), FN (BD Bioscience), or LN (BD Bioscience) at a concentration of 10 μg/ml at 37 °C for 1 h. All plates were rinsed with PBS and blocked with 1% heat-inactivated bovine serum albumin (BSA) for 1 h at 37 °C. Plates were again rinsed with PBS and air-dried. Cells were seeded at 5 × 10⁴ cells/well in 200 μl of serum-free medium and allowed to attach for 1 h at 37 °C. Non-adherent cells were removed with a multi-channel pipette, and adhered cells were gently washed twice with PBS. An MTT assay was performed to determine the number of adhered cells (26). Briefly, 200 μl of 5 μg/ml MTT in serum-free MEM was added to each well and incubated for 2 h at 37 °C. All MTT solution was removed, and 200 μl of dimethyl sulfoxide was added to solubilize the formazan crystals for 20 min at 37 °C. Samples were transferred to a 96-well plate, and the absorbance was measured at 600 nm in a Vmax microtiter plate reader (Molecular Devices). In the competition studies for MDA-MB-435 cell adhesion to VN and FN surfaces, human wt-PAI-1 was from Molecular Innovations, and human plasma PAI-3 was from Affinity Biologicals.

Immunofluorescent Staining—For F-actin staining, cells were plated at a density of 20,000/12 mm² on sterile glass coverslips previously coated with either VN or FN (50 μg/ml). The coverslips were coated according to the above-mentioned procedure for 48-well plates. Cells were allowed to adhere in the absence of serum for 1 h at 37 °C. After gently removing the non-adherent cells, coverslips were fixed as described above. Cells were stained with FITC-labeled phalloidin (Molecular Probes) at a 1:200 dilution in PBS for 30 min at room temperature. Nuclei were stained with Hoechst (1:40,000, Molecular Probes) for 1 min at room temperature. Coverslips were rinsed in PBS and mounted with 50% glycerol in PBS.

Flow Cytometry for Integrin Subunit Expression—Half a million cells were washed with PBS containing 1% BSA by centrifuging at 1000 rpm for 5 min at 4 °C. Pelleted cells were incubated with 100 μl of an ascitic fluid containing integrin antibodies diluted with serum-free medium for 1 h at 37 °C. A 1:1 dilution of 1.5 × 10⁴ events were counted for each sample. Listmode files were replayed for data analysis by using WinMDI 2.7 software.

Immunoblot Analysis—Production of uPA protein in the wt- and P14-PAI-1-expressing MDA-MB-435 cells was determined essentially as described by Ma et al. (27). MDA-MB-231 cells that constitutively express uPA were used as a positive control. From confluent cultures of wt- and P14-PAI-1-expressing MDA-MB-435 and MDA-MB-231 cells, 30 μg of total protein (by Bradford assay) from each cell type was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with an ascitic fluid monoclonal antibody (#390 from Oncogene Science, Inc.) followed by anti-mouse IgG-peroxidase conjugate. Secondary antibody detection was by enhanced chemiluminescence.

Motility Assays—Experiments were conducted using BioCoat² culture inserts (BD Bioscience) with an 8-μm pore size membrane in a 24-well companion plate. For chemotaxis (CTX), VN or FN coated inserts were incubated with 0.1% BSA (750 μl) and added to each well of the plate. Cells were seeded at 5 × 10⁴ cells (500 μl) in the culture insert in serum-free medium with 0.1% BSA and incubated for 4 h at 37 °C. For haptotaxis (HTX), the lower surfaces of the culture inserts were coated with VN or FN (50 μg/ml) in serum-free medium with 0.1% BSA for 2 h at 37 °C. Inserts were then rinsed with serum-free medium and added to the plate. Cells were incubated for 5 h at 37 °C. After the incubation period for both the CTX and HTX experiments, the media was removed from the insert, and cells on the upper surface
of MDA-MB-435 cells without the addition of recombinant protein. These experiments were performed in triplicate, and each data point represents \( n = 3 \). Each bar represents the mean \( \pm S.D \). This is a representative experiment of at least \( n = 3 \).

