Promigratory Effect of Plasminogen Activator Inhibitor-1 on Invasive Breast Cancer Cell Populations

Bénédicte Chazaud,*[†] Rémy Ricoux,*
Christo Christov,* Anne Plonquet,*[†]
Romain K. Gherardi,*[†] and
Georgia Barlovatz-Meimon*[‡]

From GERMEN,* INSERM EMI-0011,[†] and INSERM U492,[‡]
Université Paris XII Val-De-Marne, Créteil, France

The urokinase-type plasminogen activator (uPA) system is a dynamic complex in which the membrane receptor uPAR binds uPA that binds the plasminogen activator inhibitor (PAI)-1 localized in the extracellular matrix, resulting in endocytosis of the whole complex by the low-density lipoprotein receptor-related protein (LRP). High expression of PAI-1 is paradoxically associated with marked tumor spreading and poor prognosis. We previously reported a nonproteolytic role of the [uPAR:uPA:PAI-1:LRP] complex operative in cell migration. Here we explored whether matrix PAI-1 could be used as a migration support by human breast cancer cells. We showed that the uPA system and LRP are localized at filopodia of invasive cells, and that formation/internalization of the [uPAR:uPA:PAI-1:LRP] complex is required for attachment and migration of cancer cells on plastic and on a PAI-1 coat. PAI-1 increased both filopodia formation and migration of cancer cells suggesting a chemokine-like activity. Migration velocity, expression of the uPA system, use of the [uPAR:uPA:PAI-1:LRP] complex to migrate, and promigratory effects of PAI-1 paralleled cancer cell invasiveness. Phenotyping and functional analysis of invasive cancer cell subclones indicated that different cell subpopulations may use different strategies to migrate depending on both the environment and their expression of the uPA system, some of them taking advantage of abundant available PAI-1. (*Am J Pathol* 2002, 160:237–246)

The urokinase-type plasminogen activator (uPA) system is involved in extracellular matrix (ECM) proteolysis and cell migration.^{1,2} It is composed of a proteinase, uPA, a GPI-anchored receptor (uPAR) maintaining uPA at cell surface, and two specific inhibitors (PAI).^{1,2} PAI-1 is bound to ECM proteins, such as vitronectin (VN) that stabilizes PAI-1 in its active form.^{3,4} Binding of PAI-1 to uPAR-bound uPA results in internalization of the [uPAR:

uPA:PAI-1] complex via endocytic receptors of the low-density lipoprotein (LDL) receptor family such as LDL-related protein receptor (LRP).^{5–8} Endocytosis can occur because uPA binding to PAI-1 abrogates PAI:VN binding^{4,9,10} and induces a PAI-1 conformational change that exposes a cryptic high-affinity binding site for LRP.¹¹ Subsequently, intracellular degradation of the complex occurs with recycling of uPAR back to the cell surface.¹²

Cancer cell invasion involves both ECM proteolysis and cell migration.^{1,2} High tumor expression of uPAR and uPA is associated with poor prognosis of breast cancer.¹³ High PAI-1 levels also represent a strong and independent marker of cancer invasiveness and metastatic spreading,^{13,14} an unexpected finding if one considers that PAI-1 anti-proteolytic activity should potentially inhibit cancer invasion.¹ The paradox was confirmed by the report that cancer invasion and neovascularization are abolished in PAI-1-deficient mice, and can be restored by local induction of PAI-1 expression.¹⁵ In this study, PAI-1 immunoreactivity was mainly detected in peritumoral host stroma, a finding consistent with overexpression of PAI-1 at the periphery of invasive breast cancer.¹⁶

In addition to their involvement in proteolysis, the uPA system components exert nonproteolytic roles operative in adhesion and migration of various cell types.^{9,17–21} Implication of PAI-1 in cell migration was initially considered through its interplay with VN. Indeed, PAI-1 competes with VN cellular ligands, ie, β -integrins and uPAR.⁹ According to the cell type and the migration assay used, cell migration on/through VN was either inhibited^{17,18} or promoted^{9,19} by addition of PAI-1. We have recently studied the implication of PAI-1 in cell migration through its interactions with both uPAR-bound uPA and LRP. We showed that myogenic cell motility was critically dependent on integrity of the [uPAR:uPA:PAI-1:LRP] complex, both cell migration and membrane ruffling activity being abolished by truncated uPA (ATF) unable to bind PAI-1.²²

Supported by the Association pour la Recherche contre le Cancer, by the Institut Garches (to B. C.), by a INSERM/CNRS grant "adhesion cellulaires-matériaux," and by the Ministère de la Recherche, Université Paris XII (to G. B. M.).

Accepted for publication October 8, 2001.

Address reprint requests to Georgia Barlovatz-Meimon, INSERM U492, Faculté de Médecine, 8 rue du Général Sarraill, 94000 Créteil, France. E-mail: barlovatz@creteil.inserm.fr.

These results were in keeping with a mechanical role of the uPA system²³ in which the [uPAR:uPA:PAI-1:LRP] complex could transiently bridge the cell membrane to ECM.²² Ability of PAI-1 to both decrease its affinity for matrix VN and increase its affinity for endocytic receptors in response to uPA binding^{9,10} suggests that it may represent a key molecule in the rapid attachment/disattachment events occurring at the cell leading edge that are required for migration.²⁴

To investigate if and how PAI-1 is implicated in breast cancer cell migration *in vitro*, and to discriminate the respective role of cancer and host-derived PAI-1, we performed migration assays on uncoated and PAI-1-coated surfaces. Results indicate that matrix PAI-1 can behave as a migration accelerator for cancer cells that strongly express uPA system components, providing a clue to the breast cancer PAI-1 paradox.

