# SYMPOSIUM

# Unexpected Role of Plasminogen Activator Inhibitor 1 in Cell Adhesion and Detachment

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**Plasminogen activator inhibitor 1 (PAI-1) is the primary physiological inhibitor of plasminogen activation *in vivo*, and thus it is one of the main regulators of the fibrinolytic system. In this regard, individuals with elevated PAI-1 seem to have an increased risk for thrombotic disease, whereas those lacking the inhibitor develop a lifelong bleeding diathesis. Unexpectedly, recent observations demonstrate that cancer patients with high PAI-1 levels have a poor prognosis for survival. This correlation with metastatic disease may be related to the observation that high PAI-1 levels decrease the adhesive strength of cells for their substratum, and that this de-adhesive activity of PAI-1 is not related to its role as a protease inhibitor. Initial insights into potential mechanisms by which PAI-1 regulates the attachment, detachment, and migration of cells are addressed in this review.** Exp Biol Med 229:1090–1096, 2004

**Key words:** plasminogen activator inhibitor 1; vitronectin; extracellular matrix; cell attachment; cell detachment

The fibrinolytic system functions to remove pathological fibrin from the vasculature, a process that is initiated by the release of plasminogen activators (PAs) from cells, including those of the vessel wall itself (1, 2). However, the PAs are not the primary fibrinolytic enzymes. Instead, they act by cleaving circulating plasminogen at a single arginine–valine bond, converting the inactive protease into the active trypsin-like enzyme, plasmin. Plasmin then digests the fibrin that binds the platelets to each other and to the vessel wall. There are two immunologically distinct mammalian PAs: urokinase-like PA (uPA) and tissue-type PA (tPA). These PAs also differ functionally. For example, tPA binds to fibrin and activates plasminogen in the clot and is thought to be the primary fibrinolytic activator (3). In contrast, uPA has no affinity for fibrin. However, it binds to a receptor on cells (the urokinase receptor; uPAR; Refs. 4, 5), generating plasmin on the cell surface—a process that promotes extracellular matrix degradation and cell migration/invasion (6).

Precise regulation of the fibrinolytic system constitutes a critical feature not only of vascular hemostasis but also of a number of other physiological and pathological processes including wound healing, embryogenesis, tumor cell invasion, and so forth. This control is accomplished in large part by plasminogen activator inhibitors (PAIs; Ref. 7). Although five molecules with PAI activity have been identified, the primary physiological inhibitor of both tPA and uPA is plasminogen activator inhibitor 1 (PAI-1; Ref. 8). It is a single-chain glycoprotein with a molecular weight of 50,000 Da, and it appears to be important clinically because high levels of this inhibitor are associated with an increased risk for thrombosis, whereas low levels increase the risk for bleeding (9). Although PAI-1 is a trace protein in blood (its concentration is only 5–10 ng/ml), it is present in relatively high concentrations in platelets (10, 11). The release of PAI-1 from platelets may stabilize newly formed thrombi against premature lysis and thus contribute to the known resistance of platelet-rich thrombi to thrombolytic agents.

PAI-1 has a number of unusual properties for a protease inhibitor (8). Besides being a trace protein in plasma, it is regulated by a variety of hormones, cytokines, and growth factors; it is an immediate–early gene; and it binds to the adhesive glycoprotein vitronectin (VN; Refs. 12, 13). High PAI-1 levels are also associated with a poor prognosis for survival for a number of human cancers (2, 14). This

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unexpected correlation may be related to the recent observation that PAI-1 has very dramatic effects on the ability of cells to attach to (15, 16), migrate on (17, 18), and be detached from (19) their substratum. The addition of low concentrations of PAI-1 to cells leads to the rapid rearrangement of the actin cytoskeleton, the loss of focal adhesions, and the assumption of the migratory phenotype (18).