Statistical Analysis—Statistical analysis was performed using InStat, GraphPad Software, Inc. A one-way analysis of variance test was performed to quantify the number of adhered cells as described under “Experimental Procedures.” Data are represented as percent adhesion of MDA-MB-435 cells without the addition of recombinant protein. These experiments were performed in triplicate, and each data point represents \( n = 3 \). Each bar represents the mean \( \pm S.D. \) This is a representative experiment of at least \( n = 3 \).

of the membrane were removed by “scrubbing” the membrane with a cotton-tipped applicator. Cells that migrated to the lower surface of the membranes were fixed to the membrane with 100% methanol for 5 min. Inserts were then washed with PBS and stained with Hoechst (Molecular Probes) diluted in PBS to a final concentration of 500 \( \mu g/ml \) for 2 min. The membranes were excised from the insert, inverted, and mounted on glass microscope slides. The total number of nuclei was counted in each of three fields at 40× magnification using UV fluorescence microscopy.

RESULTS

Effect of Exogenously Added PAI-1 and PAI-3 on MDA-MB-435 Cell Adhesion to VN—Exogenous PAI-1 can bind to VN and release cells bound to this substratum (9). We confirmed this interaction in adhesion experiments when exogenous wt-rPAI-1 and MDA-MB-435 were added to a VN coated plate at the same time, wt-rPAI-1 inhibited cell adhesion in a dose-dependent manner (Fig. 1, upper panel). The addition of wt-rPAI-1 (50 nM) blocked MDA-MB-435 cell adhesion to VN by \(~50\%\). A PAI-1 blocking antibody added at the same time as the wt-rPAI-1 restored cell adhesion to approximately that of the untreated control cells, and exogenous wt-rPAI-1 did not block cell adhesion to FN, because PAI-1 does not bind FN (data not shown).

Because PAI-3 lacks a VN binding site (28), exogenous PAI-3 should not affect cell adhesion to VN. Confirming this prediction, in adhesion experiments when exogenous wt-PAI-3 was added with MDA-MB-435 cells at the time of plating, there was no effect on cell adhesion either to VN (Fig. 1, lower panel) or to FN (data not shown).

Expression of Transfected PAI-1 and PAI-3 Genes in MDA-MB-435 Cells—To test the effect of endogenously expressed plasminogen activator inhibitors on tumor cell adhesion and motility, MDA-MB-435 cells were stably transfected with PAI-1 and PAI-3. The parental MDA-MB-435 cells lack endogenous gene expression of PAI-1 and PAI-3 making them ideal candidates for evaluating a role for these serpins in cancer cells (Fig. 2). MDA-MB-435 cells were transfected with pcDNA3.1 mammalian expression vectors containing either wt-PAI-1, P14-PAI-1, wt-PAI-3, or P14-PAI-3 genes. At least two clones were derived for each vector. Expression of the transfected genes and protein production was verified by rtPCR (Fig. 2, upper panels) and by immunoblot (data not shown). Protein expression was also detected by immunofluorescent staining for PAI-1 in clone 1 of wt-transfected and clone 2 of P14-PAI-1-transfected cells, and for clone 2 and clone 1 for wt and P14-PAI-3, respectively (Fig. 2, lower panel). There was no visible difference in protein localization between wt- and P14-expressing clones for PAI-1 or PAI-3, respectively. In non-permeabilized transfected MDA-MB-435 cells, immunofluorescent staining verified that both PAI-1 and PAI-3 were localized to the cell surface (data not shown).

