Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins

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The binding of urokinase plaminogen activator (uPA) to its cell surface receptor (uPAR; CD87) promotes cell adhesion by increasing the affinity of the receptor for both vitronectin (VN) and integrins. We provide evidence that plasminogen activator inhibitor (PAI)-1 can detach cells by disrupting uPAR–VN and integrin–VN interactions and that it does so by binding to the uPA present in uPA–uPAR–integrin complexes on the cell surface. The detached cells cannot reattach to VN unless their surface integrins are first activated by treatment with MnCl₂. Immunoprecipitation and subcellular fractionation experiments reveal that PAI-1 treatment triggers deactivation and disengagement of uPA–uPAR–integrin complexes and their endocytic clearance by the low density lipoprotein receptor–related protein. Transfection experiments demonstrate that efficient cell detachment by PAI-1 requires an excess of matrix-engaged uPA–uPAR–integrin complexes over free engaged integrins and that changes in this ratio alter the efficacy of PAI-1. Together, these results suggest a VN-independent, uPA–uPAR-dependent mechanism by which PAI-1 induces cell detachment. This pathway may represent a general mechanism, since PAI-1 also can detach cells from fibronectin and type-1 collagen. This novel “deadhesive” activity of PAI-1 toward a variety of cells growing on different extracellular matrices may begin to explain why high PAI-1 levels often are associated with a poor prognosis in human metastatic disease.

Introduction

Plasminogen activator inhibitor (PAI)*-1 is a member of the serine proteinase inhibitor (SERPIN) family and is the primary physiological inhibitor of both tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA; Andreassen et al., 2000). It is a trace protein in plasma and circulates in complex with the adhesive glycoprotein vitronectin (VN) (van Meijer and Pannekoek, 1995; Loskutoff et al., 1999). The binding of PAI-1 to VN stabilizes the inhibitor in its active conformation, and this binding is reversed when the inhibitor forms complexes with tissue-type plasminogen activator or uPA.

Activation of pro-uPA frequently occurs when it binds to uPA receptor (uPAR; CD87) on cells (Kjoller, 2002). Active uPA on the cell surface can be inhibited by PAI-1 (Cabellis et al., 1989), and the resulting inactive PAI-1–uPA–uPAR complexes are rapidly internalized by the low density lipoprotein (LDL) receptor-related protein (LRP) (Nykjær et al., 1997; Czekay et al., 2001). Thus, PAI-1 initiates an LRP-dependent decrease in cell surface uPAR. Interestingly, uPAR is elevated on the surface of LRP-deficient cells, and this increase correlates with increased cell mobility (Webb et al., 2001). The binding of uPA to uPAR stimulates intracellular signaling (Ossowski and Aguirre-Ghiso, 2000; Kjoller, 2002) and also induces conformational changes in uPAR (Mondino et al., 1999; Wei et al., 2001) which increase its affinity for VN and promote its interaction with a variety of integrins (Chapman and Wei, 2001; Tarui et al., 2001; Kjoller, 2002). uPAR ligation also can activate certain integrins and growth factor receptors (Liu et al., 2002).

VN is relatively unique among adhesive proteins not only because PAI-1 binds to it with high affinity and specificity but also because cells can attach to it through integrins, uPAR, or both (Waltz et al., 1997; Deng et al., 2001). The high affinity binding sites for both PAI-1 and uPAR reside...
in two overlapping but distinct regions of the NH₂-terminal somatomedin B (SMB) domain of VN (Okumura et al., 2002) and are immediately adjacent to the single RGD sequence in the molecule. The affinity of PAI-1 for the SMB domain is much higher than the affinity of uPAR for this domain (Deng et al., 1996). Thus, PAI-1 can competitively inhibit the uPAR-dependent attachment of cells to VN (Deng et al., 1996; Waltz et al., 1997; Deng et al., 2001). Binding of PAI-1 to SMB also inhibits integrin-mediated cell adhesion, presumably by sterically blocking the adjacent RGD site (Stefansson and Lawrence, 1996; Okumura et al., 2002). Cells which attach to VN through uPAR can be detached by PAI-1 (Deng et al., 1996). Whether PAI-1 also can detach cells bound to VN (or other matrices) through integrins is largely unknown (Germer et al., 1998; Deng et al., 2001).

In this report, the deadhesive effects of PAI-1 on cells attached to VN via integrins are investigated in detail. Unexpectedly, the deadhesive activity of PAI-1 does not appear to require its interaction with VN. However, there is an absolute requirement for its binding to the uPA present in uPA–uPAR–integrin complexes on the cell surface. Interestingly, PAI-1 also detaches cells from fibronectin (FN) and type I collagen by a similar uPA/uPAR-dependent mechanism. These observations suggest that cell detachment by PAI-1 may represent a general mechanism for the release of cells from the extracellular matrix. Thus, PAI-1 should be added to the list of known deadhesion molecules (e.g., thrombospondin [Murphy-Ullrich, 2001]).

