The Low Density Lipoprotein-receptor-related Protein Is a Motogenic Receptor for Plasminogen Activator Inhibitor-1*

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Although plasminogen activator inhibitor-1 (PAI-1) is known to stimulate cell migration, little is known about underlying mechanisms. We show that both active and inactive (e.g. cleaved) PAI-1 can activate the Jak/Stat signaling system and stimulate cell migration in chemotaxis, haptotaxis, chemokinesis, and wound healing assays. Moreover, antibodies to the LDL receptor-related protein (LRP) and an LRP antagonist (RAP) blocked these motogenic effects of PAI-1, while a PAI-1 mutant that did not bind to LRP failed to activate the Jak/Stat signaling pathway or to stimulate cell migration. PAI-1 had no chemotactic effect on LRP-deficient cells. These results indicate that LRP is a signaling molecule, that it mediates the migration-promoting activity of PAI-1, and that this activity does not require intact, biologically active PAI-1. Activation of this LRP-dependent signaling pathway by PAI-1 may begin to explain how the inhibitor stimulates cell migration in a variety of normal and pathological processes.

The fibrolytic system not only removes fibrin deposits from the vasculature, but also has been implicated in extracellular matrix (ECM) remodeling (1–3). The primary fibrinolytic enzyme is plasmin, a potent, broadly acting serine protease that is formed when circulating plasminogen is cleaved and activated by one of two plasminogen activators (PAs), tissue-type (tPA), and urokinase-type (uPA). Until very recently, most studies of this system have focused on the biochemistry and consequences of plasmin generation in a variety of settings including fibrin degradation, tissue remodeling, inflammation, and cancer invasion. However, in recent years, it has become increasingly apparent that uPA, together with its cellular receptor, uPAR (CD87), and its primary inhibitor, PAI-1, may themselves have profound effects on cells and their association with the ECM. Unexpectedly, these very specific effects on the attachment, detachment and migration of cells do not require the presence of plasminogen or the generation of plasmin.

The binding of uPA to uPAR on the cell surface increases the adhesive properties of cells by increasing the affinity of uPAR for the ECM protein vitronectin (VN) and for integrin adhesion receptors (1, 4). These interactions also induce cell signaling (5–8). Because uPA is a glycospiphatidylinositol-anchored protein, the observed signaling activity is probably not an inherent property of uPAR itself, but rather an indirect effect of its association with integrins and/or other membrane receptors such as FPLR1 (9). The binding of uPA to free PAI-1 in solution generates an uPA/PAI-1 complex that is catalytically inactive and has no signaling activity (10). However, when PAI-1 binds to uPA on the cell surface, it induces the internalization of the resulting PAI-1-UuPA/PAI-1 complex and inhibits uPA-induced cell migration (10–12). Thus, PAI-1 can regulate both pericellular proteolysis and the concentration of uPA and uPAR on the cell surface. The binding of PAI-1 to uPA on the cell surface also leads to the deactivation and internalization of integrins that are bound to uPAR, inducing cell detachment (13). Finally, PAI-1 binds to VN and inhibits the adhesion and/or migration of cells on this ECM by blocking integrin and/or uPAR binding sites (14–16). Taken together, these observations suggest that PAI-1 may influence a variety of normal and pathological processes involving cell migration, and this seems to be the case (2, 5, 7).

The exact mechanism(s) by which PAI-1 influences cell migration remains unclear. Because of its protease inhibitory activity, PAI-1 was originally thought to be an inhibitor of cell migration and invasion (17). Indeed, PAI-1 can inhibit uPA activity and thus control the pericellular proteolysis that may be required during cell migration through the ECM. Moreover, since the addition of PAI-1 to cells leads to the internalization of the uPA/uPAR complex, PAI-1 also can decrease the levels of uPA and uPAR on the cell surface, possibly decreasing cell migration. In fact, PAI-1 appears to inhibit uPA-mediated chemotaxis by this mechanism (10). However, PAI-1 is also chemotactic for a variety of cells (10, 18), and is up-regulated in cells that migrate into the denuded area of a wounded cell monolayer (19). These observations suggest a role for PAI-1 in wound healing and cell migration. The apparently conflicting observations that PAI-1 can either inhibit or stimulate cell
migration may be related to differences in the local concentration of PAI-1 since low concentrations appear to promote angiogenesis, while high levels inhibit it (reviewed in Ref. 2).

Whatever the case, it seems likely that low density lipoprotein receptor-related protein (LRP) is involved since the internalization of the uPA/uPAR/integrin complex by PAI-1 is an LRP-dependent process (11–13). LRP is a relatively large macromolecule composed of two subunits, a 515 kDa α-chain and a smaller 85 kDa β-chain that constitute its N- and C-terminal exons, respectively (reviewed in Refs. 14, 20). Two NPYX motifs are present in the cytoplasmic tail of LRP, and these motifs not only appear to be important for endocytosis, but also are an important connection between LRP and cytoplasmic signaling molecules such as Src. The observation that LRP is the endocytic receptor involved in the internalization of uPAR (11, 12, 21) suggests that LRP can regulate uPA-dependent cell migration. The observation that PAI-1-mediated internalization of uPAR inhibits uPA-dependent cell migration and cell cytoskeleton reorganization (10) is consistent with this idea. Moreover, when the internalization of uPAR is impaired or blocked, cell surface uPAR levels increase and cell motility is enhanced (22, 23).