Materials and Methods

Materials

Culture media were from Life Technologies (Paisley, UK). Culture plastics, fibronectin (FN), VN, Matrigel, FACS permeabilizing solution, anti-CD29-PE monoclonal antibody (mAb), anti-CD51/CD61-fluorescein isothiocyanate (FITC) mAb, IgG1-PE, and IgG1-FITC were from Becton Dickinson (Bedford, MA). Cell dissociation solution, aprotinin, GRG-DTP, Fast Red were from Sigma Chemical Co. (St. Louis, MO). Blocking anti-VN mAb was from Biosource (Camarillo, CA). Blocking anti-integrin $\beta 1$ mAb (clone P4C10) was from Chemicon international (Temecula, CA). Control mouse IgGs (0.5 mg/ml) and rabbit Ig (5 mg/ml) were from Vector Laboratories (Burlingame, CA). Goat anti-mouse Ig-FITC (1.8 mg/ml), goat anti-mouse Ig-rhodamine (1.8 mg/ml), and goat anti-rabbit Ig-rhodamine (2 mg/ml) were from Coulter (Fullerton, CA). Active recombinant PAI-1 no. 1095, HMw uPA no. 124, ATF n0. 126, mAb against uPAR no. 3932 (0.5 mg/ml), uPA no. 3689 (0.5 mg/ml), and PAI-1 no. 3780 (0.5 mg/ml) used for labeling experiments, blocking mAb against uPAR no. 3936 and PAI-1 no. 3783 for inhibitory assays were from American Diagnostica (Greenwich, CT). Wild-type PAI-1, anti-LRP (clone 8G1, 0.775 mg/ml), and anti-gp330 (clone 1H2, 0.2 mg/ml) mAbs were generously provided by D. A. Lawrence and D. K. Strickland (Rockville, MD), polyclonal anti-VLDL-R (5.57 mg/ml) antibodies and RAP (receptor-associated protein) were provided by A. Christensen and A. Nykjaer (Aarhus, Denmark), and peptides P25 and P36 were provided by H. A. Chapman (Boston, MA).

Cell Culture

MCF7-AZ, MCF7/6, and MDA-MB-231 breast cancer cell lines were cultured as previously described.^{25,26} Two MDA-MB-231 clonal cell lines, isolated by end-point dilution and with divergent expressions of the uPA system, were chosen for further studies. In all experiments, cells were harvested using cell dissociation solution.

Flow Cytometry

Cells were treated with FACS permeabilizing solution, incubated with primary antibodies (1/10), further incubated with fluorescent secondary antibodies (1/100), and fixed with paraformaldehyde. Integrin labeling was performed according to the manufacturer's instructions. Controls were performed using isotypic Ig. A minimum of 5000 cells was counted in each assay. Fluorescence intensity was measured using a Coulter EPICS XL Flow Cytometry System. Percent positive cells was determined according to the marker approach using the population gate described in Current Protocols in Cytometry.²⁷ The marker approach is best suited for single or double indirect labelings according to the same reference.

Immunofluorescence

Cells were fixed by paraformaldehyde, incubated with anti-LRP (30 μ g/ml) mAb for 1 hour, washed with phosphate-buffered saline (PBS), then incubated with goat anti-mouse-rhodamine (1/100) antibodies for 1 hour and washed with PBS. Slides were further incubated with either anti-uPAR or anti-uPA (40 μ g/ml) or anti-PAI-1 (20 μ g/ml) mAbs, washed with PBS, then incubated with goat anti-mouse-FITC (1/100) antibodies, and washed with PBS. To label F-actin, fixed cells were incubated with rhodamine phalloidin (1 μ g/ml) for 30 minutes. Controls, including incubation with mouse IgG, showed no labeling. Cells were observed with a Zeiss 100 neofluar lens.

Immunohistochemistry

Immunohistochemical expression of the uPA system and the endocytic receptors was evaluated as previously described²⁸ in 16 breast ductal *in situ* carcinomas, with or without microinvasion [grade III ($n = 6$), grade II ($n = 6$), grade I ($n = 4$)] [rqsb], and 15 invasive ductal carcinomas of various grades [grade III ($n = 8$), grade II ($n = 4$), grade I ($n = 3$)].²⁹ Sections were microwaved in 100 mmol/L Tris-HCl, pH 8 (4 $\times$ 5 minutes) for uPAR, and 10 mmol/L citrate, pH 6 (3 $\times$ 5 minutes) for PAI-1 detection. Proteinase XXIV (10 minutes) was used for uPA detection. Slides were treated by bovine serum albumin 3% for 2 hours, incubated with primary antibodies (against uPAR, 40 μ g/ml; uPA, 40; PAI-1, 20; LRP, 10; VLDLR, 50; gp330, 20) at 4°C overnight, further treated using LSAB2 kit and stained with Fast Red. Controls were performed using isotypic Ig.

Cell Attachment Assay

Ninety-six-wells plates were coated for 1 hour and 30 minutes with 40 μ g/ml of PAI-1 diluted in PBS and saturated with bovine serum albumin 1% for 30 minutes. Cells (30,000 per well) were incubated for 2 hours at 37°C, washed, fixed, and stained with crystal violet. Optical density was read at 570 nm. Optical density obtained in bovine serum albumin-coated wells was subtracted. When used, effectors were added to the cells at time of

Figure 1. *In vivo* localization of uPA system components. uPAR (A), uPA (B), PAI-1 (C), and LRP (D) immunolabeling in invasive breast cancer. **Arrows**, tumor cell labeling; **arrowheads**, stromal cell labeling. Original magnifications, $\times 200$.

seeding. In some experiments, HMw uPA (5 $\mu\text{g/ml}$) was added 30 minutes before seeding.