## PAI-1 and Cell Adhesion

**Vitronectin.** Vitronectin is a single-chain glycoprotein consisting of 459 amino acids. It is divided into a number of specific domains (Fig. 1), including the 44-residue somatomedin B (SMB) domain at the amino terminus, the connecting region that contains the single RGD sequence in the molecule and that is immediately adjacent to the SMB domain, and two hemopexin-like repeats. The binding site for PAI-1 is localized to the SMB domain (20). All of the active PAI-1 in plasma circulates in complex with VN (12), and the binding of PAI-1 to VN stabilizes the inhibitor (21) and alters its specificity (22), making it a reasonable thrombin inhibitor. Thus, VN is clearly a cofactor for PAI-1. Interestingly, VN also binds uPAR (23), and the binding site for uPAR is located in the SMB domain as well (24). The two binding sites for PAI-1 and SMB are partially overlapping but not identical (24).

**uPAR.** uPAR (CD87) is a glucosyl-phosphatidyl-inositol (GPI)-anchored protein and the cellular receptor for uPA (4). Binding of uPA to uPAR not only localizes proteolytic activity to the cell surface but also has a number of other very specific effects on cells. For example, it increases the affinity of uPAR for VN (23), it leads to the formation of stable complexes between uPAR and a variety of integrins (19, 25, 26), and it induces the activation of signaling molecules that are important for cell migration (27–29). uPAR is organized into three structural domains: D1, D2, and D3. The most N-terminal domain (D1) contains the principal uPA-binding sequence (30), whereas D2 is implicated in binding to VN (31, 32). uPAR is an adhesion receptor for cells growing on VN, and these cells can therefore attach to VN through uPAR, integrins, or both (16, 33).

That the PAI-1, uPAR, and integrin binding sites are clustered so closely together at the $NH_2$ end of the VN molecule (Fig. 1) raised the possibility that PAI-1 may competitively inhibit uPAR and integrin-mediated cell attachment to VN, which appears to be the case (16). For example, PAI-1 inhibits the adhesion of MCF7 cells to VN, and these cells are known to adhere to VN through integrins (34). PAI-1 also inhibits the binding of U937 cells that adhere only through uPAR (15, 23), and it inhibits the binding of Hela and HT-1080 cells that employ both adhesion receptors.

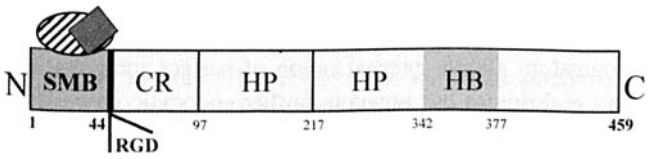


**Figure 1.** Domain structure of vitronectin (Adapted from Ref. 24). SMB, somatomedin B domain; CR, connecting region; HP, hemopexin-like repeat; HB, heparin binding domain. The RGD site is adjacent to the SMB domain, which contains overlapping binding sites for plasminogen activator inhibitor 1 (oval with diagonal lines) and urokinase-like plasminogen activator (square).

In summary, cell adhesion to VN is a unique and complex process because cells can adhere to it through uPAR, integrins, or both. The binding of PAI-1 to the SMB domain of VN seems to block both of these adhesive sites, thus preventing the attachment of cells to this extracellular matrix protein. Importantly, this is a VN-specific process because PAI-1 does not bind to other extracellular matrix proteins or inhibit cell adhesion to them.

## PAI-1 and Cell Detachment

**Cell Detachment from VN.** On the basis of the above cell attachment studies, we predicted that PAI-1 would be able to detach cells from VN by binding to the SMB domain and competitively displacing uPAR and integrins. Moreover, we predicted that this process would be specific for VN because, as mentioned, PAI-1 cannot bind to other matrix proteins. As discussed below, both of these hypotheses were incorrect. This conclusion is based on the use of two PAI-1 variants developed by Dan Lawrence (Table 1; Refs. 35, 36). The first (Q123K) no longer binds to VN ($V^-$) and thus does not block cell attachment to VN. However, it is still an effective PA inhibitor ($P^+$). The second variant no longer functions as a PA inhibitor ($P^-$) but retains its VN binding property ($V^+$). Because of this, it blocks cell attachment to VN.

