Int. J. Cancer: **71,** 116–122 (1997)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

MELANOMA CELL MIGRATION ON VITRONECTIN: REGULATION BY COMPONENTS OF THE PLASMINOGEN ACTIVATION SYSTEM

Andreas STAHL and Barbara M. MUELLER*

Department of Immunology, Scripps Research Institute, La Jolla, CA

Tumor cell migration and invasion require complex interactions between tumor cells and the surrounding extracellular matrix. These interactions are modified by cell adhesion receptors, as well as by proteolytic enzymes and their receptors. Here, we study the influence of the protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR) on melanoma cell adhesion to, and migration on, the extracellular matrix protein vitronectin (VN). Cell adhesion to VN, but not to type I collagen, is significantly enhanced in the presence of either uPA or its amino-terminal fragment (ATF). Soluble uPAR can inhibit this effect, indicating that uPA/uPAR on melanoma cells can function as a VN receptor. In the absence of bivalent cations, uPA/uPAR can promote cell attachment on VN, but not cell spreading, suggesting that the glycosylphosphatidylinositol (GPI)-anchored uPAR alone is unable to organize the cytoskeleton. Chemotactic melanoma cell migration on a uniform VN matrix is inhibited by uPA and ATF, implying that cell motility decreases when uPA/uPAR acts as a VN receptor. In contrast, plasminogen activator inhibitor 1 (PAI-1) can stimulate melanoma cell migration on VN, presumably by inhibiting uPA/uPAR-mediated cell adhesion to VN and thereby releasing the inhibition of cell migration induced by uPA. Together, our data implicate components of the plasminogen activation system in the direct regulation of cell adhesion and migration, thereby modulating the behavior of malignant tumor cells. ***Int. J. Cancer,*** **71:116–122, 1997.**
© 1997 Wiley-Liss, Inc.

Invasion and metastasis are hallmarks of tumor progression and depend on the ability of tumor cells both to degrade and to migrate through connective tissue barriers. Malignant cells, in order to become invasive, acquire and utilize a repertoire of matrix-degrading enzymes, as well as adhesion receptors, particularly of the integrin type (Mignatti and Rifkin, 1993; Ruoslahti, 1992; Ashkenas *et al.,* 1994). Although specific molecular components involved in cell migration and invasion have been identified, there is currently only a limited understanding as to the integration of different cellular functions that influence cell behavior and produce the malignant phenotype.

One proteolytic system that has been implicated in normal and pathological processes involving cell invasion is the plasminogen activation cascade (Danø *et al.,* 1985; Vassalli *et al.,* 1991). In this system, the serine protease urokinase-type plasminogen activator (uPA) converts the ubiquitous proenzyme plasminogen into plasmin which, in turn, can degrade a wide range of glycoproteins. Plasminogen activation is thought to occur on the cell surface, where a specific uPA receptor localizes and enhances uPA activity (Behrendt *et al.,* 1995) and also mediates the endocytosis of uPA complexed to specific inhibitors (Conese and Blasi, 1995).

Components of the plasminogen activation system, including uPA, its serpin-type inhibitor, PAI-1, and its cellular receptor, uPAR, are expressed by tumor and stroma cells in various malignancies. In human breast cancer tissues, for example, free and cell surface-bound uPA is associated with tumor cells and fibroblasts (Carriero *et al.,* 1994), uPAR is expressed by tumor cells, macrophages and endothelial cells (Bianchi *et al.,* 1994) and PAI-1 by tumor cells, fibroblasts, macrophages and endothelial cells (Bianchi *et al.,* 1995). A correlation between the expression of components of the uPA system and tumor progression has been described for cutaneous melanoma, where the expression of uPA, uPAR, PAI-1 and PAI-2 is not detectable in benign or early stages, but appears frequently in advanced primary and metastatic tumors (DeVries *et al.,* 1994).

Inhibiting uPA's proteolytic activity with function-blocking antibodies (Ossowski and Reich, 1983; Hearing *et al.,* 1988) or altering the proteolytic balance of tumor cells by overexpression of specific inhibitors (Mueller *et al.,* 1995; Soff *et al.,* 1995) can inhibit tumor growth, invasion and metastasis. Similarly, strategies that prevent uPA from binding to uPAR also significantly reduce tumor angiogenesis, growth, invasion and metastasis (Crowley *et al.,* 1993; Kook *et al.,* 1994; Min *et al.,* 1996). Together, these data imply a critical role for uPA and uPAR in the biology of malignant tumors.