Two-Dimensional Migration Assay

This assay was modified from that previously described.³⁰ Cells were seeded into a glass cylinder deposited on a Petri dish. At subconfluence, the cylinder was removed, and cells were gently washed and allowed to migrate in serum-free medium for 6 hours at 37°C. Strictly similar results were obtained when MDA-MB-231 cells were seeded at high density (to be used immediately at subconfluence) and shortly grown without serum (3 to 15 hours) before cylinder removal, or when MDA-MB-231 cells were seeded into the cylinder, allowed to grow in presence of serum until subconfluence (several days), and subjected to washings before starting the migration assay in serum-free conditions. The area occupied by the cells was recorded using a charge-coupled device camera attached to an inverted microscope and quantified with the Zeiss KS-300 imaging system. The radius of the area occupied by the cells, considered as a disk, was calculated. The migration score was expressed as the radius calculated after a 6-hour incubation time beyond the initial radius and was given in μm . The measurement represents net changes in the whole-cell population and not single cell movements. Increase of the area was not because of cell proliferation, because the

total cell number did not vary statistically during the whole experiment. Cell migration was also tested on supports coated with FN, VN, or PAI-1. Although active recombinant PAI-1 (no. 1095) has a given half-life of more than 500 hours at 25°C, its ability to bind uPA after a 5-day incubation was confirmed using uPA-FITC (not shown). When used, effectors were added to the cells at the beginning of the incubation at 37°C.

Three-Dimensional Migration Assay

For invasion assay,³¹ inserts with 8- μm pores for 24-well plates were coated on their upper aspect with 50 $\mu\text{g/insert}$ of Matrigel containing PAI-1 (0 to 10 $\mu\text{g/ml}$). Cells (2×10^5) were added in the upper chamber and incubated 20 hours in serum-free medium. Cells emigrated to the lower aspect of the membrane were fixed, stained with crystal violet, and counted manually at $\times 20$ magnification (5 fields per insert).

Statistical Analysis

Results are means $\pm$ SEM of experiments run in triplicate and performed using three different cultures. The Mann Whitney and Student's *t*-tests were used. *P* values are indicated in figures as follows: *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

Results

Expression of the uPA System Components by Breast Cancer Cells

In vivo expression of uPAR was not observed in ductal *in situ* carcinoma (0 of 16) but was observed in zones of diffuse infiltration of invasive ductal carcinoma (11 of 15), regardless of their grade. uPAR immunostaining was observed in cancer cells, where it was particularly strong in isolated invasive cells remote from the bulk of the tumor (Figure 1A). It was also observed in spindle stromal cells at the invasion margin and in macrophages in both neoplastic and nonneoplastic breast tissue. uPA (Figure 1B) and PAI-1 (Figure 1C) immunostainings were detected in both cancer and stromal cells of invasive ductal carcinoma (15 of 15) and ductal *in situ* carcinoma (11 of 16), cancer cell expression being invariably stronger in invasive ductal carcinoma than in ductal *in situ* carcinoma. Stromal PAI-1 expression was particularly strong in spindle cells in an immediate vicinity of invasive tumor cells (Figure 1C). Endocytic receptors were expressed in both cancer and stromal cells, regardless of staging. Virtually all cancer cells expressed VLDL-R (not shown), occasional cancer cells expressed LRP (Figure 1D), and none expressed gp330 (not shown).

Several studies have shown that MDA-MB-231 cells are more invasive than MCF7/6 cells, and that MCF7-AZ cells are virtually not invasive.^{25,32–35} It was also shown that both the expression of uPAR, uPA, and PAI-1 and the number of uPAR sites per cell correlate with cell invasiveness.^{33,35,36} Flow cytometry studies also suggested some correlation between expression of the uPA system components and cell invasiveness (Figure 2). In contrast to LRP the expression of which correlated positively with invasiveness ($P < 0.005$), that of VLDL-R correlated negatively with invasiveness ($P < 0.05$), whereas gp330 was virtually not expressed. The respective percentage of positive noninvasive MCF7-AZ cells was: uPAR, $33 \pm 7\%$; uPA, 48 ± 2.2 ; PAI-1, 45 ± 0.8 ; LRP, 17 ± 1.4 ; VLDL-R, 36 ± 5 ; gp330, 0.3; that of moderately invasive MCF7/6 cells was: uPAR, $40 \pm 10.3\%$; uPA, 50 ± 14.2 ; PAI-1, 55 ± 3.0 ; LRP, 25 ± 3.8 ; VLDL-R, 15 ± 3 ; gp330, 0.5; that of most invasive MDA-MB-231 cells was: uPAR, $48 \pm 6.7\%$; uPA, 64 ± 6.7 ; PAI-1, 65 ± 4.3 ; LRP, 55 ± 8.1 ; VLDL-R, 0.7; gp330, 2.

Most invasive MDA-MB-231 cells expressed uPAR, uPA, PAI-1, and LRP at the cell leading edge and tip of filopodia; immunolocalization showed that LRP co-localized with all of the uPA system components (Figure 3).

Inhibition of the [uPAR:uPA:PAI-1:LRP] Complex Formation Predominantly Inhibits Migration of Most Invasive Cells

Migration of the three cell lines on plastic correlated positively with their invasiveness: the distance covered in 6 hours was 31.5 ± 5.2 , 50.4 ± 8.2 , and $91.6 \pm 8.9 \mu\text{m}$ for MCF7-AZ, MCF7/6, and MDA-MB-231 cells, respectively ($P < 0.001$) (Figure 4, inset).

Figure 2. Breast cancer cell line expression of uPA system components. Cytometry analysis of uPAR, uPA, PAI-1, LRP, gp330, or VLDL-R labeling. **Gray curve**, isotypic control; **white curve**, specific staining.