**Results**

**Detachment of cells from VN by PAI-1**

Cells can attach to VN through uPAR, integrins, or both. Since uPAR-mediated cell attachment to VN can be reversed by PAI-1 and since PAI-1 also blocks the RGD sequence in VN, it is possible that excess PAI-1 may competitively displace both uPAR and integrins from their association with VN, thus detaching the cells. According to this idea, cell detachment results from the binding of PAI-1 to VN. To test this hypothesis, we examined the behavior of HT-1080 cells, since they attach to VN via both uPAR and integrins (Deng et al., 2001). The cells were first acid treated to remove endogenous uPA from uPAR, thus making available a more consistent pool of surface uPAR (Cubellis et al., 1989; Czekay et al., 2001). Little or no cell detachment was observed when the cells were incubated with uPA or PAI-1 alone compared with the untreated control (Fig. 1 A). However, if the cells were preincubated with exogenous uPA from uPAR, thus making available a more consistent pool of surface uPAR (Cubellis et al., 1989; Czekay et al., 2001). Little or no cell detachment was observed when the cells were incubated with uPA or PAI-1 alone compared with the untreated control cells (Fig. 1 A). However, if the cells were preincubated with exogenous uPA the subsequent addition of PAI-1 now detached ~65% of the cells. A similar effect was observed when the cells were subjected to sequential incubation with uPA and the PAI-1 mutant which binds to uPA but not to VN (i.e., PAI-1[ΔuPA]). However, little detachment was observed using the PAI-1 mutant which does not bind to uPA (i.e., PAI-1[ΔuPA]). Thus, cell detachment by PAI-1 seems to be dependent on the interaction of PAI-1 with uPA and not with VN. Interestingly, when the cells were incubated with the non-uPA-binding PAI-1 variant (i.e., PAI-1[ΔuPA]) in the presence of RGD peptide (but not the RGE peptide; unpublished data), the majority of the cells were again detached (Fig. 1 A). The presence of RGD peptide alone had no effect on cell detachment. Thus, disruption of VN–integrin interactions by the RGD peptide allowed the VN-binding variant of PAI-1 to detach the cells, presumably because the cells were now bound to VN only through uPAR (Deng et al., 2001).

Two experiments were performed to further investigate the uPA dependency of cell detachment by PAI-1. In the first, HT-1080 cells were incubated with the amino terminal fragment (ATF) of uPA instead of with uPA itself. Although the ATF binds to uPAR (Ploug et al., 1998), it
lacks the catalytic domain of uPA, and thus does not interact with PAI-1. The addition of PAI-1 to the ATF-pre-treated cells did not lead to cell detachment (Fig. 1 A). Thus, detachment of HT-1080 cells by PAI-1 depends on the presence of active uPA bound to uPAR.

In the second experiment, the effect of PAI-1 on cell detachment from VN$_{40-459}$ was examined. This variant of VN does not interact with PAI-1 because it lacks the SMB domain (i.e., the uPAR and PAI-1 binding sites). However, it still contains the RGD site necessary for integrin-mediated attachment. As expected, PAI-1 alone had no effect on cell detachment (Fig. 1 B). However, if the cells were first preincubated with uPA and then PAI-1 (or the PAI-1 variant which binds uPA but not VN, i.e., PAI-1$_{[P]}$) was added, the majority of the cells were detached. Again, the VN-binding variant that does not bind to uPA (i.e., PAI-1$_{[P-V]}$) had no effect on cell detachment. Thus, HT-1080 cell detachment by PAI-1 is a uPA-dependent process but not a VN-dependent process.
PAI-1 decreases the level of uPAR–αV integrin complexes on the cell surface

It is now clear that the binding of uPA to uPAR on the cell surface leads to the formation of molecular complexes between the occupied receptor and a variety of integrins and that these changes promote stronger cell adhesion to VN (Chapman and Wei, 2001; Kjoller, 2002). Since addition of PAI-1 to uPA-occupied uPAR leads to cell detachment from VN (Fig. 1), we hypothesized that the effects of PAI-1 on cell detachment were mediated by decreases in uPAR–integrin complexes at the cell surface. To test this possibility, uPA-pretreated cells were incubated with PAI-1 (or the PAI-1 variants) at 18°C to permit internalization of surface proteins. Postnuclear supernatants were prepared, immunoprecipitated using mAbs against VN-specific integrins, and then analyzed by immunoblotting for uPAR (Fig. 2).

The addition of uPA caused a fivefold increase in the amount of uPAR coimmunoprecipitated with antibodies against αVβ3 (Fig. 2 A). Subsequent addition of PAI-1 reduced the amount of immunoprecipitated uPAR to control values. Similar results were obtained when the immunoprecipitation experiments were performed using the mAb against αVβ5 (Fig. 2 B). Again, addition of the PAI-1 variant, which did not bind to uPA (i.e., PAI-1P,γ+), had no effect on the amount of precipitable uPAR–αVβ5 complexes. Together, the data in Fig. 2, A and B, indicate that PAI-1 decreases the amount of uPA–uPAR–αV integrins at the cell surface.