By ELISA, wt-PAI-1-expressing clone 1 and P14-PAI-1-expressing clone 2 secreted 12 and 8.0 \( \mu g/ml \) per \( 1 \times 10^6 \) cells, respectively. By ELISA, wt-PAI-3-expressing clone 2 and P14-PAI-3-expressing clone 1 secreted 6.1 and 8.4 \( \mu g/ml \) of protein per \( 1 \times 10^6 \) cells, respectively. The P14-PAI-1 and P14-PAI-3-expressing MDA-MB-435 cells represent a population that was not only subjected to the same transfection
method and antibiotic resistant selection as the wild-type population for both PAI-1- and PAI-3-expressing cells, but the P14 populations were transfected with an identical vector and gene sequence with the exception of a single nucleotide. Thus, changes in phenotype between the various PAI-1/PAI-3-expressing MDA-MB-435 clones should reflect biological differences between wild-type active and mutant non-inhibitory serpin effects on the MDA-MB-435 cells.

**Effect of Endogenously Expressed PAI-1 and PAI-3 on MDA-MB-435 Cell Adhesion**—Unexpectedly, we found that wt-PAI-1-expressing MDA-MB-435 cells adhered to both VN and FN 2- to 3-fold better than P14-PAI-1 and untransfected MDA-MB-435 cells (Fig. 3, upper panel). Similarly, we found that adhesion of wt-PAI-3-expressing MDA-MB-435 cells increased 3- to 4-fold to both VN and FN compared with P14-PAI-3 and control MDA-MB-435 cells (Fig. 3, lower panel). Adhesion experiments were also performed with laminin (LN) and type I collagen as the substratum. Cell adhesion to LN (Fig. 3) and type I collagen (data not shown) was significantly increased for both the wt-PAI-1- and wt-PAI-3-expressing MDA-MB-435 cells but not for their non-inhibitory P14 counterparts. In control experiments, cell adhesion to tissue culture-treated plastic or to poly-L-lysine-coated plastic was similar between the wt-PAI-1, P14-PAI-1, wt-PAI-3, P14-PAI-3, or untransfected MDA-MB-435 cells. Additionally, six clones of wt-PAI-1-expressing cells isolated and cloned in a separate transfection experiment also adhered to both VN and FN 2- to 3-fold better than untransfected control MDA-MB-435 cells (data not shown).

In adhesion experiments when exogenous wt-rPAI-1 (50 nM) was added to either PAI-1- or PAI-3-expressing MDA-MB-435 cell clones, adhesion was blocked by an average of 40% for wild-type and P14 populations (Fig. 4). Although wt-PAI-1- and wt-PAI-3-expressing cells showed 2- to 4-fold increased adhesion compared with untransfected MDA-MB-435 cells (Fig. 4), the addition of exogenous wt-rPAI-1 at time of plating can still bind VN and reduce cell adhesion compared with PAI-1- and PAI-3-expressing cells plated in the absence of exogenous wt-rPAI-1.

![FIG. 3. Stable transfection of wt-PAI-1 (upper panel) and wt-PAI-3 (lower panel) in MDA-MB-435 cells increased cell adhesion to vitronectin, fibronectin, and laminin. Cells were seeded at 5 × 10^4 cells/well in serum-free media either in the absence or presence of exogenously added wt-rPAI-1 (50 nM) and allowed to attach for 1 h at 37 °C. Non-adherent cells were removed, and the MTT assay was performed to quantify the number of adhered cells as described under “Experimental Procedures.” Data are expressed as percent adhesion of untransfected controls. The experiments were performed in triplicate, and each data point represents n = 3. Each bar represents the mean ± S.D. This is a representative experiment with at least n = 3. *, p < 0.05; **, p < 0.01.

![FIG. 4. Exogenously added PAI-1 partially blocks adhesion of stably transfected PAI-1 and PAI-3-expressing MDA-MB-435 cells to vitronectin. Cells were seeded at 5 × 10^4 cells/well in serum-free media either in the absence or presence of exogenously added wt-rPAI-1 (50 nM) and allowed to attach for 1 h at 37 °C. Non-adherent cells were removed, and the MTT assay was performed to quantify the number of adhered cells as described under “Experimental Procedures.” Open bars, untreated controls; solid bars, exogenously added wt-rPAI-1. Adhesion of the untreated clones is normalized to 100%, and data for wt-rPAI-1-treated clones is expressed as percent adhesion of the untreated control for each clone. The experiments were performed in triplicate, and each data point represents n = 3. Each bar represents the mean ± S.D. This is a representative experiment with n = 3. *, p < 0.05.