Inhibition of uPA-generated plasmin by aprotinin failed to inhibit cancer cell migration (Figure 4). However, anti-uPAR mAb that block uPA binding to uPAR inhibited migration proportionally to invasiveness (inhibition by 59.2%, 80.8, and 84.3 for MCF7-AZ, MCF7/6, and MDA-MB-231 cells, respectively, all $P < 0.005$) (Figure 4). Similarly, ATF (amino-terminal fragment of uPA), which is unable to bind PAI-1 but competes with uPA for uPAR binding, also inhibited migration [by 43.8% ($P < 0.01$), 77.7% ($P < 0.005$), and 79.4% ($P < 0.005$), respectively], as did anti-PAI-1 mAb (by 47.6%, 66.7%, and 76.2%, all $P < 0.005$) (Figure 4). Receptor-associated protein (RAP), which inhibits the binding to LDL-receptor family

Figure 3. *In vitro* co-localization of uPA system components with LRP. MDA-MB-231 cells were treated for LRP immunolabeling (red) (A–C) and for uPAR (A'), uPA (B'), and PAI-1 (C') immunolabelings (green). Scale bar, 10 μ m.

members, also inhibited migration (by 50% ($P < 0.05$), 76.2% ($P < 0.005$), and 77.7% ($P < 0.005$), respectively) (Figure 4). This effect was likely because of LRP inhibition as the strongest inhibition was observed on MDA-MB-231 cells that lack gp330 and VLDL-R.

Blocking anti-VN mAb inhibited migration in inverse proportion to invasiveness (by 81.7%, 65.8%, and 54.1% for MCF7-AZ, MCF7/6, and MDA-MB-231 cells, respectively, all $P < 0.005$), as did GRGDTP peptide, that inhibits integrin binding to ECM proteins [100%, 68.2% ($P < 0.005$), and 66.7% ($P < 0.01$), respectively] (Figure 4).

Figure 4. Breast cancer cell line migration. **Bottom:** Migration on plastic of cells incubated with or without control IgGs (5 μ g/ml), aprotinin (10 IU/ml), GRGDTP (integrin-blocking peptide) (0.1 mg/ml), ATF (amino-terminal fragment of uPA) (500 ng/ml), RAP (300 nmol/L), anti-VN (5 μ g/ml), anti-uPAR (5 μ g/ml), or anti-PAI-1 (5 μ g/ml) mAbs. **Inset:** Migration of untreated cells.

To investigate the role of the complex formed by uPAR, uPA, PAI-1, and LRP in cell migration, we first assessed attachment capacities of cancer cells on PAI-1.

Invasive Cells Can Attach to a PAI-1 Coat

MDA-MB-231 cells attached to a PAI-1 coat in a dose-dependent way ($P < 0.05$); this attachment at 0.37 μ mol/L was twofold less efficient on PAI-1 than on VN ($P < 0.05$) (Figure 5A).

Coating with latent PAI-1 or coating with active PAI-1 followed by incubation with anti-PAI-1 mAb dramatically decreased cell attachment to PAI-1 ($P < 0.05$ and $P < 0.005$) (Figure 5B). Removing uPA from uPAR by acid washing³⁷ prevented cell attachment to PAI-1 ($P < 0.01$) (Figure 5B). Addition of ATF to acid-washed cells at time of seeding did not allow attachment ($P < 0.01$) whereas in the same conditions, addition of uPA restored cell attachment up to the control value (Figure 5B). Similarly, saturation of the PAI-1 coat with uPA before seeding allowed acid-washed cells to attach, whereas untreated cells did not attach (Figure 5B). These results indicate that the complexed uPAR, uPA, and PAI-1 molecules link cell membrane to the support.

Interestingly, LRP was also required for full cell attachment to PAI-1 as RAP inhibited attachment by 70% ($P < 0.01$) (Figure 5B). This suggested that the cell-to-support molecular link was dynamic. Consistently, cells seeded on PAI-1 showed striking morphological changes. These changes consisted in marked filopodia formation whereas no stress fibers were observed (Figure 5C).

Figure 5. MDA-MB-231 cell attachment to PAI-1. **A:** Cell attachment to wild-type PAI-1 and VN coats. **B:** Attachment to wild-type PAI-1 (40 μg/ml), to recombinant active PAI-1, to latent PAI-1 (PAI-1 before incubated at 37°C) of cells incubated with or without anti-PAI-1 mAb (5 μg/ml), ATF (500 ng/ml), HMw uPA (5 μg/ml), or RAP (300 nmol/L). In some experiments, cells were acid-washed before seeding and/or the PAI-1 coat was saturated with HMw uPA (5 μg/ml) before seeding. **C:** Cells seeded on bovine serum albumin (I), VN (II), and PAI-1 (III) (1.1 μmol/L) for 2 hours were labeled using rhodamine phalloidin.

They differed from the changes observed in cells seeded on VN that mainly showed stress fiber formation and less filopodia than cells seeded on PAI-1 (Figure 5C). We then assessed the migrating capacities of cancer cells on PAI-1-coated surfaces.

PAI-1-Enriched Environment Markedly Accelerates Migration of Invasive Cells

Migration of all cancer cell lines was significantly increased on immobilized PAI-1 as compared to uncoated surfaces. But the effect of PAI-1 was more pronounced in invasive cells, the covered distances being increased by 37.8% ($P < 0.01$), 57.9% ($P < 0.005$), and 70.2% ($P < 0.005$) for MCF7-AZ, MCF7/6, and MDA-MB-231 cells, respectively (Figure 6A). Both ATF and RAP dramatically inhibited cell migration on immobilized PAI-1: ATF decreased migration by 76.4%, 90.6%, and 83.8%, and RAP by 77%, 90%, and 93.3%, respectively (all $P < 0.005$)

Figure 6. Cell migration in a PAI-1-enriched environment. **A:** Cell migration on uncoated and PAI-1-coated surfaces in the absence or the presence of ATF (500 ng/ml) or RAP (300 nmol/L). **B:** MDA-MB-231 cell migration on PAI-1, FN, and VN (1.1 μmol/L). **C:** Invasion of MDA-MB-231 cells through Matrigel that contains or not PAI-1.

(Figure 6A). MDA-MB-231 cell migration rate on PAI-1 was 1.5-fold and 15-fold higher than on FN and VN, respectively ($P < 0.05$ and $P < 0.005$) (Figure 6B). As cells can produce ECM molecules during the assay, we evaluated involvement of VN and integrins in migration on PAI-1. Neither did anti-VN mAb, GRGDTP, anti-integrin β1 mAb (that inhibited cell migration on FN by 96%), nor P25, a peptide inhibiting the lateral interaction between uPAR and integrins³⁸ affect invasive cell attachment or migration on PAI-1 (not shown).