Cells released by PAI-1 do not reattach to VN

To begin to investigate the mechanism of cell detachment by PAI-1, HT-1080 cells were detached from VN by treatment with trypsin or by sequential incubation with uPA and PAI-1 as above. The cells were then washed and allowed to reattach to VN40-459 (which lacks the uPAR binding domain), thus only permitting cell attachment via integrins. The trypsin-released control cells (i.e., uPA alone) rapidly and completely reattached to this VN variant (Fig. 3 A). This reattachment was not enhanced further by the addition of MnCl2, a treatment known to activate αV-integrins (Pampori et al., 1999) but was significantly reduced by EDTA. Thus, adhesion of these trypsinized cells did not require integrin activation. In contrast to these results, <10% of the cells that were detached by sequential treatment with uPA and PAI-1 were able to reattach to VN40-459 under these conditions. In this case, the magnitude of reattachment was dramatically increased by the addition of MnCl2. Thus, sequential treatment with uPA and PAI-1 appears to decrease the number of active integrins on the cell surface.

Binding of VN to uPA/PAI-1–treated HT-1080 cells

Experiments were performed to test the possibility that the inability of the uPA/PAI-1–treated cells to reattach to VN40-459 (Fig. 3 A) reflects the absence of active integrins. HT-1080 cells were detached nonenzymatically (see Materials and methods) and then incubated with 125I-labeled VN at 4°C (to prevent internalization of surface proteins) in the absence or presence of uPA alone or uPA followed by PAI-1. The 125I-VN bound very efficiently to control cells treated with uPA alone (Fig. 3 B). However, considerably less binding was observed when cells pretreated with uPA/PAI-1 were employed, and this binding was again stimulated by the addition of MnCl2. Together, these observations suggest that sequential treatment of HT-1080 cells with uPA and PAI-1 converts the majority of active integrins at the cell surface into an inactive state. Fig. 3 also shows that antibodies against αvβ3 and αvβ5 reduced the binding of 125I-VN to uPA–treated cells by ~40 and ~70%, respectively. LM609 and P1F6 also blocked the attachment of HT-1080 cells to VN by similar amounts (unpublished data), whereas a nonspecific IgG had no effect. Although the combined effects of the two antiantig(in) antibodies slightly exceeded 100%, this...
effect was within the variation of these experiments. Thus, these two integrins play a significant role in HT-1080 cell attachment to VN, and both are regulated by PAI-1. When considered together with the coimmunoprecipitation data (Fig. 2), these observations suggest that addition of PAI-1 to uPA-pretreated cells leads to a deactivation of $\alpha_{V}$-integrins in conjunction with a decrease in precipitable uPAR–$\alpha_{V}$-integrin complexes at the cell surface.

PAI-1 initiates LRP-mediated endocytosis of uPA–uPAR–$\alpha_{V}$-integrin complexes

Although incubation of HT-1080 cells with uPA and PAI-1 leads to the formation and internalization of PAI-1–uPA–uPAR complexes by LRP (Czekay et al., 2001), little information is available about the fate of the integrins that also are associated with these complexes (Fig. 2). To further investigate this question, HT-1080 cells were surface biotinylated and then detached from VN by incubation in non-enzymatic cell dissociation solution. The suspended cells were then incubated with uPA alone at 4°C or with uPA (4°C) followed by PAI-1 (18°C). Incubation at 4°C prevents the internalization of surface proteins, whereas incubation at 18°C (Dunn et al., 1989) allows endocytosed proteins to accumulate in early or sorting endosomes but prevents them from progressing further down the endocytic pathway (i.e., cargo transport to lysosomes for degradation; receptor recycling to the plasma membrane [PM]). Cell lysates were prepared and fractionated on Percoll gradients, and fractions containing the PMs and early endosomes (EEs) were collected and analyzed for the presence of biotinylated integrins. As indicated in Fig. 4 A, when uPA alone was added to the cells $\alpha_{V} \beta_{3}$ (panel a) and $\alpha_{V} \beta_{5}$ (panel d) were detected only in PM fractions. Thus, under these conditions $\alpha_{V}$-integrins appear to be localized to the cell surface. Subsequent addition of PAI-1 induced a significant shift of these integrins into EE fractions (Fig. 4 A, b and e), and anti-LRP antibodies greatly reduced these effects (Fig. 4 A, c and f). Thus, the PAI-1–mediated internalization of these integrins occurs via an LRP-dependent mechanism. These observations support the hypothesis that the binding of PAI-1 to uPA–uPAR–integrin complexes initiates cell detachment by deactivating and disengaging uPAR and $\alpha_{V}$-integrins and prevents cell reattachment to VN by initiating the simultaneous clearance of the two adhesion receptors.

Additional experiments were performed to determine whether $\alpha_{V} \beta_{5}$ and uPAR were internalized separately or in complex with each other. The pooled gradient fractions containing either PM or EE were immunoprecipitated with antibodies against $\alpha_{V} \beta_{5}$ (P1F6) and then analyzed for the presence of uPAR by immunoblotting after SDS-PAGE. As indicated in Fig. 4 B, when the cells were incubated sequentially with uPA and PAI-1 the majority of both $\alpha_{V} \beta_{5}$ (panel a), and uPAR (panel e) were detected in EE. The addition of receptor-associated protein (RAP) blocked the shift of $\alpha_{V} \beta_{5}$ (Fig. 4 B, b) and uPAR (Fig. 4 B, f) from the PM into EE, whereas the presence
of a nonspecific IgG had no effect (Fig. 4 B, c and g). Thus, the sequential addition of uPA and PAI-1 appears to internalize $\alpha_v$-integrins in complex with uPAR. If the cells were incubated with preformed uPA–PAI-1 complexes (instead of sequential incubation with uPA and PAI-1), the amount of $\alpha_v\beta_3$ (Fig. 4 B, d) in EE was significantly reduced. However, our earlier studies (Czekay et al., 2001) showed that under these conditions uPAR is internalized into EE.

