Is Plasminogen Activator Inhibitor-1 the Molecular Switch That Governs Urokinase Receptor-mediated Cell Adhesion and Release?

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Abstract. Induction of the urokinase type plasminogen activator receptor (uPAR) promotes cell adhesion through its interaction with vitronectin (VN) in the extracellular matrix, and facilitates cell migration and invasion by localizing uPA to the cell surface. We provide evidence that this balance between cell adhesion and cell detachment is governed by PA inhibitor-1 (PAI-1). First, we demonstrate that uPAR and PAI-1 bind to the same site in VN (i.e., the amino-terminal somatomedin B domain; SMB), and that PAI-1 competes with uPAR for binding to SMB. Domain swapping and mutagenesis studies indicate that the uPAR-binding sequence is located within the central region of the SMB domain, a region previously shown to contain the PAI-1–binding motif. Second, we show that PAI-1 dissociates bound VN from uPAR and detaches U937 cells from their VN substratum. This PAI-1 mediated release of cells from VN appears to occur independently of its ability to function as a protease inhibitor, and may help to explain why high PAI-1 levels indicate a poor prognosis for many cancers. Finally, we show that uPA can rapidly reverse this effect of PAI-1. Taken together, these results suggest a dynamic regulatory role for PAI-1 and uPA in uPAR-mediated cell adhesion and release.

TUMOR invasion and metastasis depend upon the coordinated expression and temporal regulation of a series of proteolytic (Chen, 1992; Dano et al., 1985; Mignatti and Rifkin, 1993) and adhesive (Nesbit and Herlyn, 1994) events. Urokinase-type plasminogen activator (uPA)[1] is one of the proteases frequently implicated in these processes (Dano et al., 1985; Mignatti and Rifkin, 1993; Cohen et al., 1991; Vassalli et al., 1991). It is a serine protease that catalyzes the conversion of plasminogen into plasmin. Plasmin itself is a broadly acting trypsin-like enzyme that not only degrades fibrin and a variety of extracellular matrix (ECM) proteins, but also may activate metalloproteinases (Mignatti and Rifkin, 1993; He et al., 1989). Plasminogen (Plow et al., 1995) and uPA (Huarte et al., 1985; Roldan et al., 1990) bind to specific receptors, localizing plasmin activity to the cell surface where it can be employed by migrating cells to degrade and/or modify tissue barriers during a variety of normal and pathologic processes (Mignatti and Rifkin, 1993). It is not surprising that many tumors express increased uPA and uPA receptor (uPAR) (Del Vecchio et al., 1993; Delbaldo et al., 1995) since these molecules may provide the tumor cells with the necessary proteolytic activity for invasion (Heiss et al., 1995).

It is difficult to reconcile this simple paradigm of surface-associated proteolytic activity and invasiveness, with two recent observations. First, uPA actually promotes the adhesion of U937 tumor cells to the adhesive glycoprotein vitronectin (VN) (Waltz et al., 1993; Waltz and Chapman, 1994) rather than releasing them from their attachment to this ECM substratum. This activity depends on the binding of uPA, its amino-terminal fragment (ATF; residues 1-143), or its epidermal growth factor domain (EGF; residues 1-48), to uPAR (Waltz and Chapman, 1994), and occurs in the presence of RGD peptides and/or EDTA (i.e., in an integrin-independent manner) (Waltz and Chapman, 1994). uPA alters the conformation of uPAR (Ploug et al., 1994), increasing its affinity for VN (Waltz et al., 1993). Second, many tumor cells express increased levels of PA inhibitor-1 (PAI-1) (Heiss et al., 1995; Sier et al., 1994) the primary endogenous inhibitor of uPA. Unexpectedly, this potent inhibitor of plasminogen activation is necessary for optimal invasiveness of cultured lung cancer cells (Liu et al., 1995), and an increasing number of studies demonstrate that high PAI-1 levels indicate a poor prognosis for survival of patients with breast (Grondahl-Hansen et al., 1993), lung (Pedersen et al., 1994), gastric (Nekarda et al., 1994), ovarian (Kuhn et al., 1994), and cervical (Kobayashi et al., 1994) cancer. These observations suggest that PAI-1

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1. *Abbreviations used in this paper*: PA, plasminogen activator; PAI-1, PA inhibitor-1; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VN, vitronectin.

may play a critical role in tumor cell invasion. The mechanisms by which uPA promotes cell adhesion and PAI-1 facilitates tumor cell invasion, remain to be delineated.

Cells employ a variety of adhesion receptors to attach to the ECM, including proteoglycans (i.e., syndecans; Bernfield et al., 1993), integrins (Hynes and Lander, 1992), and uPAR. Under most circumstances, the integrins appear to be the more important of the ECM adhesion receptors (Gumbiner, 1996). They consist of a large family of heterodimeric transmembrane proteins composed of different combinations of α and β subunits which largely determine their ligand specificity (Hynes and Lander, 1992). In this context, the exact role and biological significance of uPAR, a glycosylphosphatidylinositol (GPI)-anchored membrane protein (Behrendt et al., 1995; Ploug et al., 1991), in controlling cell adhesion remain to be determined. This alternative adhesion pathway may assume importance in some pathological situations. For example, recent studies show that the expression of various integrins is downregulated in many tumor cells (Howlett et al., 1995; Hauptmann et al., 1995; Roussel et al., 1994; Boukerche et al., 1994), and tumor cells frequently fail to deposit a fibronectin matrix around themselves (Rouslahti, 1994). The fact that the uPAR system may be upregulated in migrating tumor cells (Behrendt et al., 1995; Gladson et al., 1995; Sier et al., 1994; Mignatti and Rifkin, 1993) suggests that these cells may use this alternative adhesion pathway as a response to the disturbed expression of normal cell adhesion proteins.

The above observations indicate that the plasminogen activating system may provide both surface-associated protease activity and a novel adhesion mechanism for cells. However, in spite of the critical importance of uPAR in controlling these processes, the nature of the molecular "switch" that determines which response will dominate remains to be defined. The possibility that PAI-1 itself is involved in these processes is suggested by the observations that this inhibitor binds to both uPA and VN but does so by quite distinct mechanisms. For example, PAI-1 binds to and inhibits uPA, but is inactivated in the process (Kruithof, 1988; Loskutoff, 1991; Van Meijer and Pannekoek, 1995). Although PAI-1 also binds to VN with high affinity, it retains its activity and is, in fact, stabilized by this interaction. PAI-1 complexed with VN can still bind to and inhibit uPA (Knudsen et al., 1987) and tissue-type PA (tPA) (Declerck et al., 1988; Mimuro and Loskutoff, 1989). Interestingly, the resulting PA/PAI-1 complexes have little affinity for VN, and are rapidly released from it.

In this report we demonstrate that the binding site for uPAR on VN is contained within residues 1-41 at the amino terminus of the molecule, the somatomedin B (SMB) domain (Deng et al., 1995; Jenne and Stanley, 1985; Van Meijer and Pannekoek, 1995). The SMB domain is also the high affinity binding site for active PAI-1 in VN (Seiffert and Loskutoff, 1991*a*). We also show that PAI-1 effectively competes with uPAR for VN binding, that the interaction between PAI-1 and SMB prevents the adhesion of U937 cells to VN, and that low concentrations of PAI-1 rapidly release U937 cells from their attachment to VN. These observations are incorporated into a model in which PAI-1 is suggested to play a central regulatory role in uPAR mediated cell adhesion and release. This model may explain why high PAI-1 levels correlate with a poor prognosis for many cancers.