Three-dimensional migration assay was conventionally performed to assess cancer cell invasion in a PAI-1-enriched environment. Low (0.1 μg/ml) PAI-1 concentration did not affect cancer cell invasion whereas higher PAI-1 concentrations (1 to 10 μg/ml) enhanced cell invasion up to 75% ($P < 0.05$) (Figure 6C).

According to Their Expression of uPAR, uPA, PAI-1, and LRP, Invasive Cell Clones Use Either Integrins or the uPA System to Migrate

Expression of the uPA system components by MDA-MB-231 cells was heterogeneous, showing two or three different subpopulations (Figure 2). End-point dilution technique provided two clonal cell lines, called MDA^{low} and MDA^{high} in respect with their markedly divergent expression of uPA system components. The respective percentage of positive cells were: for uPAR 17 ± 2.5 versus 59 ± 4.2% ($P < 0.005$), for uPA 27 ± 2.3 versus 60 ± 2% ($P < 0.005$)

Figure 7. High and low MDA-MB-231 cell subclones. **A:** Cytometry analysis of uPAR, uPA, PAI-1, LRP, gp330, VLDL-R, β1, or αvβ3 labeling. **Gray curve**, isotypic control; **white curve**, specific staining. **B:** Cell migration on plastic with or without GRGDTP (0.1 mg/ml), ATF (500 ng/ml), or RAP (300 nmol/L). **C:** Cell migration on PAI-1, VN, or FN coats (1.1 μmol/L). **D:** Cells seeded on VN and PAI-1 (1.1 μmol/L) for 2 hours were labeled using rhodamine phalloidin.

0.05), for PAI-1 41 ± 4.2 versus $71 \pm 1.4\%$ ($P < 0.01$), for LRP 38 ± 3 versus $68 \pm 0.6\%$ ($P < 0.01$), for gp3302 versus 4%, for VLDL-R and 0.3 versus 1% (Figure 7A). Invasion assays through Matrigel indicated that MDA^{high} subclone was threefold more invasive than the parental MDA-MB-231 cell line and that MDA^{low} subclone was twofold less invasive than MDA-MB-231 cells (not shown), a finding consistent with previous studies indicating a correlation between expression of uPA system components and cell invasiveness.^{33,35,36}

Two-dimensional migration of MDA^{low} cells on plastic was markedly affected by integrin inhibition (GRGDTP inhibition, 88%; $P < 0.005$) and less affected by inhibition of the uPA system (ATF inhibition, 24%; $P < 0.05$; RAP inhibition, 2%; NS) (Figure 7B). Conversely MDA^{high} cell migration was poorly affected by integrin inhibition (GRGDTP inhibition, 27%; NS) and strongly inhibited by both ATF (71%, $P < 0.01$) and RAP (62%, $P < 0.01$) (Figure 7B).

The two cell lines exhibited markedly divergent migration rates on PAI-1, MDA^{high} cells migrating at very high velocity on this support (Figure 7C) ($P < 0.005$). They had similar migration rates on FN (Figure 7C), a finding in keeping with a similarly high (99%) expression of $\beta 1$ integrin (Figure 7A). In addition, the two clones had different migration rates on VN (Figure 7C), despite a similar and moderate ($14 \pm 5\%$) expression of $\alpha v \beta 3$, the main integrin for VN (Figure 7A). VN accelerating effect on MDA^{low} was mild (migration rate less than twofold that on plastic and similar to that on FN) whereas PAI-1 accelerating effect on MDA^{high} was marked (migration rate more than fivefold that on plastic and more than twofold that on FN) (Figure 7C).

Finally, the two cell lines showed divergent reorganizations of their actin cytoskeleton; on VN, MDA^{low} cells became polarized whereas MDA^{high} cells remained rounded (Figure 7D). Conversely, on PAI-1, MDA^{low} cells did not polarize whereas MDA^{high} cells developed a polarized migrating phenotype (Figure 7D).

Discussion

In the present study, interference with the [uPAR:uPA:PAI-1:LRP] complex formation induced a marked decrease of breast cancer cell migration, as previously observed in myogenic cells.²² Moreover, PAI-1 was shown to mediate cancer cell attachment through uPAR-bound uPA, supporting that the uPAR:uPA:PAI-1 complex can bridge the cell membrane to ECM.²¹ As blockade of LRP strongly inhibited cell attachment to PAI-1, this likely resulted from permanent renewal of transient cell to matrix links supported by high-affinity uPAR:uPA:PAI-1 molecular interactions.

Both dynamic links of cell membrane to ECM and signal transduction occur at the leading edge of migrating cells.²⁴ The uPA system components were detected at the leading edge of invasive cells and at the tip of filopodia. Interrelations of the uPA system with molecules that regulate lamellipodia cytoskeletal organization, such as myosin light chain kinase,³⁹ rac-1, and RhoA,⁴⁰ have

been recently described. Association of the uPA system with membrane-ruffling activity was also mentioned,⁴¹ and, indeed, microcinematography has previously shown that ATF suppresses filopodia, membrane-ruffling activity, and motility of myogenic cells.²²