Figure 2A shows that PAI-1 detaches cells from VN. In these initial experiments, we examined the behavior of HT-1080 cells because they attach to VN via both uPAR and integrins. The figure shows that most of the cells remained attached when the cultures were incubated with uPA alone or with PAI-1 alone. However, when the cells were sequentially incubated with uPA and PAI-1, the majority were rapidly detached. A similar effect was observed when

**Table 1.** Variants of Human Plasminogen Activator Inhibitor 1 (PAI-1) with Altered Binding Characteristics for Urokinase-Like Plasminogen Activators (P) and Vitronectin (V)

| | Mutation | Designation | Blocks attachment to vitronectin (somatomedin B) |
|---|---|---|---|
| Active PAI-1 (14-1b) | Q123K | PAI-1 ($P^+V^-$) | No |
| Active PAI-1 (14-1b) | T333R A335R | PAI-1 ($P^-V^+$) | Yes |

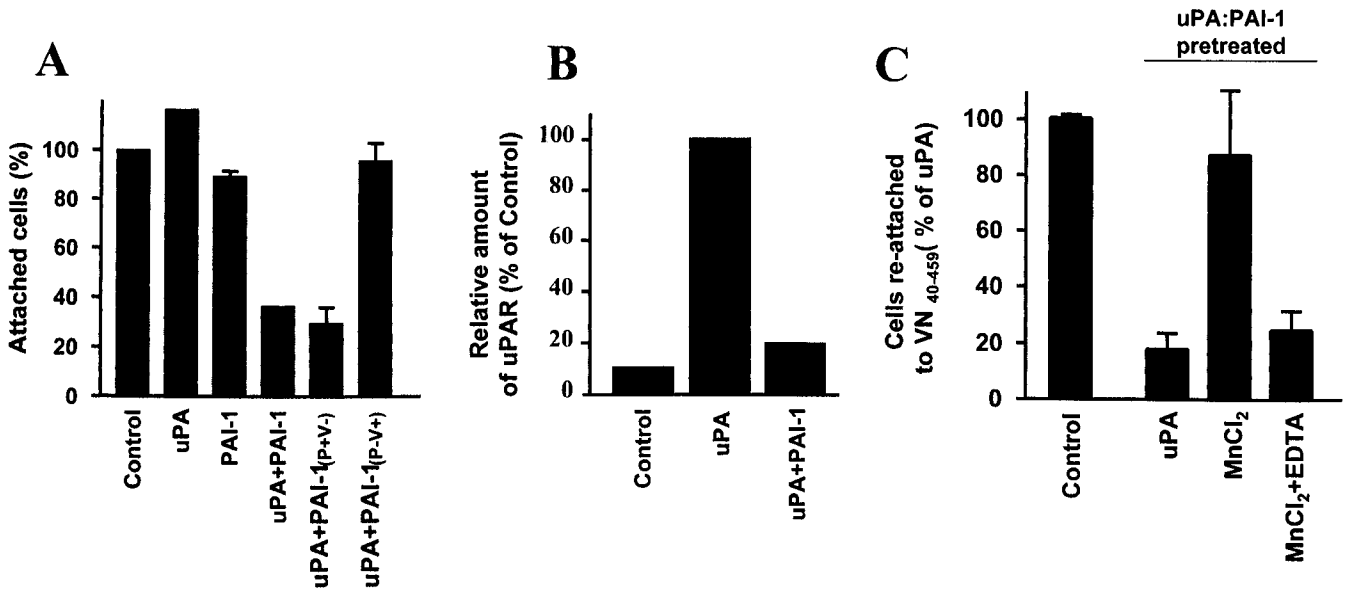


**Figure 2.** Effects of plasminogen activator inhibitor 1 (PAI-1) on cell detachment from vitronectin (VN). (A) Detachment of urokinase-like plasminogen activator (uPA)-pretreated HT-1080 cells requires binding of plasminogen activator inhibitor 1 to surface bound uPA but not to VN. (B, C) Binding of plasminogen activator inhibitor 1 to uPA:urokinase receptor (uPAR):integrin complexes decreases the amount of uPAR at the cell surface (B), and leads to deactivation of integrins (C). (Data adapted from Ref. 19).

the cells were subjected to sequential incubation with uPA and the PAI-1 mutant that binds to uPA but not to VN. However, little detachment was observed using the PAI-1 mutant that binds to VN but not uPA. These observations demonstrate that cell detachment by PAI-1 occurs in a VN-independent manner.

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 (19, 25, 26) and to an increase in cell attachment.(33). We thus hypothesized that the de-adhesive effects of PAI-1 may reflect a PAI-1 mediated dissociation of these complexes. To test this possibility, cells were pretreated with uPA at 4°C, which allows receptor binding but no internalization. PAI-1 was then added at 18°C, a temperature that permits the internalization of surface receptors (e.g., uPAR, integrins) into early endosomes, but not their recycling. Cell extracts were then prepared and immunoprecipitated using monoclonal antibodies against $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (not shown), and then the immunoprecipitates were analyzed by