Materials and Methods

Materials

Recombinant human PAI-1 was purified from *Escherichia coli* strain JM105 transformed with the PAI-1 expression plasmid pMBL11 as described previously (Seiffert et al., 1994). Latent PAI-1 was generated by incubating active recombinant PAI-1 for 24 h at 37°C (Deng et al., 1996). Human high molecular weight two-chain uPA was from CalBiochem (La Jolla, CA). VN was purified from human plasma by heparin affinity chromatography in the presence of urea (Yatohgo et al., 1988), and iodinated to a specific activity of 8.9×10^7 cpm/μg by using the IODO-GEN system (Pierce, Rockford, IL). The expression and purification of recombinant SMB and SMB mutants have been described previously (Deng et al., 1996). The EGF-like domain of human uPA (residues 1-48) was expressed and purified from recombinant yeast as described (Stratton-Thomas et al., 1995). Biotinylated uPAR was labeled according to Kaufman et al. (1993) using soluble recombinant human uPAR expressed and secreted from baculovirus-infected Sf9 insect cells as described (Goodson et al., 1994). ATF (residues 1-143 of uPA) and unlabeled soluble recombinant human uPAR were the kind gifts of Dr. Jieyi Wang (Abbott Laboratories, Abbott Park, IL). The anti-VN mAbs (1244 and 153) were developed and purified as described (Seiffert et al., 1994). GRGDSP peptide was from Peninsula Laboratories (Belmont, CA).

Binding Assays

The binding of uPAR to immobilized VN was investigated by using a uPAR microtiter plate assay. Briefly, microtiter wells were coated with VN by incubating them in 100 μl of a solution containing 1 μg/ml of VN in PBS at 4°C overnight. The wells were washed and then blocked by incubation with 5% casein in PBS for 1 h. Biotinylated uPAR (10 nM), uPA (10 nM) or the EGF domain of uPA (10 nM), and various competitors were added to the wells and the plate was incubated at 37°C for 1 h. The amount of bound uPAR was determined by employing streptavidin-alkaline phosphatase (Zymed Labs., So. San Francisco, CA) and the ELISA amplification system (GIBCO BRL, Gaithersburg, MD). The change of color was determined at 495 nM, and the O.D. reading from duplicate wells was corrected by subtracting values for the wells incubated in the absence of biotinylated uPAR.

The binding of iodinated VN to U937 cells was quantified as follows. U937 cells at 10^6 cells/ml were incubated for 16 h in RPMI 1640 (BioWhittaker, Walkersville, MD) containing 10% FBS (GIBCO BRL), human transforming growth factor β (TGF-β1) (Sigma Chem. Co., St. Louis, MO; 1 ng/ml) and 1α,25-dihydroxy-vitamin D3 (CalBiochem; 50 nM) (Waltz and Chapman, 1994). The cells were then washed twice with serum-free RPMI containing 0.02% BSA (Sigma) and resuspended at 5×10^6 cells/ml in the same media. The cells (0.2 ml) were then incubated with iodinated VN at 4°C in the presence or absence of various competitors, and after 90 min, aliquots (50 μl) were removed, layered on 0.3 ml of 20% sucrose, and centrifuged for 3 min at 12,000 rpm. The amount of iodinated VN associated with the cell pellet was determined in a γ counter. Triplicate determinations were made for each condition, and data are expressed as mean ± S.D.

Cell Adhesion Assay

U937 cells previously activated by culturing them in the presence of human TGF-β1 and vitamin D3 (as above), were washed twice, resuspended in serum-free media containing 0.02% BSA, and plated at 2.5×10^5 cells/well in 96-well tissue culture plates precoated with 100 μl VN (2 μg/ml), and blocked with 5% BSA. The cells were incubated in the VN-coated wells for 3 h at 37°C in 5% CO_2, and then the plates were washed three times to remove nonadherent cells. Each step of the washing procedure included gentle agitation for 1–2 min in fresh media using a microtiter plate mixer. Triplicate wells were then photographed at both low (40×) and high (200×) magnification.

Results

Active PAI-1 Inhibits the Binding of uPAR to VN

Recent studies suggest that PAI-1 inhibits the adhesion of U937 cells to VN by binding to uPA and promoting the removal of both uPA and uPAR from the cell surface (Waltz

et al., 1993). However, PAI-1 also binds to VN with high affinity (Deng et al., 1995; Salonen et al., 1989; Seiffert and Loskutoff, 1991*b*; Van Meijer and Pannekoek, 1995), raising the possibility that it may inhibit cell adhesion through its interaction with VN rather than through its interaction with uPA. A series of experiments was performed to distinguish between these possibilities. Fig. 1 shows that purified recombinant PAI-1 does indeed inhibit the binding of VN to U937 cells, that it does so in a dose-dependent manner, and that 20 nM of active PAI-1 completely inhibited this interaction. Interestingly, preincubation of the cells with PAI-1 to presaturate the uPA did not inhibit the subsequent binding of iodinated VN to the cells (Fig. 1, *shaded bar*). In fact, essentially all of the VN-binding sites were still available after this treatment. In contrast, preincubation of VN with purified PAI-1 prevented it from subsequently binding to purified uPA/uPAR complexes (Fig. 2, *open triangle*). Moreover, active PAI-1 directly inhibited the binding of purified uPAR to VN immobilized onto microtiter wells, with an IC_{50} of ~10 nM. In these experiments, the EGF domain of uPA (i.e., residues 1-48) was employed instead of uPA itself. The EGF domain of uPA cannot bind to PAI-1, but still binds to uPAR and enhances its interaction with VN (Waltz and Chapman, 1994). Inactive latent PAI-1, which does not bind to uPA or VN (Declerck et al., 1988; Wiman et al., 1988; Mimuro and Loskutoff, 1989), showed no inhibition in this assay. The demonstration that both purified VN and purified uPAR inhibit the interaction (Fig. 2), indicates that these molecules specifically interact in this assay system. In separate experiments we demonstrated that ^{125}I-labeled VN binds to both uPA/uPAR and uPA/uPAR/PAI-1 complexes immobilized on microtiter wells (data not shown). Taken together, these results indicate that PAI-1 inhibits the binding of uPAR to VN by a mechanism that depends upon its interaction with VN, and not with uPA.

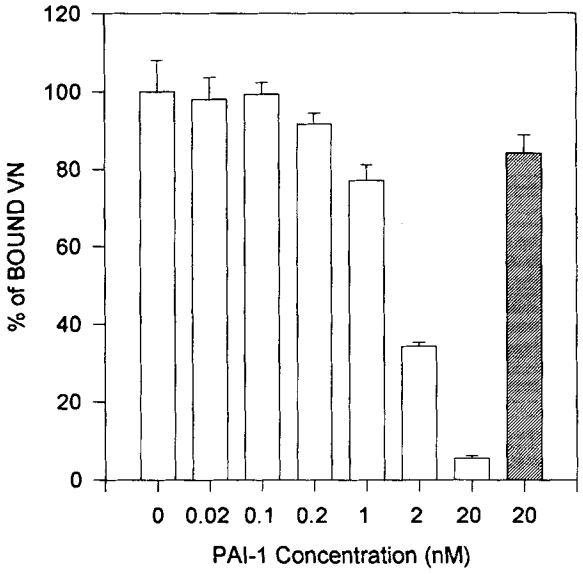

Figure 1. PAI-1 inhibits the binding of VN to U937 cells. U937 cells were stimulated overnight with TGF-β1/vitamin D3, washed extensively, and then incubated on ice for 90 min in the presence of ^{125}I-labeled VN and increasing amounts of purified PAI-1 (*open bars*). The cells were then centrifuged through 20% sucrose, and cell-associated VN was determined in a γ counter. In one experiment, the cells were preincubated with PAI-1 (20 nM) for 30 min, washed, and then the ^{125}I-labeled VN was added for 90 min (*shaded bar*). The latter data were not adjusted for cell loss due to the extra washing step. In these experiments, 100% of bound VN represented 5,900 cpm.