**Detachment of HT-1080 cells from FN and type-1 collagen by PAI-1**

The effects of PAI-1 on cell detachment are VN independent, raising the possibility that the inhibitor can also detach cells from other matrix proteins which do not contain binding sites for PAI-1 and/or uPAR. This hypothesis is supported by the observation that the sequential addition of uPA and PAI-1 (or PAI-1$_{uPA-WT}$) to HT-1080 cells attached to FN or type-1 collagen (Coll-I) detached $\sim$35–40% of the cells (Fig. 5).

Comparison of Figs. 1 and 5 indicates that the sequential addition of uPA and PAI-1 induced significantly less cell detachment from FN and Coll-I than from VN. Although FN and Coll-I contain multiple binding sites for integrins, there is only one binding site (i.e., RGD sequence) present in VN (Okumura et al., 2002). Thus, it is likely that the engagement of a larger number of integrins by cells attached to FN and Coll-I (versus VN) may lead to stronger adhesion and less effective detachment by PAI-1. In this regard, there are no published reports showing a direct effect of PAI-1 on integrins. Figs. 1–4 show that PAI-1 alone has no effect on free integrins, i.e., it only internalizes those integrins associated with uPA–uPAR complexes. Thus, it may be the ratio of matrix-engaged uPA–uPAR–integrin complexes to total engaged integrins that determines the efficiency of PAI-1–induced cell detachment. If this hypothesis is correct, then increasing the amount of surface uPAR should increase the proportion of active integrins in complex with uPAR and lead to more pronounced cell detachment by PAI-1. To test this hypothesis, wild-type (WT) CHO cells, which express endogenous $\alpha_v\beta_3$ (Zaffran et al., 2000) and uPAR (Fowler et al., 1998), and two CHO cell clones, which overexpress either human $\alpha_v\beta_3$ (Pampori et al., 1999) or human uPAR (Tarui et al., 2001), were allowed to attach to VN (Fig. 6 A) and FN (Fig. 6 B). These cells were especially appropriate for these studies since the $\alpha_v\beta_3$-overexpressing cells produce $\sim$40–50× more $\alpha_v\beta_3$ integrin than their WT counterparts (Pampori et al., 1999), whereas the CHO-uPAR cells express 65–70-fold more uPAR than the CHO-WT cells ($\sim$47,000 sites/cell versus $\sim$700 sites/cell; unpublished data).

As expected, neither uPA nor PAI-1 alone detached CHO-WT cells or CHO cells that overexpress $\alpha_v\beta_3$ from VN (Fig. 6 A). However, upon sequential incubation of CHO-WT cells with uPA and PAI-1 nearly 50% of the cells were detached. Importantly, this detachment was not observed in the CHO cells that overexpressed $\alpha_v\beta_3$ integrins. Thus, increasing the ratio of $\alpha_v\beta_3$ to uPAR seemed to inhibit PAI-1–mediated cell detachment from VN. In parallel experiments, CHO-WT cells and CHO cells overexpressing human uPAR were allowed to attach to FN (Fig. 6 B). Again, neither uPA nor PAI-1 alone could detach the cells. In this case, the CHO-WT cells could not be detached from FN by sequential incubation with uPA and PAI-1. However, if the CHO cells were overexpressing uPAR, $>50$% of the cells were detached. Thus, increasing the ratio of uPAR over active $\alpha_v$-integrins facilitates PAI-1–mediated detachment of CHO cells. Together, these results suggest that the efficiency of cell detachment by PAI-1 depends largely on the fraction of the total matrix-engaged integrin pool that is actually bound to uPA–uPAR complexes and can in turn bind PAI-1.

**Table 1. Detachment of various cell lines from VN and FN by PAI-1**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment of cells attached to vitronectin</th>
<th>Treatment of cells attached to fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (uPA)</td>
<td>uPA + PAI-1</td>
</tr>
<tr>
<td>HT-1080</td>
<td>100 ± 3.1</td>
<td>31.4 ± 0.2</td>
</tr>
<tr>
<td>MCF-7</td>
<td>100 ± 2.9</td>
<td>78.7 ± 0.7</td>
</tr>
<tr>
<td>HeLa</td>
<td>100 ± 3.1</td>
<td>38.9 ± 3.3</td>
</tr>
<tr>
<td>AoSMC</td>
<td>100 ± 3.1</td>
<td>39.9 ± 0.9</td>
</tr>
</tbody>
</table>

HT-1080, MCF-7, HeLa, and AoSMCs were allowed to attach to VN or FN for 1.5 h at 37°C and then were incubated with uPA alone, or with uPA or uPAsuPAR followed by PAI-1 as described in Materials and methods. After incubation, the cells were vigorously agitated, and the floating cells were removed by washing. The relative number of attached cells was quantitated by crystal violet staining (as described in Materials and methods). Each column reflects the average of triplet samples ± SD and is expressed as a percentage of the control (media + uPA, 100%).
Effect of soluble uPAR on PAI-1–mediated cell detachment from FN, Coll-I, and VN