Vitronectin (VN) is a common component of the extracellular matrix, particularly the basement membrane (Preissner, 1991), and has also been demonstrated in the matrix of human tumors (Gladson and Cheresh, 1991). Cells adhere to VN primarily by using VN receptors of the integrin type, particularly $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Gladson and Cheresh, 1994). Expression of the integrin $\alpha_v\beta_3$ has been also linked to malignant progression of melanoma (Albelda *et al.,* 1990), and metastatic melanoma cells coordinately express $\alpha_v\beta_3$ and uPAR (Nip *et al.,* 1995). uPA/uPAR has been described as modulating the interactions between cells and VN-containing matrices. For example, on FG pancreatic carcinoma cells, $\alpha_v\beta_5$ is not sufficient to support haptotactic migration on VN; rather, these cells need uPAR expression and occupancy with catalytically active uPA to migrate on a VN gradient (Yebra *et al.,* 1996). Further, uPA/uPAR colocalizes with the integrin $\alpha_v\beta_5$ on HaCaT keratinocytes adhering to VN, and uPA-mediated activation of plasminogen leads to an abrogation of VN adhesion of these cells, presumably by degradation of the integrin binding site on VN (Reinartz *et al.,* 1995). In contrast, cytokine-stimulated U937 monocytic cells do not adhere to VN, but become adhesive to this substrate in the presence of uPA or diisopropylfluorophosphate-inactivated uPA (Waltz *et al.,* 1993). Further investigation of the latter effect revealed the presence of a high-affinity (K_D approximately 30 nM) cation- and Arg-Gly-Asp-independent VN binding molecule on the surface of stimulated monocytic cells, the activity of which is tightly coupled to uPAR occupancy (Waltz and Chapman, 1994). This VN receptor was subsequently identified as uPAR (Wei *et al.,* 1994). The observation that uPAR functions as a VN receptor only upon binding of uPA suggests that uPAR, like adhesion molecules of the integrin type (Schwartz *et al.,* 1995), exists in an active and inactive conformation with regard to VN binding (Wei *et al.,* 1994). An additional modulator of uPA/uPAR-mediated adhesion to VN is PAI-1, which downregulates uPA-

Abbreviations: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VN, vitronectin; ATF, amino-terminal fragment of uPA; GPI, glycosylphosphatidylinositol; PAI-1, type-1 plasminogen activator inhibitor; FBS, fetal bovine serum; MDCM, matrix-dependent chemotactic migration.

Contract grant sponsor: National Institutes of Health, contract grant number CA59692; Contract grant sponsor: American Cancer Society.

*Correspondence to: Barbara M. Mueller, Department of Immunology, Scripps Research Institute, 10550 N. Torrey Pines Road, IMM13, La Jolla, CA 92037.

Received 5 November 1996

dependent adhesion of U937 myeloid cells to VN (Waltz *et al.*, 1993). Here, we report on the effect of uPA on the interactions between tumor cells and a VN matrix, utilizing a human melanoma cell line that expresses high levels of uPAR. We implicate uPA/uPAR as a VN adhesion receptor on human melanoma cells and further demonstrate that uPA/uPAR, acting as an adhesion receptor, inhibits chemotactic melanoma cell migration on a VN matrix. Finally, we demonstrate that PAI-1 can overcome the uPA/uPAR-mediated inhibition of chemotactic migration, thus implying a promigratory role for PAI-1.

MATERIAL AND METHODS

Cells and cell culture

The human melanoma cell line M24met has been described previously (Stahl and Mueller, 1994). M24met cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were passaged using versene (0.5 mM EDTA, 0.15 M NaCl and 20 mM HEPES).

Matrix proteins, enzymes and antibodies

Urea-purified VN was prepared from human plasma following a standard protocol (Yatahogo *et al.*, 1988). Type I collagen was purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant human single-chain uPA and the purified amino-terminal fragment (ATF) of human uPA were kind gifts of Drs. J. Henkin and B. Credo (Abbott Laboratories, Abbott Park, IL). Soluble recombinant uPAR was generously provided by Dr. S. Rosenberg (Chiron, Emeryville, CA). Recombinant human PAI-1 and an anti-catalytic anti-human uPA mouse monoclonal antibody (394) were purchased from American Diagnostica (Greenwich, CT).