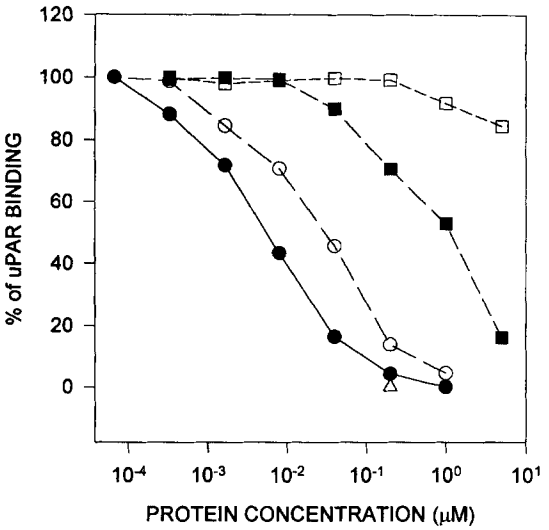

Figure 2. PAI-1 inhibits uPAR binding to VN. Biotin-labeled uPAR (10 nM) and the EGF domain of uPA (10 nM) were added to VN-coated microtiter wells in the presence of increasing concentrations of various competitors. The amount of bound uPAR was then determined by the uPAR microtiter plate assay (see Materials and Methods). Competitors used in the assay include active PAI-1 (●), VN (○), uPAR (■), and latent PAI-1 (□). In one experiment, the VN-coated wells were preincubated with PAI-1 (200 nM) for 1 h, the wells were washed, and then biotin-labeled uPAR and the EGF domain of uPA were added as above (△).

The Balance between uPA and PAI-1 Governs the uPAR/VN Interaction and Cell Adhesion

The kD for the VN/PAI-1 interaction is ~0.3 nM (Seiffert and Loskutoff, 1991*b*), while the VN/uPAR interaction is 10 nM (Waltz and Chapman, 1994). The 30-fold higher affinity of PAI-1 over uPAR for VN suggests that the inhibitor may be able to detach cells from their VN substratum. Cell adhesion assays were performed in order to test this hypothesis (Fig. 3). U937 cells were incubated in VN-coated wells in serum-free media for 3 h, and then PAI-1 (40 nM) was added. After another 1 h of incubation, the wells were washed three times to remove nonadherent cells, and then photographed. Fig. 3 shows that the presence of excess PAI-1 caused the vast majority of cells to become nonadherent and to be released from their VN substratum. This effect occurs in a PAI-1 dose-dependent manner (data not shown), in agreement with the results shown in Fig. 1. PAI-1 also caused the release of nontreated or PMA-treated U937 cells from VN. Incubation of these cells in media containing 0.5 mM GRGDSP peptide or 10 mM EDTA did not release the cells from VN (not shown).

It is well established that PAI-1 inhibits uPA (Vassalli et al., 1991; Van Meijer and Pannekoek, 1995) and that the PAI-1 in the resulting uPA/PAI-1 complexes no longer

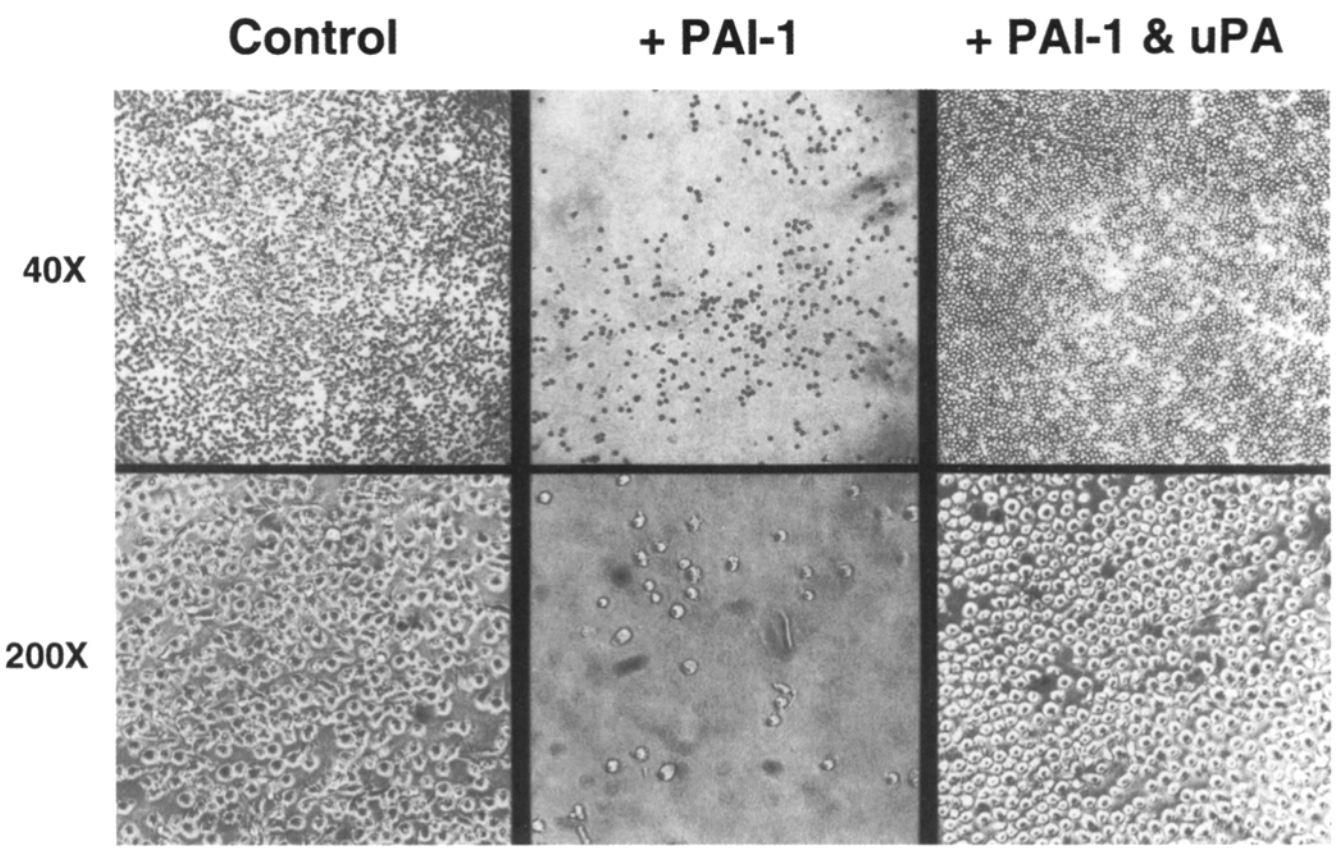

Figure 3. PAI-1 detaches U937 cells from VN coated wells. U937 cells were stimulated with TGF-β1/vitamin D3 overnight, and then seeded onto VN-coated cell culture wells in triplicate in serum-free media containing 0.02% BSA. 3 h later, media alone (*left panels*), media containing PAI-1 (*middle panels*), or media containing PAI-1 and uPA (*right panels*) was added. The final concentration of PAI-1 and uPA in the wells was 40 nM and 100 nM, respectively. The wells were incubated for an additional 60 min and then agitated and washed three times to remove nonadherent cells as described in Materials and Methods. Representative wells from each set were then photographed at low and high magnification (*top panels* and *bottom panels*).

binds to VN (Knudsen et al., 1987). In fact, the addition of uPA or tPA to VN, specifically releases any PAI-1 previously bound to it. Therefore, similar experiments were performed to investigate the effect of uPA on cell adhesion and release. Fig. 3 (*right panels*) graphically demonstrates that uPA prevents the PAI-1–mediated release of U937 cells from VN. This effect is uPA dose-dependent (data not shown), and excess uPA (i.e., 2–5-fold) is able to completely prevent the cell release by PAI-1. Similar excesses of uPA and tPA completely inhibit and/or reverse the binding of PAI-1 to VN (Knudsen et al., 1987; Mimuro and Loskutoff, 1989). Interestingly, the uPAR binding fragment of uPA (i.e., ATF) did not prevent this PAI-1–mediated release of cells from VN (not shown). Although ATF binds to uPAR and enhances uPAR/VN binding (Waltz and Chapman, 1994), it cannot bind to PAI-1 (Cubellis et al., 1989). In separate experiments, U937 cells were allowed to bind to ^{125}I-labeled VN in the absence or presence of PAI-1 and/or uPA (Fig. 4 *A*). In these experiments, uPA (lane *2*) and ATF (lane *3*) slightly increased the binding of ^{125}I-labeled VN to the cells (compare to lane *1*), while 2 nM PAI-1 inhibited it by ~50% (lane *4*). The subsequent addition of uPA reversed this effect of PAI-1 (compare lanes *4* and *6*), but ATF did not (compare lanes *4* and *5*). Fig. 4 *B* demonstrates that tPA can also reverse the anti-adhesive effects of PAI-1. This observation is significant because tPA does not bind to uPAR (Roldan et al., 1990; Vassalli et al., 1991; Behrendt et al., 1995). Moreover, the PAI-1 in tPA/PAI-1 complexes, like that in uPA/PAI-1 complexes, no longer binds to VN (Declerck et al., 1988; Mimuro and Loskutoff, 1989).