In the present study, we further investigate the role of PAI-1 in cell migration. We demonstrate that PAI-1 is a potent chemotactant molecule, which induces cell migration with corresponding changes in cell morphology, cytoskeleton reorganization, and phosphorytrosine redistribution. Using several PAI-1 mutants and specific inhibitors, we show that the chemotactic activity of PAI-1 does not depend on its interactions with uPA, tPA, or VN, but rather with LRP. This interaction leads to the activation of the Jak/Stat signaling pathway and the induction of cell migration.

EXPERIMENTAL PROCEDURES

Materials—Placenta-derived human LRP was a generous gift from Dr. S. K. Moestrup (University of Aarhus, Denmark). A plasmid encoding glutathione S-transferase fused to RAP (GST-RAP) was kindly provided by Dr. J. Kuiper (Leiden University, The Netherlands), and used for expression of GST-RAP in Escherichia coli DH5α as described previously (24). As the GST tag does not interfere with the binding properties of RAP (24), GST-RAP was used throughout the present study and is referred to as RAP. The BIACORE®X3000 biosensor system, reagents, and CM sensor chips (research grade), from Biacore AB (Uppsala, Sweden). Human fibroentin was from Roche Applied Science. Nonspecific rabbit and mouse IgG, as well as collagen I, formylated peptide fMLP, FITC-, and TRITC-phalloidin were from Sigma. Rabbit anti-phosphotyrosine polyclonal antibody, and anti-mouse IgG rhodamine conjugated Fab’2 fragment secondary antibody, were from Chemicon (Temecula, CA). Mouse anti-human Jak1 and anti-human Stat1 monoclonal antibodies were from BD Biosciences, Transduction Laboratories (Lexington, KY). The Jak inhibitor AG-490 was from Bio-tnol (Plymouth Meeting, PA). VN was purified from human plasma as described (15). A rabbit polyclonal antibody against human PAI-1 was generated in rabbits by standard procedures. The polyclonal antibody against human LRP was from RDI, Research Diagnostics (Flanders, NJ), and further purified using the affinity chromatography kit MabTrap G II (Amersham Biosciences).

PAI-1 Mutants—The cDNAs for the PAI-1 mutants used in this study were kindly provided by Dr. D. Lawrence (American Red Cross, Rockville, MD), and expressed and purified as described (13). The stable PAI-1 mutant (14-1B) behaved like wild-type PAI-1 in the cell migration assays (data not shown). Thus, PAI-1 14-1B was used throughout this study and is referred to as PAI-1. Cleaved PAI-1, which was obtained by limited proteolysis of PAI-1 with elastase (25), was catalytically inactive and did not bind to VN (data not shown). It migrated with a molecular weight of ~39,000 daltons (not shown) indicating that it contained a reactive center located in residues 123 (25). The PAI-1 mutants Q123K, T333R/A335R, and R346M/M347S have been described previously (25, 26). Final endotoxin levels were evaluated using the Limulus Amebocyte Lysate kit QCL-1000 (Bio-Whittaker, Walkersville, MD). Only protein preparations with an endotoxin level of less than 0.14 EU/ml were used.

Cell Culture—Human smooth muscle cells (AoSMC, CASMC), human endothelial cells (HAE, HCAEC) from the aortic and coronary arteries, respectively, and human dermal microvascular endothelial cells, neonatal (HMVEC-d neo), were cultured according to the supplier (Clonetics, Charlotte, NC). Rat smooth muscle cells (RSMC) were a kind gift of Dr. Marco Bertulli (Bayer Research Laboratory, Milan, Italy). MEF-1 are wild-type murine embryonic fibroblasts derived from the same mouse strain as MEF-2, is generated after treatment with the reagent LRP (27). BSMC, HIT-1080 (highly invasive human fibrosarcoma cells), IPF (non-invasive human melanoma cells that do not express uPA), MEF-1, and MEF-2 cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum.

Migration Assays—Chemotaxis assays were performed as previously described (28), using modified Boyden chambers. Briefly, ~50,000 cells suspended in 2 μl of medium were added to the upper wells of Boyden chambers, and the molecules to be tested were added to the lower well in serum-free medium. Antibodies or inhibitors were added to both wells. Haptotaxis assays were performed under the same conditions, except that the filters were washed with serum-free Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin, and then preincubated with the indicated amounts of PAI-1 or fibroentin in the Dulbecco’s modified Eagle’s medium solution for 3 h at 37 °C. The filters were washed, and then serum-free medium was added to both the upper and lower wells. Wounding assays were performed as previously described (29). Briefly, confluent monolayers were scraped with a pipette tip, and the number of cells migrating into the wound over the next 24 h was then determined. Chemokinesis assays were performed as described (29), except that the wells were stimulated for increasing times with 2 ng PAI-1 to be tested, and the presence or absence of 5 μg/ml RAP, and were only labeled with phal-loidin. The cells were then counted using an Olympus UplanFL 100 lens. Quantification of the actin cytoskeleton reorganization was performed by taking low magnification photographs and counting the resting cells (those that exhibit numerous stress fibers and a non-polarized morphology) and non-resting cells (polarized cell shape with reorganized actin cytoskeleton due to a decrease in stress fibers, and increased membrane ruffling and actin semi-rings). All experiments were performed at least twice in triplicate. Results are the mean ± S.E. of the number of cells counted in ten high power (×40) fields per filter and expressed as fold over control. Random cell migration (i.e. migration in the absence of chemotactant) was given the arbitrary value of 100%.