immunoblotting for uPAR. As expected (33), the addition of uPA caused a 5- to 10-fold increase in the amount of uPAR "pulled down" in complex with these integrins, compared to the untreated control (Fig. 2B). More important, the subsequent addition of PAI-1 to these cells led to a dramatic decrease in the amount of the coimmunoprecipitated uPAR. Although not shown, the variant of PAI-1 that bound to uPA but not to VN, worked as well as PAI-1 itself. However, the variant that bound to VN but not to uPA had no effect. Thus, cell detachment by PAI-1 is associated with a decrease in the concentration of uPAR/integrin complexes at the cell surface, and this process requires the binding of PAI-1 to uPA but not to VN. Figure 2C shows that compared to control cells, cells released by sequential treatment with uPA and PAI-1 at 4°C cannot reattach to VN. However, if the released cells were first treated with $MnCl_2$, a treatment known to activate $\alpha_v$-integrins, significant cell attachment was observed. Thus, the sequential treatment of cells with uPA and PAI-1 at 18°C leads to a decrease in uPAR/integrin complexes at the cell surface and to the inactivation of the majority of active integrins at the cell surface.

It is known that incubation of cells with uPA and PAI-1 leads to the formation and internalization of the resulting PAI-1:uPA:uPAR complexes by the low-density lipoprotein receptor–related protein (LRP; Refs. 37, 38). Because Figure 2B shows that $\alpha_v$-integrins are also associated with these complexes, we wondered whether the loss of active integrins on the cell surface was also a result of their internalization by LRP. To investigate this question, intact HT-1080 cells were biotinylated to label their surface proteins and then detached from VN by incubation in nonenzymatic cell dissociation solution. The suspended cells were then incubated with uPA alone at 4°C for 1 hr, and then with PAI-1 at 18°C. Remember, the latter temperature allows internalization of surface integrins into early endosomes but prevents further endocytic processing. The cells were broken open, plasma membrane and early endosome fractions were prepared by centrifugation through Percoll gradients, and the various fractions were then analyzed for the presence of biotinylated integrins. When uPA alone was added to the cells, $\alpha_v\beta_3$ (Fig. 3A) and $\alpha_v\beta_5$ (not shown) were detected only in plasma membrane