In the present study, we used an *in vitro* model with minimal implication of the VN-dependent migration systems to delineate the role of PAI-1 per se. Admittedly, *in vivo*, PAI-1 interacts with ECM macromolecules, such as VN, and might participate in cell migration through a coordinated combination of molecular events. These may include: 1) high-affinity ligation of cell membrane uPAR-bound uPA to matrix PAI-1; 2) PAI-1 detachment from ECM; 3) subsequent internalization of the complex through LRP ligation and, possibly, stable links of integrins to ECM. Myriads of transient sequences of membrane to ECM attachment/disattachment at the leading edge could well participate in membrane-ruffling activity, and groping of filopodia along ECM aimed at cell guidance through selection of appropriate sites for cell attachment.²² This view is supported by the evidence that PAI-1 increases both filopodia formation, migration, and invasion of very invasive cancer cells. Interestingly, uPAR is co-localized with chemokine (CK) receptors at the leading edge of leukocytes and other fast-migrating cells^{42,43} and numerous studies have identified chemotactic roles for uPA and for uPAR.^{41,44–47} Chemotaxis/haptotaxis relies on sensing the extracellular concentration of appropriate ligands and coupling of chemotactant receptor occupation to cytoskeletal changes leading to cell polarization.⁴⁸ Although structurally unrelated to CKs and their receptors, the uPA system has the characteristic properties of a CK system: 1) as CKs,^{49,50} PAI-1 is adsorbed on ECM; 2) as CK receptors,^{43,51} uPAR-bound uPA is localized at the leading edge and filopodia of migrating cells; 3) similarly to CK-enriched environments,^{52,53} PAI-1-enriched environment boosts cell migration; 4) as CK/CK receptor complexes,^{54,55} the uPAR-bound uPA:PAI-1 complex is rapidly internalized; 5) as CK receptors,^{42,56} uPAR/adaptor may transduce intracellular signals implying heterotrimeric G proteins.⁴¹

Invasive capacities of breast cancer cell lines paralleled migration velocity, expression of the uPA system components and of LRP, implication of the [uPAR:uPA:PAI-1:LRP] complex in migration, and promigratory effects of PAI-1. This was in keeping with the marked membrane ruffling of invasive breast cancer cells.²⁵ Fast-migrating cells do not develop strong cell-matrix interactions, as assessed by lack of both focal contact and stress fiber formation.⁴⁸ Most invasive MDA-MB-231 cells harbored such a phenotype when seeded on PAI-1; in contrast, they developed stress fibers when seeded on VN. This was indicative of various populations among MDA-MB-231 cells. These included a clonal population (MDA^{high}) that expressed high amounts of the uPA system components and used the uPA system preferentially to integrins for their migration, and another clonal population (MDA^{low}) showing weak expression of the uPA system and using the integrin-mediated migration pathway. Admittedly, the two clonal cell lines may differ in more ways than just the markers analyzed. Nevertheless,

the two clones had migration rates on PAI-1 and FN in accordance with their expression of uPA system components and $\beta 1$ integrins, respectively. In contrast, they had different migration rates on VN, despite a similar expression of $\alpha v\beta 3$, the main VN ligand. This was consistent with previous reports on PAI-1 effects on adhesion/migration on VN, that operate through modulation of PAI-1:VN interactions.^{9,17–19} However, PAI-1:VN interplay could not be directly instrumental in the fast cell migration observed on PAI-1 coat. Moreover, on MDA^{high} cells, the PAI-1 accelerating effect markedly exceeded the VN decelerating effect. This suggests that, when cells displaying the appropriate uPA system expression maneuver in a PAI-1-enriched milieu, the promigratory effect directly operated by matrix PAI-1 overcomes the inhibitory effects of PAI-1. This view was supported by the accelerating effect of matrix PAI-1 observed in the three-dimensional invasion assay.

Breast cancer tissue expression of the uPA system mainly included uPAR expression by cancer cells in invasive, but not *in situ*, tumors, and expression of uPA and PAI-1 by both cancer and stromal cells that parallel tumor aggressiveness.^{14,16,28} Endocytic receptors were also expressed by both cancer and stromal cells.^{28,57} Taken together with our *in vitro* experiments, these results could suggest that invasive cancer cells may use the uPA system in multiple ways *in vivo*. At the initial stage of breast cancer, when PAI-1, but not uPAR, is expressed in and around the tumor, PAI-1 anti-proteolytic activity may inhibit cancer cell invasion. At the invasion stage, cancer cells displaying uPAR at their surface,²⁸ can exert directional proteolysis of ECM that is counteracted by the presence of increasing levels of PAI-1 in the environment. The finding of divergent cancer clones in MDA-MB-231 cells indicates that, subsequently to mutations, the tumor cells may use different strategies to migrate depending on the availability of the relevant ligands in the cell environment. Thus, eventually, some cells might take advantage of the high amounts of PAI-1 present in the environment to migrate, using a novel function of the [uPAR:uPA:PAI-1:LRP] complex. This could explain the apparent paradox of poor prognosis of invasive breast cancers expressing high PAI-1 levels.

Acknowledgments

We thank Eric Fernandez and Lily Wan for excellent technical assistance, and Dr. D. A. Lawrence for stimulating discussions.