Immunofluorescence Microscopy—Cells were cultured, fixed, stained, and mounted as described (28), except that in some experiments, cells were pretreated for 5 min with RAP (5 μg/ml). Cells were stained either with anti-phosphotyrosine, anti-Jak1, or anti-Stat1 antibody, and then were double-stained with phalloidin for visualization of filamentous actin. In some cases, the above cells were triple stained by employing the additional nucleus probe DAPI (4′,6-diamidino-2-phenylindole, Roche Applied Science). Fluorescence photographs were taken using an Olympus BX60 microscope coupled to a DVC camera using Olympus UplanFL 100 lens, and analyzed with C-view and Image pro-plus software.

Surface Plasmon Resonance—Binding studies were performed by using the BIACORE®X3000 biosensor system, and surface plasmon resonance analysis was done as described (20). In these experiments, LRP was immobilized on a CMS sensor chip at a density of 5.21 fmol/mm², using the amine-coupling kit as instructed by the supplier. A control channel was activated and blocked in parallel, using the amine-coupling reagents in the absence of protein. Binding to the coated channel was then detected by binding of coated or uncoated PAI-1 (less than 5% of the binding to coated channels). Surface plasmon resonance analysis was performed in 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM Ca Cl2, 0.005% (v/v) Tween-20 at 25 °C with a flow rate of 5 μl/min. For qualitative measurements of the binding of the PAI-1 variants to immobilized LRP, 100 nm of each was injected in duplicate over the sensor chip surface. Different preparations of each variant were tested. BIA-OS analysis was performed using the BIACore Data analysis software. The sensor chip surface was accomplished by incubating the chip with 100 mM H3PO4 for 2 min at a flow of 5 μl/min.

Real-time Quantitative PCR—Total RNA was isolated from cells using the RNeasy mini kit (Qiagen Inc., Valencia, CA). Subsequently, cDNA was prepared from 0.3 μg of total RNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) in a final reaction volume of 20 μl. Real-time PCR amplifications were performed from 2.5 μl of cDNA diluted...
1:2 using LRP primers LRP-F (5′-TCAATGGGTGGATGTACTGGA-3′) and LRP-R (5′-AATCTTCCGGTCTGTGCCAT-3′) and β-actin primers β-actin-F (5′-TGGAACTCTGTTGCCATCCTAGAAAC-3′) and β-actin-R (5′-TAAAACGCAGCTCAGTAACAGTCCG-3′). These primer sets were employed at 150 nM in a final volume of 25 μl using the SYBR® Green Master Mix (Applied Biosystems). All PCR reactions were performed in an iCycler (Bio-Rad). Relative LRP mRNA levels were calculated after normalization to β-actin mRNA levels.

Co-immunoprecipitation and Western Blotting—Cells were incubated with 930 nM PAI-1 (or the PAI-1 variants) for 1 h in the presence or absence of RAP (6.7 μg/ml; 10 μM). The cells were lysed in 10 mM CHAPS in 20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM CaCl₂, and the resulting lysates were incubated for 18 h at 4°C in the presence of both protein G-agarose beads and the IgG fraction of the monoclonal antibody against LRP (11H4) as described (13). The beads were washed, the proteins were extracted in reducing sample buffer, fractionated by SDS-PAGE using 7.5% acrylamide gel, and then analyzed by Western blotting (13). Briefly, the separated proteins were transferred to polyvinylidene difluoride Immulon-P membranes (Millipore, Bedford, MA), the membranes were blocked by incubation in TBS, pH 7.2, 5% calf serum, 0.1% Tween-20 for 1 h, and then were incubated for 1 h with anti-PAI-1 or anti-LRP polyclonal antibodies. Blots were processed with the enhanced chemiluminescence (ECL) detection system (Pierce) using horseradish peroxidase-coupled donkey anti-rabbit (H+L) depleted of cross-reactivity to mouse IgG (Jackson ImmunoResearch, West Grove, PA). PAI-1 migrated with an apparent Mr 43 kDa while LRP (β-chain) had an apparent Mr ~85 kDa.

Statistical Analysis—Statistical analysis was performed using Prism software. The Student’s t test was employed for pair-wise comparisons of treatments, while an ANOVA model was used for the evaluation of treatments with increasing doses of a reagent.