Metabolic labeling of M24met cells

M24met cells were harvested with versene, and approximately 5×10^6 cells were resuspended in a volume of 20 ml RPMI supplemented with 10% FBS and containing 200 µCi of ^{3}H-thymidine (Amersham, Arlington Heights, IL). Cells were placed onto tissue culture plastic and allowed to grow overnight. Prior to the assay, the radioactive supernatant was removed, and the cell monolayers were rinsed several times and harvested with versene. β-scintillation counting of defined cell numbers indicated an average specific labeling of 0.5–1.0 cpm/cell.

Matrix-dependent chemotactic migration (MDCM) assay

We developed an assay to measure the influence of uPA on the directed migration of cells in the presence of a uniform matrix. The assay was performed in modified Boyden chambers (tissue culture-treated, 6.5-mm diameter, 8-µm pore size Transwell chambers from Corning-Costar, Cambridge, MA). The upper and lower sides of the polycarbonate membranes in the Transwell chambers were coated with 100 µl of a 10-µg/ml solution of VN or collagen type I in Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.8). After 1 hr incubation at room temperature, excess matrix solution was aspirated from the upper and lower sides of the membrane. RPMI with 10% FBS (500 µl) was added to the lower chamber as a chemo-attractant. ^{3}H-thymidine-labeled M24met melanoma cells in RPMI without FBS were admixed with uPA and other proteins, as indicated, and the cell density was adjusted to 100,000 cells/ml. Cell suspensions (100 µl) were transferred into the upper compartments of the chambers and incubated overnight in a humidified CO_2-incubator at 37°C. Cells were harvested separately from the upper and lower sides of the membrane using trypsin/versene (Biowhittaker, Walkersville, MD) at 37°C. Radioactivity was measured in a β-scintillation counter. The percentage of migration was calculated for each well as the ratio between cpm in the lower compartment and total cpm, and then averaged for quadruplicate samples. For MDCM of untreated M24met cells on a VN matrix, we found that typically 6–8% of the cells migrated to the lower compartment within a 16-hr period.

Adhesion assay

Cell adhesion assays were performed in 96-well cluster plates that were coated for 1 hr at 37°C with 10 µg/ml VN or collagen type I in TBS and subsequently blocked with 3% BSA in TBS for 1 hr at 37°C. To determine nonspecific binding, wells were only blocked with 3% BSA. ^{3}H-thymidine-labeled M24met cells were suspended in either RPMI or in RPMI containing 3 mM EDTA, admixed with uPA or other proteins, as indicated, and the cell concentration was adjusted to 50,000 cells/ml. Cell suspensions (100 µl) were added to matrix protein-coated wells and incubated for 1 hr at 37°C in a humidified CO_2 incubator. Nonadherent cells were removed by gentle rinsing with either RPMI or RPMI with 3 mM EDTA. Adherent cells were quantified either by counting under an inverted microscope or lysed by freezing and thawing, and the radioactivity was determined by β-scintillation counting. Both quantification techniques gave comparable results. Data points were calculated from quadruplicate wells and expressed as the mean ± SD.

RESULTS

We previously showed that the human melanoma cell line M24met expresses a high number of uPAR molecules, and that uPA binding to its receptor can stimulate these cells to invade through and migrate toward reconstituted basement membrane (Matrigel) (Stahl and Mueller, 1994). uPA can specifically mediate the attachment of monocytic cells to the matrix protein VN (Waltz *et al.*, 1993) and uPAR, on these cells, can act as a uPA-dependent VN-receptor (Wei *et al.*, 1994). These findings prompted us to investigate the effects of uPA on melanoma cell adhesion and migration on VN. Initially, we used a standard adhesion assay in which ^{3}H-thymidine-labeled M24met cells in RPMI were allowed to adhere to VN-coated plastic for 1 hr. Under these conditions, M24met adhesion was >90% inhibited by a mixture of function-blocking antibodies against integrins $\alpha_v\beta_3$ and β_5 (data not shown). The adhesion of M24met cells to VN was significantly increased in the presence of uPA (Fig. 1), with the highest uPA concentration (100 nM) tested resulting in a >3-fold increase in cell adhesion.