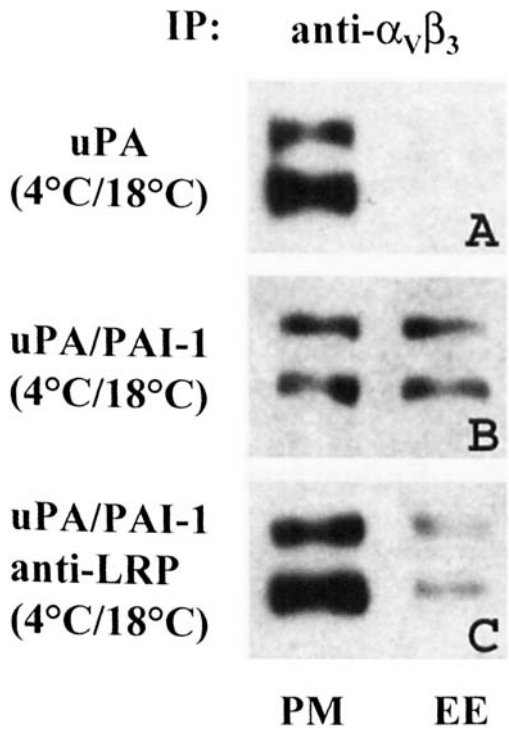


**Figure 3.** Lipoprotein receptor-related protein (LRP)-mediated endocytosis of urokinase receptor (uPAR):integrin complexes. Incubation of HT-1080 cells with urokinase-like plasminogen (uPA) alone (A) had no effect on the localization of uPAR at the plasma membrane (PM). Incubation of uPA-pretreated cells with plasminogen activator inhibitor 1 (PAI-1) (B) leads to internalization of both uPAR and integrins into an early endosomal (EE) intracellular compartment. In the presence of antibodies against LRP (C), internalization is blocked, and uPAR and integrins remain at the PM. (Data adapted from Ref. 19).

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 both integrins into early endosome fractions (Fig. 3B), and anti-LRP antibodies greatly reduced these effects (Fig. 3C). Thus, PAI-1 promoted the internalization of these integrins by an LRP-dependent mechanism. Taken together with the data in Figure 2, these observations indicate that the binding of PAI-1 to uPA:uPAR:integrin complexes initiates cell detachment by first inactivating and disengaging uPAR and $\alpha_v$-integrins from their association with ECM and then by clearing the adhesion receptor complexes by this LRP-dependent process.

**Cell Detachment from Other Matrix Protein.** As demonstrated in Figure 2A, the effects of PAI-1 on cell detachment, unlike those on cell adhesion, occur in a VN-independent manner. This observation raised the possibility that the inhibitor can also detach cells from other matrix proteins that do not contain binding sites for PAI-1. This appears to be the case because the sequential addition of uPA and PAI-1 to HT-1080 cells also detached the cells from fibronectin and type I collagen (19). However, significantly fewer cells were detached from these matrices than from VN. It turns out that fibronectin and type I collagen contain multiple binding sites for integrins but that there is only one binding site (i.e., RGD sequence) present in VN. Thus, it is likely that a larger number of integrins are engaged by cells attached to FN and type I collagen than to VN. Cell detachment by PAI-1 is associated with the inactivation (Fig. 2C) and internalization (Fig. 3B) of uPAR:integrin complexes. Because there are more engaged integrins, it may be that uPAR is limiting in the case of cells attached to FN or type I collagen. If true, then increasing the amount of surface uPA:uPAR at the cell surface should increase the number of active uPAR:integrin complexes and lead to more pronounced cell detachment by PAI-1. Again, this appears to be the case because the addition of soluble uPA:uPAR complexes to the cells greatly increased the efficiency of PAI-1 (19). Soluble uPA-uPAR complexes alone had no effect. Moreover, the sequential addition of uPA and PAI-1 to cells that do not express any uPAR did not detach them. However, if soluble uPAR was included, or if the cells were transfected with a uPAR cDNA, then the cells rapidly detached when PAI-1 was added.