RESULTS

PAI-1 Stimulates Cell Migration

Experiments were performed to investigate the effects of PAI-1 on the migration of a variety of cells as determined using...
well known chemotaxis, haptotaxis, and wound-healing assays. In preliminary experiments, we examined the chemotactic effects of PAI-1 using Boyden chambers. Fig. 1A shows that PAI-1 stimulates the migration of normal RSMC in a dose-dependent manner, with a half-maximal response at 0.02–0.2 nM and a plateau at 2–20 nM. Similar data were obtained using invasive HT-1080 and non-invasive IF6 cells (data not shown).

PAI-1 also increased the haptotactic activity of RSMC (Fig. 1B), and the rate of RSMC (Fig. 1C) and HT-1080 cell (not shown) migration into wounds, and did so at similar concentrations (compare Fig. 1, A–C).

Experiments were performed to determine the specificity of the effects of PAI-1 on cell migration. The chemotactic effect of PAI-1, but not that of the well characterized attractant fMLP, was completely blocked by neutralizing polyclonal antibodies against PAI-1 (Fig. 1D). The migration-promoting effect of PAI-1 in wound healing assays was also inhibited by the addition of anti-PAI-1 antibodies (Fig. 1E). In both cases (Fig. 1, D and E), treatment with the antibody alone, or with nonspecific IgG gave results that did not differ significantly from the untreated control. Thus, these effects are specific for PAI-1 and not caused by contaminants in the PAI-1 preparation.

The Migration-promoting Effects of PAI-1 Do Not Require Its Interaction with VN, uPA, or tPA

PAI-1 binds to and inactivates uPA and tPA, and it binds to VN and blocks its ability to function as a cell adhesion molecule. In preliminary experiments (not shown), we were unable to block the chemotactic effects of PAI-1 using monoclonal antibodies that bind avidly to VN and block PAI-1 binding (30). Moreover, a PAI-1 mutant, which cannot bind to VN (i.e. PAI-1 Q123K), stimulated RSMC migration (Fig. 2A) in a manner that was similar to that of PAI-1 itself (see Fig. 1A). Thus, the induction of cell migration by PAI-1 does not appear to depend on the binding of PAI-1 to VN.

Experiments were performed to determine whether the chemotactic activity of PAI-1 depended on its interactions with uPA or tPA. In these experiments, several variant forms of PAI-1 were compared including those which have no inhibitory activity against uPA/tPA (i.e. PAI-1 T333R/A335R and PAI-1 R346M/M347S), and an elastase cleaved form of PAI-1, which does not inhibit PAs and does not bind to VN. Unexpectedly, most of the PAI-1 variants, including the completely inactive cleaved form, were able to stimulate cell migration as well as PAI-1 itself.
and as well as bFGF and fMLP (Fig. 2B). However, the double mutant, PAI-1 R346M/M347S, failed to stimulate cell migration (Fig. 2B) even when employed at very high concentrations (up to 4 μM) (not shown). Similar data were obtained using the wound-healing assay (Fig. 2C). The observation that cleaved PAI-1 retained full chemotactic activity suggests that PAI-1-mediated cell migration does not require active PAI-1. This conclusion is supported by the observation that an intact and highly purified (i.e. reversed-phase chromatography) form of PAI-1, which lacked detectable PA inhibitory activity and did not bind to VN, retained full chemotactic activity (not shown).

**PAI-1 Induces Changes in Cell Morphology, Cytoskeleton Organization, and the Distribution of Phosphorylated Tyrosines**

Cell motility requires cytoskeleton reorganization and cell shape changes (32, 33). To investigate the effects of PAI-1 on cellular morphology, subconfluent cultures of RSMC were stimulated for 30 min with PAI-1. Actin filaments were then labeled with FITC-phalloidin, and the distribution of phosphorylated tyrosines (known to be associated with changes in the activation state of cellular signaling pathways) was visualized by immunofluorescence using anti-phosphotyrosine antibodies (Fig. 3). In control cultures (top panels), phosphotyrosine staining was observed predominantly in the cytoplasm around but not over the nucleus, and most cells showed a high number of stress fibers revealing a well-developed actin cytoskeleton. Upon stimulation with PAI-1 (bottom panels), phosphotyrosines were now detected in the nucleus (arrow) and at the leading edge of the cell membrane (arrowheads). In addition, the cells developed a more polarized, elongated morphology reflecting the spatial rearrangement of the actin cytoskeleton. Membrane ruffling and an actin semi-ring structure were now observed at the leading edge of the cells. PAI-1 induced similar changes in HT-1080 cells (not shown).

Chemokinesis experiments were performed to determine whether these changes in the morphology of PAI-1-treated cells represented a dynamic transition from the resting state to a more motile state. Cells were stimulated with PAI-1 for increasing times and changes in cell morphology were recorded.
as in Fig. 3. The cells were then classified either as “resting” or “non-resting” as described under “Experimental Procedures.” The effect of PAI-1 on cell morphology and cytoskeleton reorganization was rapid but transient (Fig. 4A). For example, within 5 min after the addition of PAI-1, the proportion of non-resting cells had increased from 45 to 60%, reaching its maximum (80%) within 15–30 min. After 30 min, the number of non-resting cells slowly decreased to the level observed in unstimulated control cultures (Fig. 4A). Thus, PAI-1 challenge rapidly leads to the reorganization of the actin cytoskeleton and to changes in cell shape, two responses connected to cell migration.