**Integrin Profiles of PAI-1 and PAI-3-expressing MDA-MB-435 Cells**—The expression profiles of various integrin subunits on the surface of the wt-PAI-1- and wt-PAI-3-expressing MDA-MB-435 cells were determined to begin to investigate a mechanism for the increased cell adhesion observed. The levels of α5, α2, α3, α4, α6, αβ1, β3, and βα integrins were assessed by flow cytometry. The left side of Fig. 6 shows the integrin profiles for α2, α3, α4, and β3 in the control, wt-PAI-1- and...
P14-PAI-1-expressing MDA-MB-435 cells, respectively. The levels of these integrin subunits were increased on the surface of wt-PAI-1-expressing MDA-MB-435 cells compared with the P14-PAI-1-expressing cells and the control MDA-MB-435 cells. Similar results were observed for wt-PAI-3 compared with P14-PAI-3-expressing MDA-MB-435 cells (Fig. 6, right side). There were also increased levels of the $\alpha_1$ and $\alpha_5$ integrin subunits detected on the wt-PAI-3-expressing MDA-MB-435 cells (data not shown). We detected no change in the integrin profiles for $\alpha_2$, $\alpha_6$, $\alpha_v$, $\beta_3$, or $\beta_4$ subunits on wt-PAI-1-, P14-PAI-1-, or P14-PAI-3-expressing MDA-MB-435 cells compared with control MDA-MB-435 cells.

Because the prototypic VN receptor is $\alpha_5\beta_3$, we were surprised that we did not detect increased levels of these integrin subunits on the surface of the wt-PAI-1- and wt-PAI-3-expressing cells. Further flow cytometry analysis with an antibody that detected the $\alpha_5\beta_3$ heterodimer was performed. There was no detectable difference in the expression of $\alpha_5$, $\beta_3$, or $\alpha_5\beta_3$ subunits on the surface of the wt-PAI-1-expressing cells, or on the P14-PAI-1-expressing and control populations.

To investigate whether the increased expression of the $\beta_1$ integrin subunit was at least partially responsible for the increased adhesion of the wild-type-expressing cells, we conducted a series of adhesion experiments with a $\beta_1$-blocking antibody to substrates that require integrin adhesion through $\beta_1$ subunits, namely FN and LN, but not VN. Addition of an anti-human $\beta_1$ antibody significantly decreased wt-PAI-1-expressing MBA-MB-435 cell adhesion to both FN and LN, but the antibody had no effect on adhesion to VN (Fig. 7). In control experiments, a nonspecific IgG did not significantly alter wt-PAI-1-expressing MBA-MB-435 cell adhesion to any of the protein substrates (data not shown).

Expression of Plasminogen Activator System Components in PAI-1- and PAI-3-expressing MDA-MB-435 Cells—Because the phenotype of the MDA-MB-435 cells expressing either wt-PAI-1 or wt-PAI-3 was different with regard to adhesion and integrin expression compared with control and their P14-PAI-1/PAI-3 counterparts, we questioned whether gene expression of the plasminogen activation system components had been altered. We compared the mRNA expression of uPA, tPA, uPAR, and annexin-II in the MDA-MB-435 cells. MDA-MB-435 cells do not express uPA (29, 30), and likewise, we did not detect uPA in control MDA-MB-435 cells or P14-PAI-1/P14-PAI-3-expressing cells (Fig. 8, upper panel). Interestingly, transfection of MDA-MB-435 cells with wt-PAI-1 and wt-PAI-3 induced uPA mRNA expression (Fig. 8, upper panel). By immunoblot analysis, we verified that uPA protein was synthesized in wt-PAI-1-expressing MDA-MB-435 cells but not in P14-PAI-1-expressing MDA-MB-435 cells (Fig. 8, lower panel). The other components of the plasminogen activation system
were apparently constitutively expressed and not altered in the MDA-MB-435 cells, regardless of PAI-1/PAI-3 expression (Fig. 8).