Thus, it appears that PAI-1 cannot detach cells from the extracellular matrix unless uPA is present on the cell surface bound to uPAR. We next asked whether simply having uPAR on the cell surface was sufficient for PAI-1–mediated cell detachment, or whether cell detachment actually required the binding of uPAR to integrins. To address this question, we took advantage of a peptide prepared by Hal Chapman and his collaborators (25). This peptide has the same sequence as the binding site in $\alpha_3$ integrins recognized by uPAR and was shown to block the binding of uPA/uPAR complexes to $\alpha_3$ integrins. The peptide, termed $\alpha_3$ 25, completely blocked cell detachment initiated by the sequential addition of uPA and PAI-1 to uPAR-expressing cells. The control scrambled peptide had no effect, indicating the specificity of this effect.

Thus, cell detachment by PAI-1 does require the binding of uPA/uPAR complexes to integrins. Now, we showed earlier (Fig. 3) that the addition of PAI-1 to uPA-pretreated cells caused the internalization of the resulting PAI-1/uPA/uPAR/integrin complexes into early endosomes, and that this internalization occurred through an LRP-dependent process. Importantly, the uPA-pretreated cells could also be detached by PAI-1 when the whole experiment was performed at 4°C (not shown); that is, under conditions that do not allow internalization. These observations raise the possibility that cell detachment by PAI-1 does not require the actual internalization of these complexes by LRP. To address this question, we examined the effects of LRP inhibitors on cell detachment by PAI-1. Although not shown, neither receptor associated protein, a specific inhibitor of ligand binding to all members of the LRP gene family, nor neutralizing antibodies developed to LRP were able to block cell detachment. Thus, even though cell detachment by PAI-1 is associated with the LRP-dependent endocytic clearance of uPAR:integrin complexes, this clearance is not actually required to release