Experiments were performed to further test the hypothesis that an excess of uPAR over integrins is necessary for efficient cell detachment by PAI-1. HT-1080 cells attached to FN or Coll-I were incubated with either uPA alone or with uPA in the presence of increasing amounts of soluble uPAR (suPAR) at 4°C. This soluble uPAR was able to bind to uPA and form complexes with purified α5β1-integrins and with integrins at the surface of HT-1080 cells (unpublished data). Addition of uPA–suPAR alone had no effect on cell detachment from either FN (Fig. 7, lane 3) or Coll-I (Fig. 7, lane 7) compared with controls (uPA alone, Fig. 7, lanes 1 and 6). However, subsequent addition of PAI-1 to the uPA–suPAR-pretreated cells released >80% of cells from these matrices (Fig. 7, lanes 5 and 8). These effects of PAI-1 on cell detachment increased with the concentration of suPAR employed (Fig. 7, lane 4 compared with 5) and were significantly stronger than the effects observed if only endogenous uPAR was present (Fig. 7, lane 2 compared with 5). The addition of suPAR to a variety of cells seeded onto VN or FN matrices also increased the number of cells detached by sequential incubation with uPA and PAI-1 (Table I). These observations indicate that increasing the surface pool of uPAR leads to more efficient cell detachment by PAI-1.

Discussion

Cell migration is a critical component of many normal and pathological processes including wound healing and cancer, and it is clear that regulated changes in the affinity state of adhesion receptors (e.g., integrins and uPAR) are essential for optimal cell motility (Lauffenburger and Horwitz, 1996). It is also clear that this process is influenced by PAI-1 (De Gryse et al., 2001; Stefansson et al., 2001). The movement of cells appears to involve at least three interdependent functional events, including (1) attachment of the leading edge of the cell, (2) cell contraction, and finally (3) detachment of the trailing edge (Huttenlocher et al., 1995). Although considerable information is available about cell attachment and contraction, relatively little is known about mechanisms of cell detachment. What is clear is that several extracellular proteins can reverse the cell adhesion process by promoting deadhesion events (Murphy-Ullrich, 2001). These proteins, including thrombospondin, tenasin, and osteonectin (SPARK), can disrupt the link between the cytoskeleton and the focal adhesion plaque, resulting in the loss of stress fibers and a decrease in the strength of integrin–ligand interactions. The signals that initiate these events remain to be determined. The experiments in this report not only demonstrate that PAI-1 should be added to this growing list of deadhesion molecules but also provide insights into underlying mechanisms. These experiments were initiated to specifically investigate the influence of PAI-1 on the detachment of HT-1080 cells from extracellular matrices composed of VN. However, it soon became apparent that this inhibitor also can promote the detachment of a variety of cells growing on different extracellular matrices.

Cells adhere to VN by engaging uPAR, integrins, or both (Chapman and Wei, 2001; Deng et al., 2001). These adhesive events are initiated in part by the binding of uPA to uPAR and can be prevented by PAI-1 binding to SMB (Chapman and Wei, 2001; Deng et al., 2001). Interestingly, these effects of PAI-1 seem to be specific for VN since the inhibitor does not bind to FN or other matrix proteins, and it does not block integrin-mediated cell adhesion to matrices composed of these molecules (Loskutoff et al., 1999). Based on these observations, we hypothesized (Deng et al., 2001) that PAI-1 also can detach cells from VN by binding to SMB and displacing uPAR and integrins. It is now clear that the story is much more complex than this and that PAI-1 can detach cells by two separate but related mechanisms, depending on the nature and quantity of the adhesion receptors actually engaged by the attached cells. In the case where cells bind to VN through uPAR alone (e.g., U937 cells) and integrins are not engaged, PAI-1 detaches the cells by directly competing with uPAR for the binding site in the SMB domain (Deng et al., 2001). Since neither PAI-1 nor uPAR bind to other matrix proteins, this mechanism of competitive displacement is specific for cells growing on VN and will not affect cells growing on FN or Coll-I. In the case where cells adhere to VN either through integrins alone or through integrins and uPAR (i.e., most other cells), this displacement of uPAR by PAI-1 is not sufficient to detach the cells, since they remain firmly anchored via integrins (Deng et al., 2001). Thus, the integrin–VN interaction also has to be disrupted to release the cells, and this reversal of integrin-mediated cell adhesion by PAI-1 does not appear to require VN. This conclusion is supported by several observations. For example, PAI-1 can detach cells from matrices that do not contain VN (e.g., matrices composed of FN or Coll-I [Figs. 5–7 and Table I]). In addition, the PAI-1 variant that does not bind to VN (i.e., PAI-1[P V]) efficiently detaches cells from VN (Figs. 1, 2, and 5). Finally, PAI-1 can detach cells from a variant of VN that lacks the PAI-1 binding domain (i.e., VN40–459 [Fig. 1 B]).

The mechanism by which PAI-1 disrupts integrin-mediated cell attachment was investigated in detail. The fact that the PAI-1 variant that bound uPA but not VN retained full detachment activity (Figs. 1, 2, and 5), whereas the variant that bound VN but not uPA was inactive (Figs. 1 and 2) indicates that the presence of active uPA on the cell surface is critical for the deadhesive effects of PAI-1. This conclusion is supported by the observation that the ATP of uPA does not support cell detachment by PAI-1 (Fig. 1 A). This fragment of uPA binds to uPAR but lacks the binding site for PAI-1 (Cubells et al., 1989).