Evidence that uPAR/uPA Complexes Bind to the SMB Domain of VN

The fact that PAI-1 binds to the SMB domain of VN (Seiffert and Loskutoff, 1991*a*) and simultaneously blocks VN binding to uPAR (Fig. 2), suggests that uPAR may also bind to the SMB domain. If this hypothesis is true, then purified SMB itself should competitively inhibit VN binding to uPAR. To test this possibility, we compared the ability of wild-type recombinant SMB and a non–PAI-1 binding mutant of SMB (i.e., SMB_{Y28A}; Deng et al., 1996) to compete with VN for uPAR binding in the microtiter plate assay (Fig. 5 *A*). The recombinant SMBs employed in these experiments contain residues 1 through 41 of VN, and therefore do not include the RGD sequence (Jenne and Stanley, 1985). As shown in Fig. 5 *A*, active wild-type

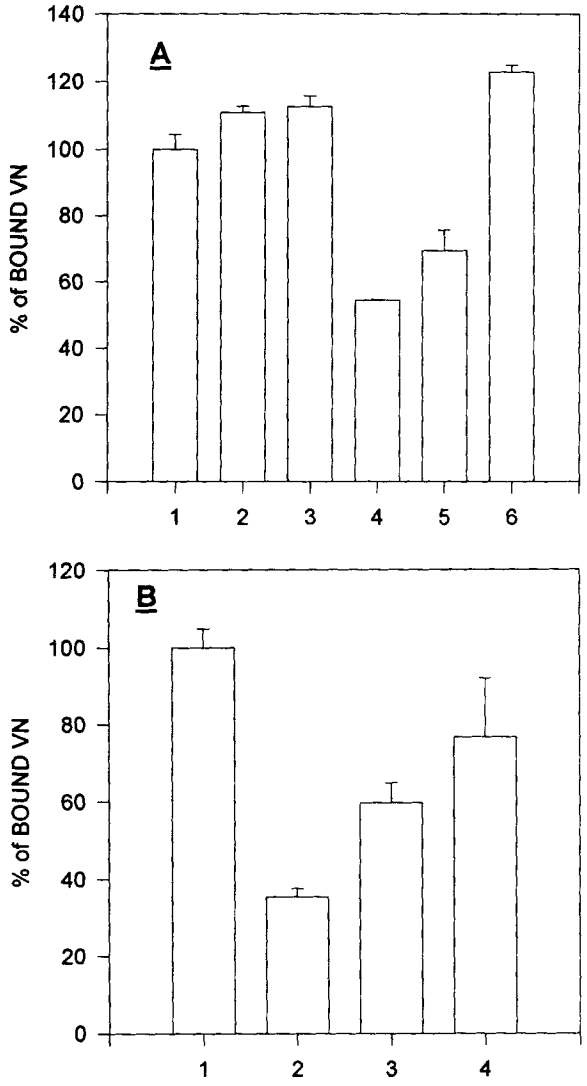

Figure 4. Evidence that it is the balance between uPA and PAI-1 that governs the binding of uPAR to VN. (*A*) ^{125}I-labeled VN was incubated with U937 cells for 90 min on ice as in Fig. 1 in the presence of serum-free media containing 0.02% BSA alone (lane *1*), or supplemented with 10 nM uPA (lane *2*), 10 nM ATF (lane *3*), 2 nM PAI-1 (lane *4*), 2 nM PAI-1 and 100 nM ATF (lane *5*), and 2nM PAI-1 and 10 nM uPA (lane *6*). (*B*) ^{125}I-labeled VN was incubated with U937 cells in media alone (lane *1*), or media containing 2 nM PAI-1 (lane *2*), 2 nM PAI-1 and 10 nM tPA (lane *3*), or 2 nM PAI-1 and 100 nM tPA (lane *4*).

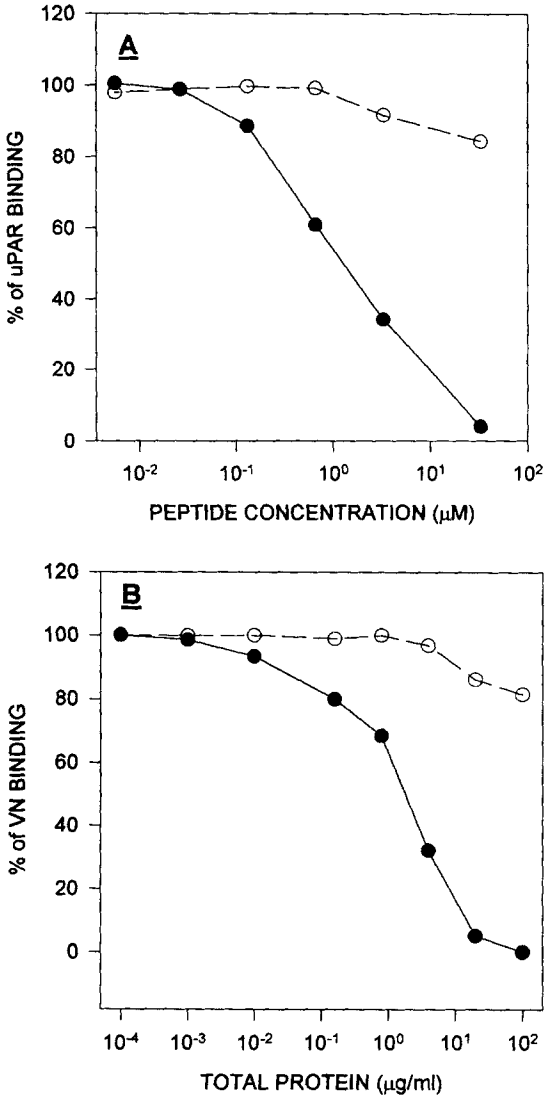

Figure 5. Evidence that uPAR binds to the SMB domain of VN. (*A*) Increasing concentrations of recombinant SMB (●) or of recombinant SMB_{Y28A} (○) were added to VN-coated microtiter wells in the presence of uPA and biotinylated uPAR, and the amount of bound uPAR was determined as in Fig. 2. (*B*) Equal concentrations of uPAR, 2 chain uPA, and PAI-1 (40 nM each) were mixed together and coated onto the wells of a microtiter plate by incubation at 4°C overnight. The wells were blocked, washed, and ^{125}I-labeled VN was added in the presence of increasing concentrations of an mAb specific for the SMB domain (i.e., mAb153; ●), or an mAb whose epitope is located outside of this domain in the connector region of VN (i.e., mAb1244; ○). The amount of bound VN was determined in a γ counter. In these experiments, 100% binding corresponds to 7,200 cpm.