Evidence that LRP Mediates the Chemotactic Signal of PAI-1

**RAP Blocks the Chemotactic Effects of PAI-1**—To induce cell migration, PAI-1 must interact with a cellular receptor that will mediate its migratory signal. LRP is known to bind a variety of ligands including PAI-1 (12, 20), and it may have signaling capacities (34, 35). Therefore, we examined the effects of RAP, a molecule that binds to LRP and blocks its interactions with all known ligands, on PAI-1-induced cell migration (Fig. 4). The addition of RAP alone did not alter the chemokinetic activity of the cells, but it completely abolished the effect of PAI-1 (Fig. 4A). RAP also completely blocked the chemotactic effect of PAI-1 on RSMC (Fig. 4B) and on HT-1080 (not shown). This was a very specific effect, since RAP did not inhibit VN-induced cell migration (Fig. 4B). VN stimulates migration through αvβ3, not through LRP (33). Finally, RAP inhibited the migration-promoting effects of the various PAI-1 variants in the chemotaxis (Fig. 4C) and wound-healing assays (not shown), and a polyclonal antibody against LRP blocked PAI-1-induced cell migration (Fig. 4D). This antibody had no effect on cell migration induced by the chemokine peptide fMLP, and nonspecific IgG did not alter RSMC migration induced by either PAI-1 or fMLP.

**PAI-1 Binds to LRP on the Cell Surface**—The fact that RAP blocked PAI-1-induced cell migration suggested that the induction required the binding of PAI-1 to LRP. A variety of experiments were performed to demonstrate that PAI-1 binds to LRP on the cell membrane. We initially performed “pull-down” experiments in which RSMC containing LRP on their surface were mixed with PAI-1 or PAI-1 variants in the absence or presence of RAP. The cells were lysed, immunoprecipitated using a monoclonal antibody against LRP, and then Western-blotted for either LRP or PAI-1. We detected both PAI-1 itself and cleaved (inactive) PAI-1 in the immunoprecipitates from the cells incubated in the absence of RAP, but neither was detected in the presence of RAP (Fig. 5A). The cleaved form of PAI-1 lacks the C terminal portion of the molecule (25) and migrates slightly faster than intact PAI-1. PAI-1 R346M/M347S was not detected in these pull-down experiments suggesting that it bound poorly to LRP, and that its failure to induce cell migration was related to its inability to bind to this receptor. This hypothesis is supported by direct binding studies using surface plasmon resonance (Fig. 5B). In these experiments, purified LRP was immobilized on the biosensor chip, and then the various PAI-1 variants were injected, and their interactions with the immobilized LRP were recorded. The corresponding kinetic constants were derived from the resulting association and dissociation curves (Table I). All PAI-1 variants that demonstrated chemotactic activity were able to bind to LRP with similar affinity (KD = 80–160 nM), while the variant, which was not chemotactic (i.e. PAI-1 R346M/M347S) showed little specific binding to the immobilized LRP (KD = 16 μM).

**PAI-1 Does Not Stimulate the Migration of LRP-deficient Cells**—To further test the hypothesis that LRP is a chemotactic receptor for PAI-1, we investigated the effects of PAI-1 on the migration of LRP-deficient cells (Fig. 6). Although, PAI-1 stimulated the migration of LRP expressing MEF-1 (Fig. 6A, column 1 and 2), it failed to stimulate the migration of LRP−/− MEF-2 (Fig. 6A, columns 3 and 4). Only the migratory response to PAI-1 was altered in these cells since their rates of random cell migration were similar (compare lanes 1 and 3). Thus, LRP deficiency appears to be responsible for the lack of the chemotactic response to PAI-1. This conclusion is supported by the observation that even at relatively high concentrations, PAI-1 did not stimulate the migration of endothelial cells (EC) (Fig. 6A, lanes 5–8), and these cells are known to be naturally devoid of LRP (36, 37). In this regard, control real-time quantitative RT-PCR experiments revealed that HMVEC-d neo do not express detectable amounts of LRP mRNA (not shown). The failure of PAI-1 to stimulate the migration of these cells was not due to the intrinsic inability of HMVEC-d neo to migrate since bFGF significantly stimulated their migration (Fig. 6A, column 9). Finally, we directly compared the migratory properties of EC and SMC from two major arteries (Fig. 6B). Again, neither EC from the coronary artery (lanes 1–3) nor the aorta (not shown) migrate in response to PAI-1 challenge (compare lane 1 versus 2), although they were induced to migrate when stimulated with bFGF (lane 3). In contrast, SMC from the coronary...
cell by activating intracellular signaling pathways. Because a variety of chemoattractants use the Janus kinase/signal transducer and activator of transcription (Jaks/Stat) pathway to regulate cell migration (38), we investigated the effects of PAI-1 on components of this pathway. In general, the inactive forms of Jaks and Stats are present in the cytoplasm. The binding of specific ligands to their receptors induces the translocation of Jaks to the plasma membrane and their subsequent activation. The activated Jaks in turn activate the Stat proteins, which then translocate into the nucleus. Fig. 7 shows the effects of PAI-1 on the distribution of Jak1, which is mainly cytoplasmic in unstimulated control cells (Fig. 7A). After stimulation with PAI-1 (Fig. 7B), a subpopulation of Jak1 localized to the cell membrane particularly in the area of membrane ruffling at the leading edge of the migrating RSMC. This change was most apparent in the merged panel where Jak1 appeared to colocalize with actin in areas of membrane ruffling (Fig. 7B, merge). As a control to firmly establish that this change in the cellular distribution of Jak1 was truly due to PAI-1, we examined the distribution of Jak1 in migrating cells not stimulated with PAI-1 (Fig. 7C). In this case, despite a clear polarized motile cell shape, Jak1 was not observed in the membrane at the leading edge of the cell (compare merged panels B and C), but instead was localized primarily to the cytoplasm. This distribution was similar to that of resting control cells (Fig. 7A).