Fig. 2 shows that the interaction of uPA with uPAR promotes complex formation between the occupied receptor and α5β1-integrins and that subsequent addition of PAI-1 decreases the number of these complexes. This decrease appears to result from the PAI-1–mediated disengagement of uPAR–integrin complexes from the extracellular matrix, leading to the cointernalization of these complexes into EEs (Fig. 4). This endocytosis requires LRP since anti-LRP antibodies and RAP block this internalization. This is the first report to our knowledge that PAI-1 can initiate disengagement of integrins from the extracellular matrix and can promote their endocytic clearance. Interestingly, when the cells were incubated with preformed uPA–PAI-1 complexes, the
neous clearance of the two cell adhesion receptors promotes complexes before the disengagement of these complexes at 4°C. Since sequential treatment of cells with uPA and PAI-1 integrins before their internalization. This appears to be the case, since the uPA–uPAR–integrin complexes leads to deactivation of the integrins and cell detachment. The presence of PAI-1 also mediates the binding of these complexes to LRP, their redistribution into clathrin-coated pits (Step 4), and their LRP-mediated endocytosis into EEs (Step 5). The uPA–PAI-1 complexes dissociate from uPAR and LRP and (Step 6) traffic through late endosomes (LE) to lysosomes for degradation. (Step 7) The unoccupied LRP–uPAR–integrin complexes also dissociate, and (Step 8) return to the cell surface via recycling vesicles (RV). This last step makes uPAR and integrins available at the cell surface for reattachment to the matrix.

The endocytic clearance of uPAR and integrins can only occur after their disengagement from VN. Therefore, we addressed the question of whether the binding of PAI-1 to uPA–uPAR–integrin complexes leads to deactivation of the integrins before their internalization. This appears to be the case, since sequential treatment of cells with uPA and PAI-1 at 4°C also decreased VN binding (Fig. 3 B). Incubation of cells at this temperature prevents endocytosis and keeps uPAR and integrins at the cell surface. Thus, the binding of PAI-1 to uPA seems to initiate the rapid and specific inactivation of the αvβ3 integrins present in uPA–uPAR–integrin complexes before the disengagement of these complexes from the matrix and their endocytosis by LRP. This simultaneous clearance of the two cell adhesion receptors promotes the subsequent detachment of HT-1080 cells from VN. Fig. 8 also emphasizes that uPAR is the surface receptor which transmits the effects of PAI-1 to integrins. Since the attachment of cells to a specific matrix is determined by the presence of specific cell surface integrins and since PAI-1 detaches cells from a variety of matrices (Table I), we must conclude that PAI-1 can somewhat indiscriminately transmit the effects of PAI-1 to the specific integrins involved in the adhesive event. This conclusion is supported by observations showing that uPAR can associate with a variety of integrins at the cell surface (Chapman and Wei, 2001).

Importantly, the uPA–uPAR-dependent mechanism of cell detachment is not unique to HT-1080 cells growing on VN. In fact, PAI-1 can also detach CHO, MCF-7, HeLa, and aortic smooth muscle cells (AoSMCs) from VN, FN, and Coll-I by a similar mechanism (Figs. 5–7 and Table I). Interestingly, the ability of PAI-1 to detach cells varies with the composition of the matrix (Figs. 1, 5, and 6 and Table I). This variation is probably related to differences in the number of integrin binding sites in each matrix. For example, the variation observed between VN, FN, and Coll-I may reflect the fact that FN and Coll-I contain more binding sites for integrins than VN. It is likely that a larger portion of the surface integrin pool can engage these adhesive glycoproteins, thus leading to stronger cell adhesion.

The ability of PAI-1 to detach cells also varies considerably from cell type to cell type (Figs. 1 and 6 and Table I), and we speculate that this variation is due to large differences in the expression of surface uPAR (Deng et al., 2001).

More specifically, we hypothesize that it is the ratio of cell surface uPAR to the total pool of engaged integrins that determines the number of uPAR–integrin complexes that can...
be affected (i.e., deactivated) by PAI-1. According to this idea, an increase in cell surface uPAR over total engaged integrins would enhance the deadhesive effects of PAI-1. This hypothesis is supported by the observation that overexpression of uPAR in CHO cells (i.e., increased ratio of uPAR to integrins) led to a pronounced increase in the efficiency of PAI-1 to detach the cells from FN (Fig. 6 B). However, when the surface pool of αβ3 integrins was enlarged to decrease this ratio (i.e., in CHO cells overexpressing αβ3 [Fig. 6 A]), PAI-1 treatments failed to detach the cells, especially from VN. Based on these observations, we hypothesized that cell surface uPAR might be limiting in some cells and that incubation of these cells with soluble uPAR in the presence of uPA would promote complex formation with a larger portion of the total active integrins. This change would again allow PAI-1 to associate with and inactivate a higher proportion of the total engaged integrin pool, increasing its efficiency of cell detachment. This hypothesis is supported by the observation that when HT-1080 cells were incubated with soluble uPAR in the presence of uPA, a dose-dependent increase in the amount of cells detached from FN and Coll-I by PAI-1 was observed (Fig. 7). Additional tests demonstrated that in the presence of uPA, soluble uPAR was able to form immunoprecipitable complexes with purified αβ3-integrins (unpublished data; Wei et al., 2001). The addition of soluble uPAR (in the presence of uPA) also enhanced PAI-1–mediated detachment of HT-1080, MCF-7, HeLa, and AoSMC cells from VN and FN (Table I).