Integrin function depends on the presence of bivalent cations (Loftus *et al.*, 1994). To characterize the uPA-dependent VN adhesion of M24met cells in the absence of functional integrins, we performed further adhesion assays in RPMI containing 3 mM

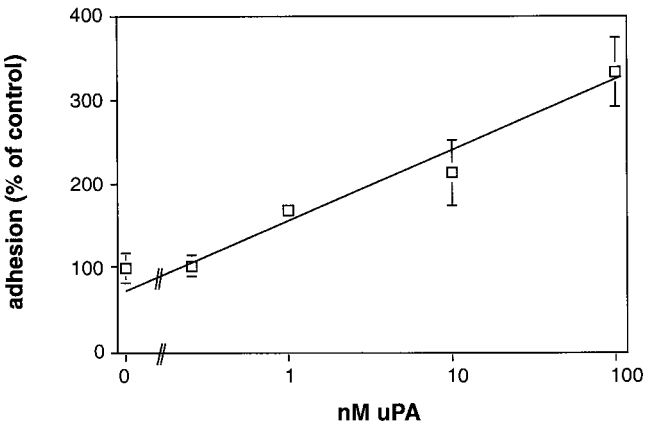

FIGURE 1 – Influence of uPA on M24met cell adhesion to VN. The adhesion of M24met cells in RPMI to plastic-bound VN was determined in the presence of different uPA concentrations (0, 0.1, 1, 10 and 100 nM). ^{3}H-thymidine-labeled cells were allowed to adhere for 1 hr at 37°C and adherent cells were quantified by determining the bound radioactivity by scintillation counting. Data points represent the mean of quadruplicate determinations (± SD) and are expressed as percentage of control.

EDTA. Under these conditions, M24met cells adhered to VN only in the presence of uPA (Table I). Soluble uPAR inhibited uPA-mediated cell adhesion to VN, indicating that uPA needs to bind to cell surface uPAR in order to promote cell adhesion (Table I). In contrast, uPA's catalytic activity was not required to mediate cell adhesion to VN. The amino-terminal fragment of uPA, that contains the uPAR-binding growth factor domain of uPA, but lacks the catalytic domain, was as potent as uPA in mediating M24met cell adhesion to VN. Similarly, an excess of an anti-catalytic anti-uPA antibody did not abolish the effect of uPA on M24met cell adhesion (Table I). These effects were specific for cell adhesion to VN, since M24met cell adhesion to type I collagen was not enhanced in the presence of uPA (Table I).

PAI-1 is a potent and specific inhibitor of uPA and is also known to bind to VN (DeClerck *et al.*, 1988; Mimuro and Loskutoff, 1989). Thus, adhesion assays were performed to test the effect of PAI-1 on uPA/uPAR-mediated M24met cell adhesion on VN. Equimolar concentrations of PAI-1 completely abolished the uPA effect and brought cell adhesion back to background values (Table I). The observation that PAI-1 is also able to abolish ATF-induced M24met cell adhesion to VN indicates that PAI-1 does not inhibit adhesion by virtue of being a uPA inhibitor, but rather by interfering directly with the molecular interaction between uPA/uPAR and VN.

Cell adhesion to components of the extracellular matrix can be viewed as a two-step process, *i.e.*, initial attachment to the substrate and subsequent cell spreading. We wanted to clarify whether uPA/uPAR could support one or both of these events in the absence of functional VN-specific integrins. To this end, M24met human melanoma cells in RPMI or RPMI/3 mM EDTA were allowed to adhere to VN-coated plastic for 1 hr in the presence or absence of 25 nM uPA. In the presence of bivalent cations, almost all cells were spread after 1 hr, regardless of the presence of uPA (Fig. 2). In RPMI/3 mM EDTA, the cells were unable to adhere to VN in the absence of exogenous uPA. In the presence of uPA, cells attached to the VN substrate, but did not spread (Fig. 2), even after prolonged incubation periods (up to 4 hr). Incubation of M24met cells for 4 hr in 3 mM EDTA did not affect cell viability. These data suggest that although uPA/uPAR can mediate attachment to VN, functional integrin-type VN receptors are necessary to mediate cell spreading.