SMB inhibited the binding of uPAR to VN, but the inactive SMB mutant did not. We previously showed that monoclonal antibody (mAb) 153 blocks the binding of PAI-1 to VN (Seiffert et al., 1994) and this mAb also blocked the binding of VN to uPA/uPAR/PAI-1 complexes immobilized on plastic surfaces (Fig. 5 *B*). In contrast, a second anti-VN mAb which did not inhibit PAI-1 binding (i.e., mAb 1244; Seiffert et al., 1994), had no effect on this interaction. These results indicate that the uPAR binding site in VN, like the high affinity PAI-1 binding site (Seiffert and Loskutoff, 1991*a*), is located in the SMB domain.

uPAR on U937 Cells Also Binds to the SMB Domain of VN

Experiments were performed to test the possibility that the binding of VN to U937 cells also occurs through the SMB domain. Fig. 6 demonstrates that purified recombinant SMB decreases the binding of VN to U937 cells in a dose-dependent manner, while the inactive Y28A SMB mutant had no effect. Moreover, inhibitory mAb 153

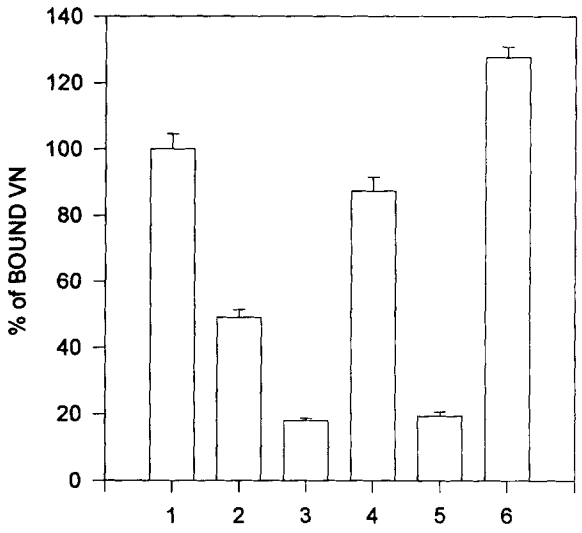

Figure 6. Evidence that uPAR on U937 cells binds to the SMB domain of VN. ^{125}I-labeled VN was incubated with U937 cells for 90 min at 4°C in the presence of serum-free media alone (lane *1*), or media containing 2 μM SMB (lane *2*), 8 μM SMB (lane *3*), 3 μM SMB mutant Y28A (lane *4*), 10 μg/ml of mAb 153 (lane *5*), or 10 μg/ml of mAb 1244 (lane *6*). In all instances, the amount of cell-associated, ^{125}I-labeled VN was determined as in Fig. 1.

blocked the binding of VN to U937 cells, but inactive mAb 1244 did not. Thus, the interaction between VN and U937 cells, like the interaction between VN and purified uPAR, is mediated by the binding of SMB to uPAR.

Identification of Critical Residues in the SMB Domain for uPAR Binding

Domain swapping and mutagenesis studies were performed to more precisely identify the uPAR binding sequence within the SMB domain. The SMB domain of VN is a cysteine-rich peptide (Fig. 7 *A*, *top*), and we previously showed that all eight cysteines are critical for maintaining the functional (i.e., PAI-1 binding) tertiary structure of the peptide (Deng et al., 1996). These cysteines appear to be organized into disulfide bonds, and substitution of any one of them with alanine resulted in the formation of inactive peptides that no longer bind to PAI-1 (Deng et al., 1996). Based on these observations, mutagenesis experiments were performed under conditions that did not change the cysteines. We initially tested the hybrid molecules created to define the PAI-1 binding region in SMB (Deng et al., 1996), and demonstrated that the uPAR binding motif, like the PAI-1 binding motif (Deng et al., 1996), is located in the central region of the molecule (data not shown). This region is indicated by the overline in the sequence shown in Fig. 7. We then converted the charged amino acids in this region into alanines and tested the resulting peptides for uPAR binding activity in an assay similar to that used in Fig. 2. When either of the negatively charged residues, Asp22 or Glu23, were converted into alanine, the resulting peptides no longer bound uPAR. However, when positively charged residues within this region (i.e., Lys17 and Lys18) were changed into alanines, the resulting mutant peptides still bound to uPAR (Fig. 7 *A*).

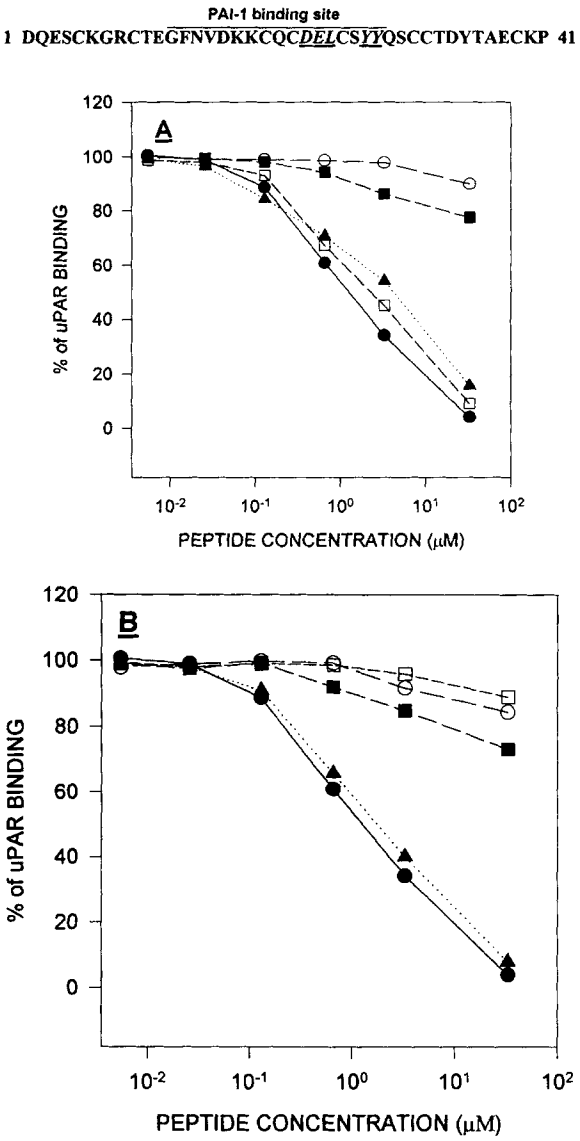

Figure 7. Mutational analysis of the uPAR binding site in SMB. Alanine scanning mutagenesis was employed to determine the role of charged residues (*A*) and polar/hydrophobic residues (*B*) within SMB for uPAR binding. The uPAR binding activity of the following mutants was tested. (*A*) Wild-type SMB (●); D22A (○); E23A (■); K6A,R8A (□); and K17A,K18A (▲). The amino acid sequence of wild-type SMB is shown across the top of this figure. The PAI-1 binding region is overlined and critical residues for uPAR binding are underlined. (*B*) Wild-type SMB (●); L24A (□); Y27A (■); Y28A (○); and Y35A (▲). All assays were performed in the uPAR microtiter plate assay as described in Materials and Methods.

In addition to the cysteines and the negatively charged residues, several polar and hydrophobic amino acids also were shown to be important in PAI-1 binding (Deng et al., 1996). These residues, along with a number of others in the central region of SMB, also were individually converted into alanines and then assayed for uPAR binding (Fig. 7 *B*). Among these residues, Leu24, Tyr27, and Tyr28 were found to be essential for uPAR binding.