Fig. 8 shows the effect of PAI-1 on the location of Stat1. Stat1 was mainly cytoplasmic in unstimulated control RSMC (Fig. 8A, left panels), and treatment of the cells with PAI-1 induced its translocation into the nucleus (as confirmed by the DAPI staining of the nucleus and the merged image). Although PAI-1 R346M/M347S failed to induce this translocation (Fig. 8A), Stat 1 was redistributed by the other PAI-1 variants (not shown). RAP completely blocked the effects of PAI-1 on Stat1 location, and on cell shape and actin cytoskeleton organization (Fig. 8A, right panels). Thus, these effects of PAI-1 on Stat1 also appear to be LRP-dependent. Fig. 8B shows that the induction of cell migration by PAI-1 was dose-dependently blocked by the addition of AG-490, an inhibitor of Jaks. However, random cell migration was not affected by these same concentrations of the inhibitor. Taken together, these data show that the Jak/Stat pathway is activated by the binding of PAI-1 to LRP, and that it is involved in the regulation of PAI-1-induced cell migration.

**DISCUSSION**

PAI-1 is the physiological inhibitor of uPA and tPA (2). It is present in plasma and can be released from platelets, monocytes, macrophages, EC and SMC during thrombotic and/or inflammatory processes. Furthermore, the secretion of PAI-1 from cells is enhanced by several factors including transforming growth factor beta and cytokines released by platelets (18, 39). Thus, PAI-1 is expected to accumulate at sites of injury and/or inflammation, and to become associated with the thrombus (40). In this regard, PAI-1 has been shown to be a potent regulator of the interaction between cells and the ECM. Thus, it can bind to VN and block the ability of cells to attach to and

**Table 1**

| PAI-1 variant | \(k_{\text{off}}\) (10^{-3} M^{-1} s^{-1}) | \(k_{\text{on}}\) (10^{-4} M s^{-1}) | \(K_D\) (nM) |
|--------------|-----------------|----------------|---------|
| 14-1B        | 5.50 ± 1.16     | 5.88 ± 1.01    | 93 ± 25 |
| Cleaved      | 1.35 ± 0.12     | 9.75 ± 0.53    | 138 ± 14 |
| Q123K        | 1.14 ± 0.53     | 7.02 ± 1.26    | 162 ± 81 |
| T333R/A335R  | 6.56 ± 0.94     | 7.84 ± 1.54    | 81 ± 20  |
| R346M/M347S  | 4.08 ± 0.69     | 2.48 ± 0.22    | 16.45 ± 3.14 |

**Fig. 6.** PAI-1 does not stimulate the migration of LRP-deficient cells. Migration assays were performed as described in the legend to Fig. 1. A, the chemotactic effect of PAI-1 (2 nm) on LRP-expressing MEF-1 cells (lanes 1 and 2) was compared with its effect on LRP-deficient MEF-2 cells (lanes 3 and 4). Lanes 5 and 6 show the chemotactic effect of 2 ng/ml (lane 5) and 200 ng/ml (lane 6) of PAI-1 on the migration of LRP-deficient HMVEC-d neo. Lanes 7 and 8 show the effect of 2 ng/ml and 200 ng/ml, respectively, of PAI-1 on the haptotactic activity of HMVEC-d neo, while lane 9 shows the effect of bFGF (50 ng/ml) on the chemotactic activity of HMVEC-d neo. B, comparison of the chemotactic effects of PAI-1 on human ECs (lanes 1–3) and SMCs (lanes 4–6) from the coronary artery. Lanes 3 and 6 show that both cell types respond to bFGF. In panels A and B, the symbol * indicates treatment where the migratory response is statistically different from the no addition control (p < 0.01 in Student’s t test).