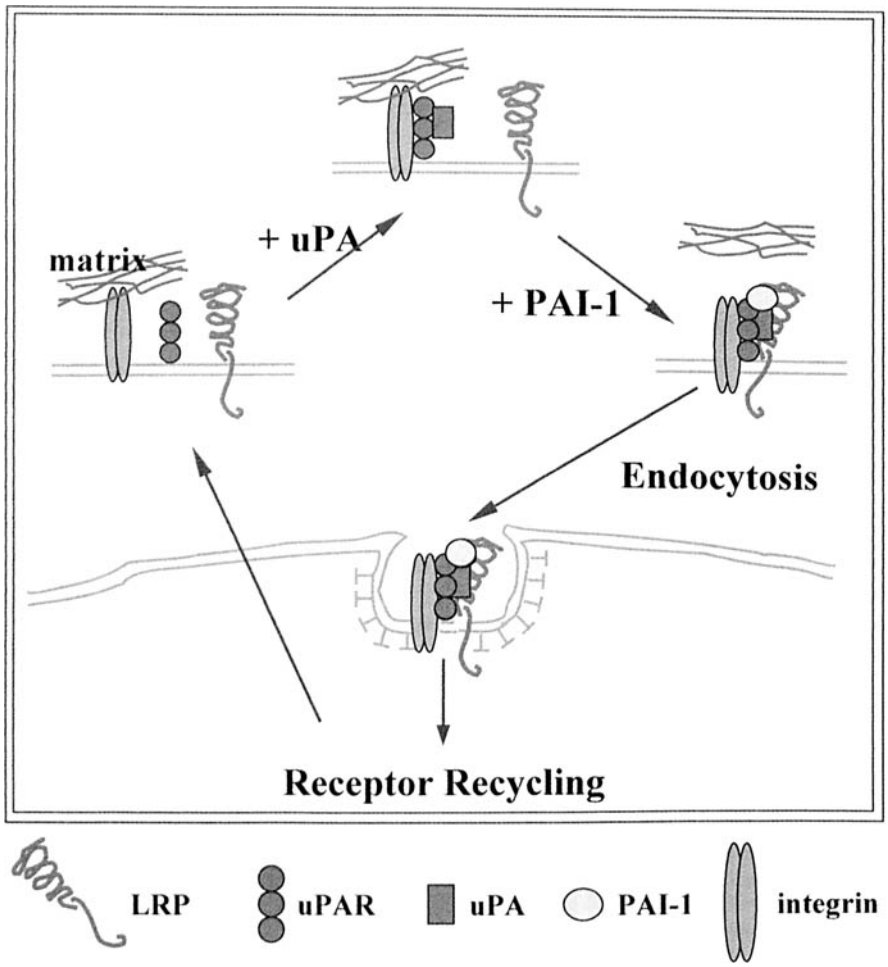


**Figure 4.** Model depicting proposed events in plasminogen activator inhibitor 1 (PAI-1)–induced cell detachment (see text for details; adapted from Ref. 19). LRP, lipoprotein receptor-related protein; uPAR, urokinase receptor; uPA, urokinase-like plasminogen

the cells. This means that under these conditions, the cells must be released because the matrix-engaged integrins are somehow inactivated. This inactivation clearly involves some change in the relationship between uPAR and the integrins.

Finally, preliminary studies (data not shown) raise the possibility that uPAR may only target those integrins on the cell surface that are specifically engaged with the extracellular surface. This conclusion is based on the use of a cell line that expresses both $\alpha_v$ integrins, and a variant of $\alpha_3$ that can no longer bind to uPAR. Thus, although these cells bind and spread on laminin 5, one of the ligands for $\alpha_3$, they cannot interact with uPAR because of this mutation. These cells could not be detached from laminin 5 by PAI-1, even in the presence of soluble uPAR.

These experiments again show that uPAR must interact with integrins. However, if the cells were plated on VN, they were rapidly detached by the inhibitor. Thus, uPAR seems to transmit the de-adhesive effects of PAI-1 specifically to the matrix-engaged integrins. Cell detachment by PAI-1 depends on the binding of uPAR to matrix-engaged integrins.

## Conclusion

These observations indicate that PAI-1–induced cell detachment requires the presence of uPA:uPAR complexes on the cell surface, that it depends on the formation of complexes between integrins and the occupied uPAR (whether present on the cell or provided exogenously), and that LRP-mediated endocytosis is not required. Finally, these observations indicate that uPAR mediates the de-adhesive activity of PAI-1 by preferentially binding to matrix-engaged integrins. Figure 4 shows the whole detachment cycle as we now understand it. The addition of exogenous uPA to resting cells, or its induction by various cytokines, leads to a conformational change in uPAR (4), causing it to bind to matrix-engaged integrins. The addition of exogenous PAI-1 to these cells, or its induction by various cytokines, results in the disengagement of the two adhesion receptors from the ECM and their internalization via an LRP-dependent process. Finally, the PAI-1 and uPA are degraded in lysosomes, whereas uPAR, integrins, and LRP are recycled to the cell surface.

One of the most interesting conclusions from these studies is that PAI-1 is an important new member of the

class of extracellular proteins termed "de-adhesion molecules" (39). This is a new activity for this versatile protein, and it may help to explain the observations that PAI-1 is an immediate early gene and that it is highly regulated by changes in the physiologic state of cells, whether in response to cytokines, growth factors, or hormones (2, 8). Obviously, these cytokine-induced changes may, in turn, contribute to the many specific changes observed when cells migrate during wound healing, tissue remodeling, and so forth. The de-adhesive properties of PAI-1 may also begin to account for the observation that patients with high PAI-1 levels in their tumors have a poor prognosis for survival in a variety of metastatic cancers (2, 14). As we now know, these tumors, or the stromal cells around them (40), may also express high uPA and uPAR, thus possibly activating this de-adhesion system, decreasing the strength of cell adhesion sites, and promoting cell migration and possibility metastasis.


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