In summary, when cells bind to VN via uPAR and integrins (e.g., HT-1080 cells [Deng et al., 2001]) and the uPAR to integrin ratio is high PAI-1 detaches the cells via a uPA-dependent mechanism that leads to deactivation and disengagement of uPAR and integrins (Fig. 8). When cells bind to other matrix proteins (i.e., FN and Coll-I) through integrins alone, PAI-1 can detach them by the same uPA-dependent and uPAR-mediated mechanism as long as the ratio of the engaged uPA–uPAR–integrin complexes to the total pool of engaged integrins is high. In cells where the surface pool of active integrins exceeds the uPAR pool (i.e., MCF-7 [Deng et al., 2001]), PAI-1 cannot detach cells from VN. Thus, if integrin-mediated adhesion is not accompanied by sufficient surface expression of uPAR, PAI-1 will be unable to detach the cells.

It is tempting to speculate that the deadhesive effects of PAI-1 are related to the observation that high PAI-1 levels are associated with a poor prognosis for survival in several metastatic human cancers (Andreasen et al., 2000; Foekens et al., 2000). In fact, the prognostic relevance of high tumor expression of PAI-1, as well as uPA and uPAR, for cancer invasiveness, metastatic spreading, and poor relapse-free survival is now well established. Moreover, recent reports indicate that high plasma levels of soluble uPAR are associated with a continuously increasing risk of mortality in breast cancer (de Witte et al., 2001). PAI-1–induced cell detachment is enhanced by the presence of soluble uPAR (Fig. 7 and Table I). Thus, Fig. 8 also emphasizes the central role of uPAR as a transmitter of the deadhesive effects of PAI-1 through the rapid redistribution and reutilization of adhesion receptors at the leading edge of these cells. Thus, we can envision an in vivo scenario where tumor-derived uPA and soluble uPAR will form complexes which bind to integrins on the cancer cells, thus facilitating the deadhesive effects of PAI-1 produced by the tumor and/or stromal cells. This effect might then enhance the dissemination of these cells from the tumor. This hypothesis remains to be tested.

Materials and methods

Reagents

Protein G–agarose, heparin sepharose, and Percoll were from Amersham Biosciences, RGD peptide (GRGDSP) was from Peninsula Laboratories, and Bis–Y-XX was purchased from Molecular Probes. NaCl was purchased from PerkinElmer, iodinated proteins were from Pierce Chemical Co., and the AKT kit for detecting biotinylated proteins was from Vector Laboratories. BSA was from HyClone, RPMI and DME cell culture media and trypsin/EDTA cell dissociation solution were from Invitrogen, and nonenzymatic cell dissociation solution and CHAPS were from Sigma–Aldrich. All other chemicals and buffers were of the highest analytical grade available.

Proteins

uPA, ATF, and uPAR. Active two-chain uPA (human) was purchased from American Diagnostica. The ATF (residues 1–135) of human uPA was a gift from Dr. Michael Ploug (Finsen Laboratory, Copenhagen, Denmark), and soluble human uPAR (Tarui et al., 2001) was supplied by Dr. Douglas Cines (University of Pennsylvania, Philadelphia, PA). RAP, RAP was provided by Dr. J.G. Neels from our laboratory. It was expressed and purified as a GST fusion protein (Herz et al., 1991). PAI-1. The recombinant stable active form of PAI-1 (14–1b [Berkenpas et al., 1995]) was a gift from Dr. Dan Lawrence (American Red Cross, Rockville, MD) and is referred to throughout this article as PAI-1. Two recombinant PAI-1 mutants were constructed on this 14–1b background by performing oligonucleotide–directed site-specific mutagenesis (QuickChange Site-Directed Mutagenesis kit; Stratagene). The first mutation, Q123→K, generated a PAI-1 variant with no detectable affinity for VN but with normal inhibitory activity against PAs (Lawrence et al., 1994). This mutant is referred to as PAI-1[Q123→K]. The second variant contained two mutations (T333→R and A335→R) which resulted in the loss of the PA inhibitory activity of PAI-1 but did not affect its binding affinity for VN (Stefansson et al., 2001). This mutant is referred to as PAI-1[P333→R]. All PAI-1 variants were expressed in Escherichia coli strain BL21 (DE3) pLysS (Stratagene), and their sequences were confirmed by DNA sequencing. Protein expression was induced by incubating the bacteria with 0.2 mM IPTG for 4–5 h at 30°C. The resulting PAI-1 variants were purified (Kvassman and Shore, 1995) and tested both for their affinity for PAs (Strandberg and Madison, 1995) and for VN (Okumura et al., 2002). Protein concentrations were determined by the BCA method (Pierce Chemical Co.).