**Fig. 7.** Jak1 and Stat1 localization in PAI-1 stimulated cells. A, expression of Jak1 in RSMC from the coronary artery (lanes 4–6) and aorta (not shown) were stimulated to migrate in response to both PAI-1 (compare lanes 4 and 5) and bFGF (lane 6) challenge. Taken together, the results in Fig. 6 demonstrate that PAI-1 cannot stimulate the migration of LRP-deficient cells, and suggest that the expression of LRP on the surface of cells is an important requirement for their migratory response to PAI-1. These observations are again consistent with the role of LRP as the chemotactic receptor for PAI-1.

**PAI-1 Activates the Jak/Stat Signaling Pathway**—Induction of cell migration requires the binding of the chemoattractant to a cellular receptor, which mediates the migratory signal to the...
migrate on this ECM component (14, 15), and it can detach cells from VN and a variety of other matrix proteins including type I collagen and fibronectin (13). Finally, PAI-1 has been shown to both stimulate (1, 10, 18, 41) and inhibit (14, 16) cell migration. These apparently inconsistent results may reflect differences in the concentrations of PAI-1 employed (10, 15), with low doses stimulating migration and high doses inhibiting it (2). The observations in this report confirm and extend the observations that low concentrations of PAI-1 stimulate cell migration.

Chemoattractant Properties of PAI-1—Four independent cell migration assays, including those specific for chemotaxis, haptotaxis, wound healing, and chemokinesis, were employed to demonstrate that PAI-1 is a powerful motogenic molecule. We also attempted to relate the migration-promoting effect of PAI-1 to several cellular parameters of cell migration including changes in cell shape, in the organization of the actin cytoskeleton, and in the distribution of phosphorylated signaling molecules. Indeed, cell motility requires cytoskeleton reorganization and cell shape changes (32). Our results show that PAI-1
stimulates cell migration in chemotaxis and wound-healing assays (Figs. 1 and 2), and it promotes changes in cell morphology and cytoskeleton organization (Fig. 3) that resemble those described previously for bFGF, fMLP, and other chemotactants (28, 33). These effects are specifically due to PAI-1 and not to potential contaminants in the PAI-1 preparations, because they were inhibited by anti-PAI-1 antibodies. Finally, the chemotactic activity of PAI-1 does not require its interaction with VN, uPA, or TPA (Fig. 2). In this regard, cleaved PAI-1, a molecule that can no longer bind to VN or inhibit PAs, was as chemotactic as the intact molecule, indicating that the chemotactic effect does not require biologically active intact PAI-1. The identity of the chemotactic sequence in PAI-1 remains to be determined.

**LRP Is the Cellular Receptor for PAI-1 and Mediates Its Chemotactic Effects**—LRP is well known as an endocytic receptor, and it binds a wide variety of ligands and mediates their internalization (20, 27). However, recent reports suggest that LRP may also mediate neurite outgrowth and calcium influx, and regulate cell migration and survival (reviewed in Refs. 12 and 42). In this regard, LRP may function as a co-receptor for the PDGF receptor, and it has been implicated in the regulation of PDGF-controlled SMC migration (34, 43). Finally, LRP was reported to be phosphorylated by members of the c-Src family of kinases, and to associate with other signaling molecules such as the adaptor protein Shc (12, 43). Thus, LRP appears to be part of a complex signaling system that regulates cell adhesion, migration, and survival (34, 35). Here we provide evidence that LRP also mediates PAI-1-induced cell migration. For example, both RAP (the LRP antagonist) and anti-LRP antibodies blocked PAI-1-directed cell migration in the chemotaxis, wound healing, and chemokinesis assays (Fig. 4). Moreover, co-immunoprecipitation and surface plasmon resonance studies demonstrate that PAI-1 binds to LRP on the cell surface (Fig. 5 and Table I), and a mutant of PAI-1 (PAI-1 R346M/M347S), which has no chemotactic activity, does not bind to LRP. Finally, LRP−/− MEF-2 isolated from LRP knock-out mice, and LRP-deficient EC (i.e. HMVEC-dneo), do not migrate in response to PAI-1 challenge (Fig. 6). It was previously reported that ECs do not express LRP (36, 37). Thus, the observation that EC from the coronary artery and the aorta do not respond to PAI-1 while SMC from these same vessels do respond (Fig. 6B), strengthens the conclusion that these differences in responsiveness reflect the presence or absence of LRP. Although EC generally do not express LRP (see however Orr et al., Ref. 35), they do express the very low-density lipoprotein receptor (VLDL-R, Ref. 44). These considerations suggest that VLDL-R cannot transduce the migratory signal induced by PAI-1. Whether other members of the LRP family of endocytic receptors (e.g. megalin; Ref. 45) can also mediate the chemotactic effects of PAI-1 remains to be determined.