Motility of PAI-1- and PAI-3-expressing MDA-MB-435 Cells—To investigate whether the increased cell adhesion affected the in vitro cell motility of the MDA-MB-435 cells, we measured chemotaxis (CTX) and haptotaxis (HTX) (31). Cell movement toward VN and FN was assessed using a modified Boyden chamber assay for both CTX and HTX. CTX measures cell migration toward a soluble chemoattractant gradient, whereas HTX measures cell migration in response to a bound ligand (31). Some debate exists as to whether there are fundamental differences between CTX and HTX. Taraboletti et al. (32) found that separate domains of the thrombospondin molecule were responsible for CTX and HTX and that antibodies specific to each domain could block CTX and HTX independent of the other domain. This suggested that at least in vitro, functional differences exist between CTX and HTX.

Wild-type PAI-1-expressing MDA-MB-435 cells had significantly increased CTX in response to both VN and FN compared with P14-PAI-1-expressing MDA-MB-435 cells and control MDA-MB-435 cells (Fig. 9, upper panel). Overall, wt-PAI-1-expressing MDA-MB-435 cells had a better chemotactic response to VN than to FN by an average of five cells per field. The chemotactic response was specific for VN and FN, because when FN was used as the chemoattractant, the wt-PAI-1-expressing MDA-MB-435 cells, the P14-PAI-1-expressing MDA-MB-435 cells, and the control MDA-MB-435 cells had similar rates of motility (data not shown). In negative control experiments, BSA was used as the chemoattractant and migration was not stimulated in the wt-PAI-1, P14-PAI-1, or control MDA-MB-435 cells. Compared with wt-PAI-3-expressing MDA-MB-435 cells, the P14-PAI-1-expressing MDA-MB-435 cells had a significantly increased CTX response to VN and FN compared with P14-PAI-1-expressing and control MDA-MB-435 cells (data not shown). Functional differences between CTX and HTX may exist between the PAI-1 and PAI-3-expressing MDA-MB-435 cells and control MDA-MB-435 cells, but P14-PAI-3-expressing MDA-MB-435 cells had a significantly increased HTX response to VN and FN compared with wt-PAI-3, P14-PAI-3, or control MDA-MB-435 cells.

Wild-type PAI-1-expressing MDA-MB-435 cells had significantly increased CTX in response to both VN and FN compared with P14-PAI-1-expressing MDA-MB-435 cells and control MDA-MB-435 cells (Fig. 9, lower panel). Overall, wt-PAI-1-expressing MDA-MB-435 cells had a better chemotactic response to VN than to FN by an average of five cells per field. The chemotactic response was specific for VN and FN, because when FN was used as the chemoattractant, the wt-PAI-1-expressing MDA-MB-435 cells, the P14-PAI-1-expressing MDA-MB-435 cells, and the control MDA-MB-435 cells had similar rates of motility (data not shown). In negative control experiments, BSA was used as the chemoattractant and migration was not stimulated in the wt-PAI-1, P14-PAI-1, or control MDA-MB-435 cells. Compared with wt-PAI-3-expressing MDA-MB-435 cells, the P14-PAI-3-expressing MDA-MB-435 cells also had a significantly increased CTX response to VN and FN compared with P14-PAI-3-expressing MDA-MB-435 cells (data not shown). Functional differences exist between CTX and HTX.
shown). Likewise, wt-PAI-3-expressing MDA-MB-435 cells showed significantly increased HITX in response to FN compared with P14-PAI-3-expressing and control MDA-MB-435 cells (data not shown). In negative control experiments, BSA was used as the bound stimulus, and migration was not stimulated in the wt-PAI-1/PAI-3-expressing cells, P14-PAI-1/PAI-3-expressing cells, or control MDA-MB-435 cells.