References

- Carmeliet P, Collen D: Development and disease in proteinase-deficient mice: role of the plasminogen, matrix metalloproteinase and coagulation system. *Thromb Res* 1998, 91:255–285
- Mignatti P: Extracellular matrix remodeling by metalloproteinases and plasminogen activators. *Kidney Int* 1995, 47:S12–S14
- Preissner KT, Grulich-Henn J, Ehrlich HJ, Declercq P, Justus C, Collen D, Pannekoek H, Muller-Berghaus G: Structural requirements for the extracellular interaction of plasminogen activator inhibitor 1 with endothelial cell matrix-associated vitronectin. *J Biol Chem* 1990, 265:18490–18498
- Lawrence DA, Palaniappan S, Stefansson S, Olson ST, Francis-Chmura AM, Shore JD, Ginsburg D: Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J Biol Chem* 1997, 272:7676–7680
- Moestrup SK, Nielsen S, Andreasen P, Jorgensen KE, Nykjaer A, Roigaard H, Gliemann J, Christensen EI: Epithelial glycoprotein-330 mediates endocytosis of plasminogen activator-plasminogen activator inhibitor type-1 complexes. *J Biol Chem* 1993, 268:16564–16570
- Argaves KM, Battey FD, MacCalman CD, McCrae KR, Gafvels M, Kozarsky KF, Chappell DA, Strauss JF, Strickland DK: The very low density lipoprotein receptor mediates the cellular catabolism of lipoprotein lipase and urokinase-plasminogen activator inhibitor type I complexes. *J Biol Chem* 1995, 270:26550–26557
- Heegaard CW, Wiborg Simonsen AC, Oka K, Kjoller L, Christensen A, Madsen B, Ellgaard L, Chan L, Andreasen PA: Very low density lipoprotein receptors binds and mediates endocytosis of urokinase-type plasminogen activator-type-1 plasminogen activator inhibitor complex. *J Biol Chem* 1995, 270:20855–20861
- Nykjaer A, Petersen CM, Moller B, Jensen PH, Moestrup SK, Holtet TL, Etzerodt M, Thogersen HC, Munch M, Andreasen PA, Gliemann J: Purified $\alpha 2$ -macroglobulin receptor/LDL receptor-related protein binds urokinase:plasminogen activator inhibitor type-1 complex. *J Biol Chem* 1992, 267:14543–14546
- Waltz DA, Natkin LR, Fujita RM, Wei Y, Chapman HA: Plasmin and plasminogen activator inhibitor type 1 promote cellular motility by regulating the interaction between the urokinase receptor and vitronectin. *J Clin Invest* 1997, 100:58–67
- Deng G, Curriden SA, Wang S, Rosenberg S, Loskutoff DJ: Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? *J Cell Biol* 1996, 134:1563–1571
- Stefansson S, Muhammad S, Cheng X-F, Battey FD, Strickland DK, Lawrence DA: Plasminogen activator inhibitor-1 contains a cryptic high affinity binding site for the low density lipoprotein receptor-related protein. *J Biol Chem* 1998, 273:6358–6366
- Nykjaer A, Conese M, Christensen EI, Olson D, Cremona O, Gliemann J, Blasi F: Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. *EMBO J* 1997, 16:2610–2620
- Andreasen PA, Kjoller L, Christensen L, Duffy MJ: The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997, 72:1–22
- Pappot H, Gardsvoll H, Romer J, Pedersen AN, Grondahl-Hansen J, Pyke C, Brnner N: Plasminogen activator inhibitor-type 1 in cancer: therapeutic and prognostic implications. *Biol Chem Hoppe-Seyler* 1995, 376:259–267
- Bajou K, Nol A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM: Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* 1998, 4:923–928
- Umeda T, Eguchi Y, Okino K, Kodama M, Hattori T: Cellular localization of urokinase-type plasminogen activator, its inhibitors, and their mRNAs in breast cancer tissues. *J Pathol* 1997, 183:388–397
- Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preissner KT, Ossowski L, Andreasen PA: Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. *Exp Cell Res* 1997, 232:420–429
- Stefansson S, Lawrence DA: The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_n\beta_3$ binding to vitronectin. *Nature* 1996, 383:441–443
- Stahl A, Mueller BM: Melanoma cell migration on vitronectin: regulation by components of the plasminogen activation system. *Int J Cancer* 1997, 71:116–122
- Ossowski L, Aguirre-Ghiso JA: Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr Opin Cell Biol* 2000, 12:613–620
- Planus E, Barlovatz-Meimon G, Rogers RA, Bonavaud S, Ingber DE, Wang N: Binding of urokinase to plasminogen activator inhibitor type-1 mediates cell adhesion and spreading. *J Cell Sci* 1997, 110:1091–1098
- Chazaud B, Bonavaud S, Plonquet A, Pouchelet M, Gherardi RK,