Discussion

Although the specificity of cell adhesion to components of the extracellular matrix is largely determined by the integrin composition of the individual cell (Gumbiner, 1996), integrin-independent cell adhesion may also be important. For example, malignantly transformed cells often lose the ability to express specific integrins (Boukerche et al., 1994; Roussel et al., 1994; Howlett et al., 1995; Hauptmann et al., 1995) and to assemble fibronectin around themselves (Rouslahti, 1994). Although such cells are frequently nonadhesive, they may become adhesive under certain circumstances (i.e., by using alternative adhesion molecules). The adhesive properties of U937 lymphoma cells illustrate this behavior. Although these cells do not normally adhere to ECM, Chapman and his collaborators showed that they could be induced to adhere to VN by treating them with TGF-β1 and Vitamin D3 (Waltz et al., 1993), treatments subsequently shown to upregulate uPAR, uPA, and PAI-1 (Waltz et al., 1993). In a series of intriguing papers, these authors demonstrated that this adhesion occurred in the presence of EDTA (Waltz and Chapman, 1994), eliminating the contribution of known integrins since integrins require divalent cations for activity (Gumbiner, 1996; Hynes and Lander, 1992). Adhesion required uPA and was mediated through uPAR (Wei et al., 1994), a molecule originally implicated in the localization of uPA to the surface of invading cells (Roldan et al., 1990; Behrendt et al., 1995; Estreicher et al., 1990). The conclusion that uPAR plays a central role in this process is further supported by the observation that nonadherent 293 cells became adherent to VN when transfected with uPAR cDNA (Wei et al., 1994). Finally, antibodies to PAI-1 appeared to promote the adhesion of U937 cells to VN, leading to the suggestion that PAI-1 itself would inhibit cell adhesion by binding to uPA on cells and inducing the internalization of the uPA/uPAR complex (Waltz et al., 1993; Cubellis et al., 1990). The observations in this report demonstrate directly that PAI-1 inhibits uPAR mediated cell adhesion. However, in these studies, this effect of PAI-1 appears to be due largely to its interaction with VN and not with uPA.

First, we directly demonstrate that purified, recombinant PAI-1 inhibits the binding of ^{125}I-labeled VN to U937 cells (Fig. 1), and that active but not latent PAI-1 inhibits the binding of purified uPAR to purified VN (Fig. 2). Since these latter experiments were performed using purified components (i.e., in the absence of cells), this effect of PAI-1 could not depend upon cell-mediated internalization and degradation. In this regard, preincubation of U937 cells with PAI-1 did not reduce their ability to subsequently bind to VN (Fig. 1), but preincubation of VN with PAI-1 blocked its ability to bind to purified uPAR (Fig. 2). These results suggest that PAI-1 inhibits cell adhesion in these assays by interacting with VN and not with uPA/uPAR complexes on the cell surface. The experiments shown in Fig. 1 were conducted under conditions of minimal internalization (i.e., at 4°C). Thus, PAI-1 completely blocked the binding of cells to VN in the absence of uPAR mediated internalization. PAI-1 also blocked, and in fact reversed, the interaction between cells and VN when the experiments were performed at 37°C (Fig. 3). Although this result could be due in part to PAI-1 mediated uPAR internalization, the uPA reversal experiments again suggest that uPAR is not involved. First of all, PAI-1 complexed to uPA on the receptor is no longer active and thus would not be a substrate for the added uPA. However, PAI-1 bound to VN can still bind to the excess added uPA and the resulting uPA/PAI-1 complexes would be released. These latter changes would be expected to promote cell adhesion (Fig. 8). The fact that the inactive ATF fragment does not reverse the effect of PAI-1 at 4°C (Fig. 4) or at 37°C (not shown) is consistent with this hypothesis since ATF binds to uPAR (Waltz and Chapman, 1994) but not to PAI-1 (Cubellis et al., 1989).

Experiments were performed to identify the binding site in VN for uPA/uPAR. Since the high affinity binding site for active PAI-1 in VN is the SMB domain (Seiffert and Loskutoff, 1991*a*), we initially examined this region of the molecule. Fig. 5 shows that purified, recombinant SMB also blocked the binding of uPAR to VN (Fig. 5 *A*), and that an mAb directed to the SMB domain blocked the binding of ^{125}I-labeled VN to immobilized uPA/uPAR (Fig. 5 *B*). Recombinant SMB and a mAb that blocked the binding of PAI-1 to SMB also blocked the binding of VN to activated U937 cells (Fig. 6). In control experiments, we showed that a mutant of SMB which does not bind to PAI-1 (i.e., SMB_{Y28A}), and an mAb whose epitope was mapped outside of the SMB domain (i.e., 1244), had no effect in these binding experiments. These results indicate that both PAI-1 and uPAR bind to the SMB domain of VN. Mutagenesis studies further demonstrate that these sites are overlapping but distinct (Fig. 7). In this regard, the polar/hydrophobic residues shown to be critical for uPAR binding (Fig. 7) were also critical for PAI-1 binding (Deng et al., 1996). However, when we directly compared the critical residues in SMB for PAI-1-binding (Deng et al., 1996) with those for uPAR-binding (Fig. 7), we noted one important difference: Glu23 was necessary for uPAR binding but not for PAI-1 binding. This difference may indicate that uPAR requires more negatively charged residues in SMB for binding than are required by PAI-1. These results indicate that the sequence in SMB for uPAR binding is very similar to, but not identical with, the PAI-1 binding sequence.

Based on these observations, we propose that PAI-1 plays a central regulatory role in uPAR mediated cell adhesion (Fig. 8). The three key features of this model are that uPA stimulates uPAR-dependent cell adhesion (Waltz et al., 1993), that uPAR binds to the SMB domain of VN (Figs. 5–7), and that PAI-1 also binds to SMB but with considerably higher affinity than does uPAR. This difference in affinity suggests that the interactions that govern cell adhesion and release will be regulated by the concentration of active PAI-1 available. According to this model (Fig. 8), cells will be nonadherent in the absence of either uPA or uPAR. Stimulation of cells in any way that causes the expression of both uPA and uPAR (e.g., with TGFβ1 and Vitamin D3) will lead to the binding of uPA to cell-associated uPAR, an interaction that alters the conformation of uPAR (Ploug et al., 1994) and promotes its binding to the SMB domain of VN in the ECM. These changes, which lead to the formation of stable attachments, will occur only when uPAR is available and uPA is present in excess over PAI-1. Consistent with this idea,

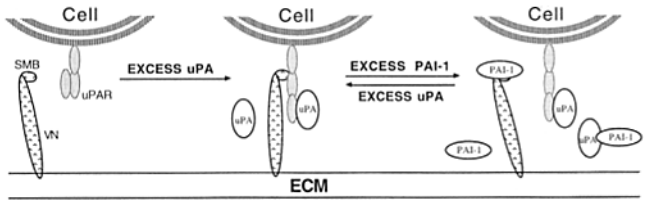

Figure 8. Model for the regulation of uPAR dependent cell adhesion and release by PAI-1 and uPA.

Western blot analysis of SDS-extracts of TGFβ1/Vitamin D3 treated adherent U937 cells revealed the presence of 15 ng of uPA per 2.5×10^5 cells and undetectable (i.e., less than 1 ng) of PAI-1 (data not shown). If these cells are now stimulated or otherwise altered to produce excess PAI-1, the inhibitor should compete with and displace uPAR from its association with SMB, releasing the cells from their VN substratum. This idea is supported by the demonstration that low concentrations of PAI-1 release U937 cells from their attachment to VN (Fig. 3). Interestingly, U937 cells attached to VN in this way cannot be released by EDTA or an RGD peptide (i.e., GRGDSP), suggesting that integrins are not involved in this process. Finally, this adhesive process is expected to be fully reversible, and excess uPA will again promote cell adhesion and invasion (Figs. 3 and 8).

This model is useful because it provides a potential mechanism and testable hypothesis to account for the unexpected observation that PAI-1 is an independent marker for poor survival in many cancers (Pappot et al., 1995). It may also help us to understand the findings of Quax et al. (1991) who noted that metastasis of human melanoma cells correlated with PAI-1 (Quax et al., 1991), and of Sier et al. (1994) who observed increased levels of PAI-1 in colorectal cancer liver metastasis (Sier et al., 1994). In these examples, the PAI-1–mediated release of cells from VN would be expected to promote the dissemination of these cells to distant tissue sites. The possibility that PAI-1 also influences the migration/invasion of these cells remains to be determined. In this regard, it should be noted that if the bound uPA is in its proenzyme form, PAI-1 will not be able to bind to and inactivate it (Andreasen et al., 1986). The released cells will be fully armed with pro-uPA and therefore with the potential protease activity necessary to invade and remodel the ECM.