DISCUSSION

The “pathological balance” between protease activity and protease inhibition in the tumor microenvironment is of paramount biological and medical relevance to tumor biology. The plasminogen activator system has been widely studied in cancer, and increased levels of either uPA or uPAR are known to be associated with a poor prognosis. Likewise, an increase in PAI-1 is also linked to a poor prognosis in different types of cancer, including breast cancer. Thus, a paradox exists between the function of PAI-1 as a serine protease inhibitor and as a negative survival factor in metastatic disease. In vitro, both exogenous and endogenous PAI-1 inhibit ECM degradation by fibrosarcoma and colon cancer cell lines (33). By contrast, in a PAI-1 knockout mouse, Bajou et al. (34) showed that host PAI-1 was important for both tumor cell invasion and angiogenesis. These and other studies have lead to the theory that PAI-1 has a role in metastatic disease independent of regulating proteolytic degradation of the ECM.

In this study, we tested the hypothesis that active PAI-1 and a non-inhibitory PAI-1 mutant would show divergent roles when expressed in the MDA-MB-435 breast cancer line. We also expressed PAI-3, another uPA inhibitor, in MDA-MB-435 cells to compare and contrast to PAI-1, because PAI-3 lacks the ability to bind VN. Many studies have evaluated the effect of exogenously added PAI-1 on cell adhesion and invasion in vivo and the consequence of immediate events (reviewed in Ref. 35). By transfecting tumor cells with active and inactive constructs of PAI-1 and PAI-3, we were able to assess the effect of constant exposure of serpin expression on a breast cancer cell milieu, albeit in an in vitro environment. Our experiments focused on two hallmarks of tumor cell invasion and metastasis, namely adhesion, and motility.

An important finding in this study was that endogenous expression of either wt-PAI-1 or wt-PAI-3 in the MDA-MB-435 breast cancer cell line increased the adhesive properties of the cells to various substrates such as VN, FN, LN, and type I collagen. Endogenous expression of wt-PAI-1 and wt-PAI-3 yielded similar increased adhesion to VN suggesting that the adhesive properties of the wt-PAI-producing MDA-MB-435 cells are independent of PAI-1/VN binding. The enhanced cell binding effect was only evident in wild-type PAI-1/3-expressing MDA-MB-435 cells, because their P14 non-inhibitory mutant counterparts did not increase adhesion more than control MDA-MB-435 cells. Serpins have a highly conserved reactive site loop region, which is responsible for both protease recognition and inhibition. Mutation in the “hinge” region at position 14 of the reactive site loop generates a non-inhibitory serpin. For PAI-1, this T333R mutation has the same conformation as active, wild-type PAI-1; it is recognized by the protease normally as the wild-type protein, but P14-PAI-1 is unable to form a stable serpin-protease complex (36). Lawrence et al. (37) found that this P14 T333R mutation reduced the inhibition activity of PAI-1 for uPA by -1000-fold. The analogous P14 T341R PAI-3 mutant also had a substantial reduction in protease inhibitory activity. Using in vitro inhibition rate constants (21), PAI-3 is a less effective uPA inhibitor when compared with PAI-1, although PAI-3-uPA complexes have been detected in vivo (19). These results suggest that a tumor cell microenvironment expressing active PAI-1 or PAI-3 could have enhanced cell adhesion properties.