- Barlovatz-Meimon G: Involvement of the [uPAR:uPA:PAI-1:LRP] complex in human myogenic cell motility. *Exp Cell Res* 2000, 258:237–244
23. Wang N, Planus E, Pouchelet M, Fredberg JJ, Barlovatz-Meimon G: Urokinase receptor mediates mechanical force transfer across the cell surface. *Am J Physiol* 1995, 268:C1062–C1066
24. Lauffenburger DA, Horwitz AF: Cell migration: a physically integrated molecular process. *Cell* 1996, 84:359–369
25. Bracke ME, Van Larebeke NA, Vyncke BM, Mareel MM: Retinoic acid modulates both invasion and plasma membrane ruffling of MCF-7 human mammary carcinoma cells in vitro. *Br J Cancer* 1991, 63:867–872
26. Cailleau R, Young R, Olive M, Reeves Jr WJ: Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst* 1974, 53:661–674
27. Robinson JP, Darzynkiewicz Z, Dean PN, Dressler LG, Rabinovitch PS, Stewart CC, Tanke HJ, Wheelless LL (eds): Phenotypic Analysis: Current Protocols in Cytometry. New York, John Wiley and Sons Inc. in affiliation with the International Society for Analytical Cytology, 1999, pp 6.2.10–6.2.15
28. Christensen L, Wiborg Simonsen AC, Heegaard CW, Moestrup SK, Andersen JA, Andreasen PA: Immunohistochemical localization of urokinase-type plasminogen activator, type-1 plasminogen activator inhibitor, urokinase receptor and α_2 -macroglobulin receptor in human breast carcinomas. *Int J Cancer* 1996, 66:441–452
29. Consensus conference on the classification of ductal carcinoma in situ. *Hum Pathol* 1997, 28:1221–1225
30. Giese A, Loo MA, Rief MD, Tran N, Berens ME: Substrates for astrocytoma invasion. *Neurosurgery* 1995, 37:294–301
31. Huang S, New L, Pan Z, Han J, Nemerow GR: Urokinase plasminogen activator/urokinase-specific surface receptor expression and matrix invasion by breast cancer cells requires constitutive p38 α mitogen-activated protein kinase activity. *J Biol Chem* 2000, 275:12266–12272
32. Couissi D, Dubois V, Remacle C, Schonne E, Trouet A: Western immunoblotting and enzymatic activity analysis of cathepsin D in human breast cancer cell lines of different invasive potential. Regulation by 17 β -estradiol, tamoxifen and ICI 182,780. *Clin Exp Metastasis* 1997, 15:349–360
33. Fauquette W, Dong-Le Bourhis X, Delannoy-Courdent A, Boilly B, Desbiens X: Characterization of morphogenetic and invasive abilities of human mammary epithelial cells: correlation with variations of urokinase-type plasminogen activator activity and type-1 plasminogen activator inhibitor level. *Biol Cell* 1997, 89:453–465
34. Gui GP, Puddefoot JR, Vinson GP, Wells CA, Carpenter R: In vitro regulation of human breast cancer cell adhesion and invasion via integrin receptors to the extracellular matrix. *Br J Surg* 1995, 82:1192–1196
35. Holst-Hansen C, Johannessen B, Hoyer-Hansen G, Romer J, Ellis V, Brnner N: Urokinase-type plasminogen activation in three human breast cancer cell lines correlates with their in vitro invasiveness. *Clin Exp Metastasis* 1996, 14:297–307
36. Li Y, Wood N, Yellowlees D, Donnelly PK: Cell surface expression of urokinase receptor in normal mammary epithelial cells and breast cancer cell lines. *Anticancer Res* 1999, 19:1223–1228
37. Stoppelli MP, Tacchetti C, Cubellis MV, Corti A, Hearing VJ, Cassani G, Appella E, Blasi F: Autocrine saturation of pro-urokinase receptors on human A431 cells. *Cell* 1986, 45:675–684
38. Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV, Chapman HA: Regulation of integrin function by the urokinase receptor. *Science* 1996, 273:1551–1555
39. Nguyen DH, Catling AD, Webb DJ, Sankovic M, Walker LA, Somlyo AV, Weber MJ, Gonias SL: Myosin light chain kinase functions downstream of Ras/ERK to promote migration of urokinase-type plasminogen activator-stimulated cells in an integrin-selective manner. *J Cell Biol* 1999, 146:149–164
40. Muller SM, Okan E, Jones P: Regulation of urokinase receptor transcription by ras- and rho-family GTPases. *Biochem Biophys Res Commun* 2000, 270:892–898
41. Degryse B, Resnati M, Rabbani SA, Villa A, Fazioli F, Blasi F: Src-dependence and pertussis-toxin sensitivity of urokinase receptor-dependent chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *Blood* 1999, 94:649–662
42. Sanchez-Madrid F, del Pozo MA: Leukocyte polarization in cell migration and immune interactions. *EMBO J* 1999, 18:501–511
43. Nieto M, Frade JMR, Sancho D, Mellado M, Martinez-A C, Sanchez-Madrid F: Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. *J Exp Med* 1997, 186:153–158
44. Gyetko MR, Todd RF, Wilkinson CC, Sitrin RG: The urokinase receptor is required for human monocyte chemotaxis in vitro. *J Clin Invest* 1994, 93:1380–1387
45. Boyle MD, Chiodo VA, Lawman MJ, Gee AP, Young M: Urokinase: a chemotactic factor for polymorphonuclear leukocytes in vivo. *J Immunol* 1987, 139:169–174
46. Busso N, Masur SK, Lazega D, Waxman S, Ossowski L: Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *J Cell Biol* 1994, 126:259–270
47. Mukhina S, Stepanova V, Traktouev D, Poliakov A, Beabealashvili R, Gursky Y, Minashkin M, Shevelev A, Tkachuk V: The chemotactic action of urokinase on smooth muscle cells is dependent on its kringle domain. *J Biol Chem* 2000, 275:16450–16458
48. Serrador JM, Nieto M, Sanchez-Madrid F: Cytoskeletal rearrangement during migration and activation of T lymphocytes. *Trends Cell Biol* 1999, 9:228–232
49. Amara A, Lorthioir O, Valenzuela A, Magerus A, Thelen M, Montes M, Virelizier J-L, Delepiere M, Baleux F, Lortat-Jacob H, Arenzana-Seisdedos F: Stromal cell-derived factor-1 α associates with heparan sulfates through the first β -strand of the chemokine. *J Biol Chem* 1999, 274:23916–23925
50. Ali S, Palmer AC, Banerjee B, Fritchley SJ, Kirby JA: Examination of the function of RANTES, MIP-1 α , and MIP-1 β following interaction with heparin-like glycosaminoglycans. *J Biol Chem* 2000, 275:11721–11727
51. Servant G, Weiner OD, Neptune ER, Sedat JW, Bourne HR: Dynamics of a chemoattractant receptor in living neutrophils during chemotaxis. *Mol Biol Cell* 1999, 10:1163–1178
52. Foxman EF, Kunkel EJ, Butcher EC: Integrating conflicting chemotactic signals: the role of memory in leukocyte navigation. *J Cell Biol* 1999, 147:577–587
53. Franitza S, Alon R, Lider O: Real-time analysis of integrin-mediated chemotactic migration of T lymphocytes within 3-D extracellular matrix-like gels. *J Immunol Methods* 1999, 225:9–25
54. Zimmermann N, Konkright JJ, Rothenberg ME: CC chemokine receptor-3 undergoes prolonged ligand-induced internalization. *J Biol Chem* 1999, 274:12611–12618
55. Solari R, Offord RE, Remy S, Aubry JP, Wells TN, Whitehorn E, Oung T, Proudfoot AE: Receptor-mediated endocytosis of CC-chemokines. *J Biol Chem* 1997, 272:9617–9620
56. Bacon KB: Chemokine-induced signal transduction in leukocytes. *Eur Cytokine Netw* 1999, 10:260–261
57. Simonsen AC, Heegaard CW, Rasmussen LK, Ellgaard L, Kjoller L, Christensen A, Etzerodt M, Andreasen PA: Very low density lipoprotein receptor from mammary gland and mammary epithelial cell lines binds and mediates endocytosis of Mr() 40,000 receptor associated protein. *FEBS Lett* 1994, 354:279–283