This demonstration that PAI-1 regulates cell adhesion and release, and that it does so by binding to VN and not by inhibiting its natural substrate (uPA), sets it apart from other serine protease inhibitors (serpins). PAI-1 also differs from other serpins because it is a trace protein in plasma, it has a relatively short half-life (i.e., 10 min), and its biosynthesis is rapidly stimulated by a variety of inflammatory mediators, growth factors, and hormones (for reviews see Loskutoff, 1991; Van Meijer and Pannekoek, 1995). Moreover, PAI-1 is an immediate-early gene (Prendergast and Cole, 1989) and has been shown to accumulate at focal points of adhesion (Ciambrone and McKeown-Longo, 1992). The short half-life and ability to be rapidly and dramatically upregulated, are the expected properties of molecules that have the potential to rapidly initiate or terminate biological processes (i.e., molecular switches). Thus, by regulating the production rate and/or activity of PAI-1, cells may be able to control their adhesiveness and movement.

We thank Drs. L. Miles and S. Hawley for helpful discussion, Drs. E. Madison and S. Shattil for critical reading of this manuscript, Dr. J. Wang for ATF, and M. McRae and J. Lapan for excellent secretarial assistance.

This work was supported in part by grant HL31950 to D.J. Loskutoff from the National Institutes of Health. G. Deng is a recipient of National Research Service Award HL09400-01, and S. Wang was supported by Training Grant HL07195-19.

Received for publication 13 May 1996 and in revised form 5 July 1996.

References

Andreasen, P.A., L.S. Nielsen, P. Kristensen, J. Grondahl-Hansen, L. Skriver, and K. Dano. 1986. Plasminogen activator inhibitor from human fibrosarcoma cells binds urokinase-type plasminogen activator, but not its proenzyme. *J. Biol. Chem.* 261:7644–7651.

Behrendt, N., E. Ronne, and K. Dano. 1995. The structure and function of the urokinase receptor, a membrane protein governing plasminogen activation on the cell surface. *Biol. Chem. Hoppe-Seyler.* 376:269–279.

Bernfield, M.R., M.T. Hinkes, and R.L. Gallo. 1993. Developmental expression of the syndecans: possible function and regulation. *Development.* 119:205–212.

Boukerche, H., M. Benchaibi, O. Berthier-Vergnes, G. Lizard, M. Bailly, and J.L. McGregor. 1994. Two human melanoma cell-line variants with enhanced in vivo tumor growth and metastatic capacity do not express the β 3 integrin subunit. *Eur. J. Biochem.* 220:485–491.

Chen, W.T. 1992. Membrane proteases: roles in tissue remodeling and tumour invasion. *Curr. Opin. Cell Biol.* 4:802–809.

Ciambrone, G.J., and P.J. McKeown-Longo. 1992. Vitronectin regulates the synthesis and location of urokinase-type plasminogen activator in HT-1080 cells. *J. Biol. Chem.* 267:13617–13622.

Cohen, R.L., X.-P. Xi, C.W. Crowley, B.K. Lucas, A.D. Levinson, and M.A. Shuman. 1991. Effects of urokinase receptor occupancy on plasmin generation and proteolysis of basement membrane by human tumor cells. *Blood.* 78:479–487.

Cubellis, M.V., P. Andreasen, P. Ragno, M. Mayer, K. Dano, and F. Blasi. 1989. Accessibility of receptor-bound urokinase to type-1 plasminogen activator inhibitor. *Proc. Natl. Acad. Sci. USA.* 86:4828–4832.

Cubellis, M.V., T.-C. Wun, and F. Blasi. 1990. Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1079–1085.

Dano, K., P.A. Andreasen, J. Grondahl-Hansen, P. Kristensen, L.S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139–266.

Declerck, P.J., M. De Mol, M.C. Alessi, S. Baudner, E.P. Paques, K.T. Preissner, G.M. Berhaus, and D. Collen. 1988. Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. *J. Biol. Chem.* 30:15454–15461.

Del Vecchio, S., M.P. Stoppelli, M.V. Carriero, R. Fonti, O. Massa, P.Y. Li, G. Botti, M. Cerra, G. D'Aiuto, G. Esposito, and M. Salvatore. 1993. Human urokinase receptor concentration in malignant and benign breast tumors by *in vitro* quantitative autoradiography: comparison with urokinase levels. *Cancer Res.* 53:3198–3206.

Delbaldo, C., M. Cunningham, J.-D. Vassalli, and A.-P. Sappino. 1995. Plasmin-catalyzed proteolysis in colorectal neoplasia. *Cancer Res.* 55:4688–4695.

Deng, G., G. Royle, D. Seiffert, and D. Loskutoff. 1995. The PAI-1/vitronectin interaction: two cats in a bag? *Thromb. Haemostasis.* 74:66–70.

Deng, G., G. Royle, S. Wang, K. Crain, and D.J. Loskutoff. 1996. Structural and functional analysis of the PAI-1 binding motif in the somatomedin B domain of vitronectin. *J. Biol. Chem.* 271:12716–12723.

Estreicher, A., J. Mühlhauser, J.-L. Carpentier, L. Orci, and J.-D. Vassalli. 1990. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. *J. Cell Biol.* 111:783–792.

Gladson, C.L., V. Pijuan-Thompson, M.A. Olman, G.Y. Gillespie, and I.Z. Yacoub. 1995. Up-regulation of urokinase and urokinase receptor genes in malignant astrocytoma. *Am. J. Pathol.* 146:1150–1160.

Goodson, R.J., M.V. Doyle, S.E. Kaufman, and S. Rosenberg. 1994. High-affinity urokinase receptor antagonists identified with bacteriophage peptide display. *Proc. Natl. Acad. Sci. USA.* 91:7129–7133.

Grondahl-Hansen, J., I.J. Christensen, C. Rosenquist, N. Brunner, H.T. Mouridsen, K. Dano, and M. Blichert-Toft. 1993. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. *Cancer Res.* 53:2513–2521.

Gumbiner, B.M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell Press.* 84:345–357.

Hauptmann, S., C. Denkert, H. Lohrke, L. Tietze, S. Ott, B. Klosterhalfen, and C. Mittermayer. 1995. Integrin expression on colorectal tumor cells growing as monolayers, as multicellular tumor spheroids, or in nude mice. *Int. J. Can-*

cer. 61:819–825.

He, C.S., S.M. Wilhelm, A.P. Pentland, B.L. Marmer, G.A. Grant, A.Z. Eisen, and G.I. Goldberg. 1989. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. *Proc. Natl. Acad. Sci. USA.* 86:2632–2636.

Heiss, M.M., R. Babic, H. Allgayer, K.U. Greutzner, K.-W. Jauch, U. Loehrs, and F.W. Schildberg. 1995. Tumor-associated proteolysis and prognosis: new functional risk factors in gastric cancer defined by the urokinase-type plasminogen activator system. *J. Clin. Oncol.* 13:2084–2093.

Howlett, A.R., N. Bailey, C. Damsky, O.W. Petersen, and M.J. Bissell. 1995. Cellular growth and survival are mediated by β 1 integrins in normal human breast epithelium but not in breast carcinoma. *J. Cell Sci.* 108:1945–1957.

Huarte, J., D. Belin, and J.-D. Vassalli. 1985. Plasminogen activator in mouse and rat oocytes: induction during meiotic maturation. *Cell.* 43:551–558.

Hynes, R.O., and A.D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell.* 68:303–322.

Jenne, D., and K.K. Stanley. 1985. Molecular cloning of S-protein, a link between complement, coagulation and cell-substrate adhesion. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3153–3157.

Kaufman, S.E., S. Brown, and G.B. Stauber. 1993. Characterization of ligand binding to immobilized biotinylated extracellular domains of three growth factor receptors. *Anal. Biochem.* 211:261–266.