**Binding of PAI-1 to LRP Activates the Jak/Stat Signaling Pathway**—We showed previously that PAI-1 does not induce MAP kinase (ERK1/2) phosphorylation and translocation into the nucleus (10). The Jak/Stat pathway is another pathway known to modulate cell proliferation, survival, differentiation, and migration (38). In this case, ligand-receptor interactions trigger the recruitment of Jak2 to the plasma membrane. Stats are latent cytoplasmic transfection factors that, once activated by Jak2, translocate into the cell nucleus. Using immunofluorescence microscopy, we show that PAI-1 induces the translocation of Jak2 to the plasma membrane at the leading edge of the cell (Fig. 7), while Stat1 is redistributed to the nucleus (Fig. 8A). This activation of the Jak/Stat pathway is a consequence of PAI-1 binding to LRP, since RAP blocks the PAI-1-mediated translocation of Stat1 into the nucleus (Fig. 8A). Furthermore, the PAI-1 mutant, R346M/M347S, which has no chemotactic effect and does not bind to LRP, failed to activate Stat1 or to induce its subsequent translocation into the nucleus. The observation that the Jak inhibitor, AG-490, blocks PAI-1-directed cell migration (Fig. 8B) is again consistent with the conclusion that the Jak/Stat pathway is directly involved in the regulation of PAI-1-mediated cell migration. The exact mechanism by which LRP signaling leads to the recruitment and activation of Jak1 remains to be determined.

**Potential Roles of PAI-1 in Cell Migration and Vascular Disease**—Based on the previous discussion, what might we expect to happen to the migratory behavior of cells if they are induced to synthesize and secrete PAI-1? The situation is clearly complex since PAI-1 can influence cell behavior in at least three different ways, depending on the activation state of the cells, the local concentration of PAI-1, and the presence or absence of VN, uPA, and uPAR. Thus, for example, if VN is present in the matrix, high concentrations of PAI-1 would bind to the VN and block cell adhesion through uPAR (15) and integrins (8, 14), thus inhibiting cell attachment and integrin-dependent migration. If active two-chain uPA is present on the cell surface bound to uPAR-integrin complexes, PAI-1 will bind to the uPA and promote cell detachment from VN and other ECM proteins (13). Finally, as shown here, low concentrations of free, non-complexed PAI-1 and its latent and cleaved forms bind to LRP and promote cell migration. This observation was initially unexpected since we (Table I) and others (46–48) have shown that these forms of PAI-1 only bind to LRP with low affinity. Complex formation between PAI-1 and either tPA or uPA results in the exposure of a high-affinity binding site in PAI-1 for LRP (46, 49). However, the binding of the uPA-PAI-1 complexes to LRP does not stimulate migration (10). Thus, there must be a second site in PAI-1, which binds LRP with low affinity and is distinct from the site in PAI-1 complexes. In any case, one possible explanation of the effectiveness of this low concentration is that PAI-1, like other heparin binding ligands of LRP (i.e. ApoE, Ref. 50; Factor IXa, Ref. 51; Factor VIII, Ref. 52), binds first to heparin sulfate proteoglycans at the cell surface, locally concentrating the inhibitor, and then is transferred to LRP. This concentrating mechanism would lead to a high local concentration of PAI-1 on the cell surface and would make the low affinity interaction between PAI-1 and LRP more likely to occur, even at the low concentrations used. Whether this ligand transfer mechanism can indeed account for our observations is under investigation.

When considered together, the above observations indicate that PAI-1 has the potential to regulate all phases of the migratory process, from cell attachment to cell detachment and migration. Since LRP is widely expressed (36), the binding of PAI-1 to LRP also may have broad pathological significance. For example, if confirmed in vivo, the observation that PAI-1 stimulates the migration of SMC but not EC, may be relevant to the proliferative response frequently observed after vascular injury induced by angioplasty (i.e. restenosis). This conclusion is based on the observation that restenosis is, in fact, the result of SMC migration from the outer layers of the vessel wall into the intima, the inner layer of the vessel wall adjacent to the lumen, where they proliferate to form a neointima (53). PAI-1 is known to be released from platelets and other cells and to accumulate at the site of injury and/or inflammation (40). Thus, according to this model, the released PAI-1 binds to LRP on SMC (but not on EC) and induces their migration into the intima. Endothelial cell damage itself leads to a burst of SMC migration and proliferation, reflecting the SMC transition from the contractile state to the synthetic state. Damaged EC (19) and SMC in the synthetic state (54) also produce PAI-1.
In summary, this study shows that the serine protease inhibitor PAI-1 can induce the migration of cells in vitro. PAI-1 also induces changes in cell shape and cytoskeleton organization that are required for cell motility. Finally, PAI-1 stimulates the redistribution of phosphotyrosines from the cytoplasm into the nucleus, and to the plasma membrane at the leading edge of the migrating cell. PAI-1 exerts these effects by binding to LRP, an interaction that leads to the subsequent activation of the Jak/Stat signaling pathway. The preliminary studies showing differences in PAI-1 responsiveness between SMC and EC raise the possibility that LRP uniquely mediates the chemotactic signal induced by PAI-1, and that LRP-deficient cells, which express other members of the LRP gene family will not migrate in response to PAI-1 challenge. This hypothesis needs to be rigorously tested in vivo. In any case, these observations provide a potential molecular mechanism by which PAI-1, by binding to LRP and activating the Jak/Stat signaling pathway, induces cell migration in a variety of normal and pathological processes.

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