Extracellular matrix proteins. Multimeric VN was purified from human plasma as described (Yatogoh et al., 1988). A truncated form of VN representing residues 40–459 (i.e., VN40–459) was constructed from the human VN cDNA (Okumura et al., 2002). This VN variant lacks the binding sites for uPAR and PAI-1 but still contains the RGD sequence for integrin binding. Human FN and human Coll-I were supplied by Drs. S. Shattil (Pampori et al., 1999) and Y. Takada (Tarui et al., 2001), respectively, from The Scripps Research Institute. A human AoSMC line and the recommended culture medium (SmGM-2) were purchased from BioWhittaker. All other cell lines were purchased from American Type Culture Collection and were cultured in DME supplemented with 10% FBS.

Cell culture

WT CHO-K1 cells and CHO-K1 cells overexpressing either human αβ3 or human uPAR were supplied by Drs. S. Shattil (Pampori et al., 1999) and Y. Takada (Tarui et al., 2001), respectively, from The Scripps Research Institute. A human AoSMC line and the recommended culture medium (SmGM-2) were purchased from BioWhittaker. All other cell lines were purchased from American Type Culture Collection and were cultured in DME supplemented with 10% FBS.

Acid treatment of cells

Unless otherwise indicated, cultured cells were acid treated (Cubells et al., 1989; Czekay et al., 2001) before incubation with exogenously added
uPA or PAI-1. Briefly, the cells were incubated in glycine buffer at pH 4.0 for 3 min at 4°C and then neutralized by incubation in TRIS buffer at pH 7.4 for 10 min. The acid-treated cells responded in a similar but more dramatic manner compared with the control cells which were not acid washed or incubated at 4°C before addition of uPA and PAI-1.

Cell detachment assay
To perform cell detachment experiments, microtiter plates were coated with various extracellular matrix proteins including VN (5 μg/mL), VNαRGD (20 μg/mL), FN (5 μg/mL), or type I collagen (5 μg/mL) for 18 h at 4°C. Cells (1.5 x 10⁵) in RPMI containing 0.02% BSA (RPMI/BSA) were added to each well and allowed to attach for 1.5 h at 37°C. The monolayers were then acid treated as above, washed twice in ice-cold RPMI/BSA, and then incubated in the absence or presence of uPA (50 nM) or AT (50 nM) for 1 h at 4°C. Unbound uPA and AT were removed by additional washing in RPMI/BSA at 4°C, and then either PAI-1, PAI-1-RGD or PAI-1-RGD (all at 40 μg/mL) was added to the cells for 30 min at 4°C in RPMI/BSA. In some cases, soluble uPAR (suPAR) and 5 μg/mL was added together with uPAR, whereas in other experiments RGD peptide (500 μM) was added at 1.5 h after incubation with 125I-labeled VN. Specificity of 125I-labeled VN binding was defined as the difference between total binding (without uPAR) and nonspecific binding measured with 125I-labeled VN and nonspecific IgG or anti-LRP IgG (both at 200 μg/mL) or RAP (10 μg/mL).

Binding of radioiodinated VN to the cell surface
VN was radioiodinated with Na-125I to a specific activity of 7,145 cpm/μg using lodo beads. Attached HT-1080 cells were acid stripped as described above and then were washed twice in PBS at 4°C and detached in enzymatically cell detachment solution. The detached cells were collected, washed, resuspended in RPMI/BSA and then incubated in RPMI/BSA with 125I-labeled VN (50 nM) at 4°C for 1 h in the presence of either uPA alone (50 nM) or uPA followed by incubation with PAI-1 (40 μg/mL) in the absence or presence of MnCl₂ (2 mM). In some experiments, cells were incubated with uPA in the presence of mAbs against αvβ₃ (LM609, 50 nM), then PAI-1, uPA followed by incubation with PAI-1 (40 μg/mL) and then αvβ₃ (LM609, 50 nM) was added to the cells for 2 h at 4°C, and then the plates were agitated and washed to remove nonadherent cells. The remaining adherent cells were quantitated as described above.

Intensification of α₅-integrins and cell fractionation
Acid-washed and surface-biotinylated HT-1080 cells were detached in enzyme-free cell detachment solution. The resuspended cells were either incubated with uPA (50 nM) for 1 h at 4°C, washed with RPMI, and then incubated in RPMI/BSA in the absence or presence of PAI-1 (40 μg/mL) for 1 h at 18°C, or were incubated for 1 h at 4°C in the presence of preformed uPA–PAI-1 complexes (Cubells et al., 1989). Cell fractionation was performed at 4°C using 20% Percoll as described previously (Czekay et al., 2001). Gradient fractions containing PM-derived material or early endosomal material were pooled and then analyzed for the presence of biotinylated αvβ₃, and αvβ₅, by immunoprecipitation with mAbs LM609 (against αvβ₃) and P1F6 (against αvβ₅), respectively, and blotting with HRP-coupled avidin followed by ECL. Some immunoprecipitates (P1F6) were also analyzed for the presence of uPAR by immunoblotting with anti-uPAR pAb followed by ECL. In some cases, binding and internalization experiments were performed in the presence of protein A–purified nonspecific IgG or anti-LRP IgG (both at 200 μg/mL) or RAP (10 μg/mL).

Statistical analysis
In general, all experiments were performed three times. Quantitative results are presented as the average of the three experiments, each performed in triplicate, ± SD.

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