Knudsen, B.S., P.C. Hapel, and R.L. Nachman. 1987. Plasminogen activator inhibitor is associated with the extracellular matrix of cultured bovine smooth muscle cells. *J. Clin. Invest.* 80:1082–1089.

Kobayashi, H., S. Fujishiro, and T. Terao. 1994. Impact of urokinase-type plasminogen activator and its inhibitor type 1 on prognosis in cervical cancer of the uterus. *Cancer Res.* 54:6539–6548.

Kruithof, E.K.O. 1988. Plasminogen activator inhibitors—a review. *Enzyme.* 40:113–121.

Kuhn, W., L. Pache, B. Schmalfeldt, P. Dettmar, M. Schmitt, F. Janicke, and H. Graeff. 1994. Urokinase (uPA) and PAI-1 predict survival in advanced ovarian cancer patients (FIGO III) after radical surgery and platinum-based chemotherapy. *Gynecol. Oncol.* 55:401–409.

Liu, G., M.A. Shuman, and R.L. Cohen. 1995. Co-expression of urokinase, urokinase receptor and PAI-1 is necessary for optimum invasiveness of cultured lung cancer cells. *Int. J. Cancer.* 60:501–506.

Loskutoff, D.J. 1991. Regulation of PAI-1 gene expression. *Fibrinolysis.* 5:197–206.

Mignatti, P., and D.B. Rifkin. 1993. Biology and biochemistry of proteinases in tumor invasion. *Physiol. Rev.* 73:161–195.

Mimuro, J., and D.J. Loskutoff. 1989. Binding of type 1 plasminogen activator inhibitor to the extracellular matrix of cultured bovine endothelial cells. *J. Biol. Chem.* 264:5058–5063.

Nekarda, H., M. Schmitt, K. Ulm, A. Wenninger, H. Vogelsang, K. Becker, J.D. Roder, U. Fink, and J.R. Siewert. 1994. Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer. *Can. Res.* 54:2900–2907.

Nesbit, M., and M. Herlyn. 1994. Adhesion receptors in human melanoma progression. *Invasion Metastasis.* 14:131–146.

Pappot, H., H. Gårdsvoll, J. Romer, A. Navrsted Pedersen, J. Grondahl-Hansen, C. Pyke, and N. Brünner. 1995. Plasminogen activator inhibitor type 1 in cancer: therapeutic and prognostic implications. *Biol. Chem. Hoppe-Seyler.* 376:259–267.

Pedersen, H., N. Brunner, D. Francis, K. Osterlind, E. Ronne, H.H. Hansen, K. Dano, and J. Grondahl-Hansen. 1994. Prognostic impact of urokinase, urokinase receptor, and type 1 plasminogen activator inhibitor in squamous and large cell lung cancer tissue. *Cancer Res.* 54:4671–4675.

Ploug, M., E. Ronne, N. Behrendt, A.L. Jensen, F. Blasi, and K. Dano. 1991. Cellular receptor for urokinase plasminogen activator: carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. *J. Biol. Chem.* 266:1926–1933.

Ploug, M., V. Ellis, and K. Dano. 1994. Ligand interaction between urokinase-type plasminogen activator and its receptor probed with 8-Anilino-1-naphthalenesulfonate. Evidence for a hydrophobic binding site exposed only on the intact receptor. *Biochemistry.* 33:8991–8997.

Plow, E.F., T. Herren, A. Redlitz, L.A. Miles, and J.L. Hoover-Plow. 1995. The cell biology of the plasminogen system. *FASEB (Fed. Am. Soc. Exp. Biochem.) J.* 9:939–945.

Prendergast, G.C., and M.D. Cole. 1989. Posttranscriptional regulation of cellular gene expression by the c-myc oncogene. *Mol. Cell. Biol.* 9:124–134.

Quax, P.H.A., G.N.P. van Muijen, E.J.D. Weening-Verhoeff, L.R. Lund, K. Dano, D.J. Ruiter, and J.H. Verheijen. 1991. Metastatic behavior of human melanoma cell lines in nude mice correlated with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. *J. Cell Biol.* 115:191–199.

Roldan, A.L., M.V. Cubellis, M.T. Masucci, N. Behrendt, L.R. Lund, K. Dano, E. Appella, and F. Blasi. 1990. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:467–474.

Rouslahti, E. 1994. Fibronectin and its $\alpha_5\beta_1$ integrin receptor in malignancy. *Invasion Metastasis.* 14:87–97.

Roussel, E., M.C. Gingras, J.Y. Ro, C. Branch, and J.A. Roth. 1994. Loss of α 1 β 1 and reduced expression of other β 1 integrins and CAM in lung adenocarcinoma compared with pneumocytes. *J. Surg. Oncol.* 56:198–208.

Salonen, E., A. Vaheri, J. Pollanen, R. Stephens, P. Andreasen, M. Mayer, K. Dano, J. Gailit, and E. Ruoslahti. 1989. Interaction of plasminogen activator inhibitor (PAI-1) with vitronectin. *J. Biol. Chem.* 264:6339–6343.

Seiffert, D., G. Ciambrone, N.V. Wagner, B.B. Binder, and D.J. Loskutoff. 1994. The somatomedin B domain of vitronectin: structural requirements for the binding and stabilization of active type 1 plasminogen activator inhibitor. *J. Biol. Chem.* 269:2659–2666.

Seiffert, D., and D.J. Loskutoff. 1991*a*. Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin B domain of vitronectin. *J. Biol. Chem.* 266:2824–2830.

Seiffert, D., and D.J. Loskutoff. 1991*b*. Kinetic analysis of the interaction between type 1 plasminogen activator inhibitor and vitronectin and evidence that the bovine inhibitor binds to a thrombin-derived amino-terminal fragment of bovine vitronectin. *Biochim. Biophys. Acta.* 1078:23–30.

Sier, C.F.M., H.J.M. Vloedgraven, S. Ganesh, G. Griffioen, P.H.A. Quax, J.H. Verheijen, G. Dooijewaard, K. Welvaart, C.J.H. Van de Velde, C.B.H.W. Lamers, et al. 1994. Inactive urokinase and increased levels of its inhibitor type 1 in colorectal cancer liver metastasis. *Gastroenterology.* 107:1449–1456.

Stratton-Thomas, J.R., H.Y. Min, S.E. Kaufman, C.Y. Chiu, G.T. Mullenbach, and S. Rosenberg. 1995. Yeast expression and phagemid display of the human urokinase plasminogen activator epidermal growth factor-like domain. *Protein Engineering.* 8:463–470.

Van Meijer, M., and H. Pannekoek. 1995. Structure of plasminogen activator inhibitor 1 (PAI-1) and its function in fibrinolysis: an update. *Fibrinolysis.* 9: 263–276.

Vassalli, J.D., A. Sappino, and D. Belin. 1991. The plasminogen activator/plasmin system. *J. Clin. Invest.* 88:1067–1072.

Waltz, D.A., and H.A. Chapman. 1994. Reversible cellular adhesion to vitronectin linked to urokinase receptor occupancy. *J. Biol. Chem.* 269:14746–14750.

Waltz, D.A., L.Z. Sailor, and H.A. Chapman. 1993. Cytokines induce urokinase-dependent adhesion of human myeloid cells; A regulatory role for plasminogen activator inhibitors. *J. Clin. Invest.* 91:1541–1552.

Wei, Y., D.A. Waltz, N. Rao, R.J. Drummond, S. Rosenberg, and H.A. Chapman. 1994. Identification of the urokinase receptor as an adhesion receptor for vitronectin. *J. Biol. Chem.* 269:32380–32388.

Wiman, B., A. Almquist, O. Sigurdardottir, and T. Lindahl. 1988. Plasminogen activator inhibitor 1 (PAI) is bound to vitronectin in plasma. *FEBS Lett.* 242: 125–128.

Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281–292.