To complement the above results, we found that endogenous expression of either wt-PAI-1 or wt-PAI-3 in the MDA-MB-435 cells had increased levels of α2, α3, α5, and β1 integrin subunits on their cell surface, which may be partly responsible for the increased adhesive properties. The change in cell surface integrin expression was not seen in either P14-PAI-1- or P14-PAI-3-expressing MDA-MB-435 cells. We speculate that the increased adhesion to FN, LN, and type I collagen is partly explained by up-regulation of α2 and β1 integrin subunits. The integrin α5β1 is known as a promiscuous receptor and has been found elevated in several metastatic tumors with increased migration and invasion (38). Mihaly et al. (39) independently reported increased integrin expression in HT1080 cells expressing PAI-1, consistent with our results. Differences in integrin expression and affinity, either increased or decreased, have been seen in many instances for invasive tumor cells compared with their non-invasive counterparts (40). Formation of the uPA-uPAR complex could be partly responsible for increased VN interactions, because uPA expression is increased in wt-PAI-1- and wt-PAI-3-expressing MDA-MB-435 cells. We examined other breast cancer cell lines and found uPA to be expressed only when PAI-1 or PAI-3 is present: for example, in MCF-7 cells (PAI-3 and uPA), MDA-MB-231 cells (PAI-1 and uPA), and MDA-MB-231 cells and BT-20 cells (neither PAI-1 nor PAI-3 and no uPA). Thus, there may be a biological significance to a tumor cell producing either PAI-1 or PAI-3 associated with uPA production (or the converse). Although we do not know the exact role uPA has in the transfected breast cancer cells, our data are consistent with PAI-3 participating in essentially the same interactions as PAI-1. PAI-1 has recently been shown to have a biological significance to a tumor cell producing either PAI-1 or PAI-3 up-regulates various integrin subunits independent of direct serpin binding to a substratum like VN, and this could modify the tumor cell phenotype.

Cell motility and adhesion are linked events coordinated by signaling pathways, receptor expression, and cytoskeletal reorganization. uPAR molecules have been shown to cluster to the leading edge, whereas integrin receptors cluster to the trailing edge and are often left behind as the cell migrates. uPA-uPAR- or uPA-uPAR-integrin-stimulated motility has been described in a variety of normal and malignant cells (reviewed in Ref. 44). We found that endogenous expression of either wt-PAI-1 or PAI-3 up-regulates various integrin subunits independent of direct serpin binding to a substratum like VN, and this could modify the tumor cell phenotype.

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we assayed motility and not invasion, we found endogenous expression of either wt-PAI-1 or wt-PAI-3 increased cell motility toward VN and FN. The increased expression of the β1 integrin subunit we found may account for this difference, because β1 is known to regulate cell movement (47). The β1 integrin subunit was shown to play a significant role in MCF-7 breast cancer cell adhesion to VN (48). Interestingly, of the additional breast cancer cell lines we examined, MCF-7 cells were the only to endogenously express PAI-3. Our results imply that a tumor cell microenvironment exposed to or up-regulating active PAI-1 or PAI-3 could have enhanced cell motility function.

The interplay between uPA, uPAR, and integrins, and their subsequent signaling events is important for tumor cell processes (see Refs. 44, 49–52, and references cited therein). Less is known about the role of PAI-1 and PAI-3 to modulate or even participate in these signaling pathways. Our data with wt-PAI-1 and wt-PAI-3-expressing MDA-MB-435 cells are consistent with recent observations that uPAR promotes integrin αβ1 interactions (49), αι and β1 integrin subunit clustering enhances uPA secretion (53), and PAI-1 modifies the signaling response of uPA for tumor cells (54). PAI-1 promotes vitronectin multimerization that then alters VN-cell adhesion functions (55). It will be interesting to extend our serpin-expressed tumor cell model system to further evaluate uPA/uPAR/integrin signaling events and how ECM proteins modulate this entire process. Collectively, these data allow us to reinforce the hypothesis that co-expression of PAI-1, uPA, and uPAR is essential for optimal carcinoma cell invasiveness (6, 7, 56). It will be interesting to extend our serpin-expressed tumor cell model system to further evaluate uPA/uPAR/integrin signaling events and how ECM proteins modulate this entire process. Collectively, these data allow us to reinforce the hypothesis that co-expression of PAI-1, uPA, and uPAR is essential for optimal carcinoma cell invasiveness (6, 7, 56).

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