Cell migration on extracellular matrix proteins is primarily a function of adhesion receptors of the integrin type. The demonstration that uPA/uPAR can act as a VN receptor on M24met cells led us to investigate the impact of uPA/uPAR on cell migration on VN-rich matrices. To this end, we used an assay that allowed us to test the effects of uPA on the chemotactic migration of cells in the presence of a specific matrix protein, and termed this a matrix-dependent chemotactic migration (MDCM) assay. For such MDCM assays, both sides of the polycarbonate filter in a Transwell chamber were coated with a matrix protein, *i.e.*, VN or collagen type I. FBS was added as a chemo-attractant to the lower chamber, and cells were allowed to migrate overnight from the upper to the lower compartment of the migration chamber. M24met cell migration in the MDCM assay on a VN matrix was integrin-dependent, since it was blocked by antibodies against the β_5, but not the β_3, subunit of integrins (data not shown). uPA at nanomolar concentrations significantly reduced the MDCM of M24met cells on a VN matrix (Fig. 3). The inhibition of MDCM by uPA required uPA binding to uPAR, since it could be overcome by an excess of soluble uPAR (Fig. 4). The uPA effect on MDCM was not observed on a collagen type I matrix and thus appeared to be specific for VN (Fig. 4). Together, these data indicate that MDCM was inhibited when uPAR contributed to cell adhesion.

Since PAI-1 was able to inhibit uPA/uPAR-mediated cell adhesion on VN, we also tested the effect of PAI-1 on chemotactic migration on a VN matrix and found that PAI-1 was able to overcome uPA-dependent inhibition of MDCM on VN. For example, 25 nM uPA caused a 50% inhibition of MDCM that was completely reversed by an equimolar concentration of PAI-1 (Fig. 5). ATF was as efficient as uPA in inhibiting M24met chemotactic migration on VN, and PAI-1 also reversed this ATF effect (Fig. 5). Thus, components of the plasminogen activation system profoundly influence chemotactic migration of M24met melanoma cells on a VN matrix. We demonstrate here that uPA binding to uPAR inhibits cell migration independent of uPA's proteolytic activity and rather depends on the ability of uPA/uPAR to function as a VN receptor. In the presence of uPA/uPAR, PAI-1 can stimulate melanoma cell migration on VN, presumably by releasing the inhibition of cell migration induced by uPA/uPAR.

TABLE I – uPA/uPAR-DEPENDENT ADHESION OF M24met CELLS IN THE ABSENCE OF BIVALENT CATIONS[1]

Matrix	Additive	Cells bound to matrix (cpm)
Vitronectin	None	95 ± 29
Vitronectin	25 nM uPA	2,984 ± 593
Vitronectin	25 nM uPA + 100 nM soluble uPAR	46 ± 18
Vitronectin	25 nM ATF	3,024 ± 200
Vitronectin	25 nM uPA + anti-uPA antibody[2]	3,630 ± 309
Vitronectin	25 nM uPA + 25 nM PAI-1	169 ± 17
Vitronectin	25 nM ATF + 25 nM PAI-1	147 ± 38
Collagen type I	None	38 ± 9
Collagen type I	25 nM uPA	59 ± 14
BSA	None	30 ± 13

[1]M24met cells (^{3}H-thymidine-labeled) were harvested in versene and resuspended in RPMI/3 mM EDTA containing uPA or other proteins, as indicated, and added to matrix protein-coated wells (5×10^3 cells/well). Cells were allowed to attach for 1 hr at 37°C and washed gently, and the number of attached cells was determined by β-scintillation counting. Data represent the mean and SD of quadruplicate determinations in a representative experiment. Similar results were obtained in independent experiments.–[2]Anti-catalytic monoclonal antibody 394 at 50 μg/ml.

DISCUSSION

We demonstrate here that uPA, uPAR and PAI-1 can profoundly influence M24met melanoma cell adhesion to and migration on VN. uPA mediates a concentration-dependent increase in M24met cell adhesion to VN that requires uPA binding to uPAR, but is independent of uPA's proteolytic activity. Furthermore, uPA induces cell adhesion that is specific for VN and independent of bivalent cations. These data indicate that uPA/uPAR can directly support adhesion to VN. This is in accordance with previously published data describing uPA/uPAR as a VN adhesion receptor on U937 cells (Waltz *et al.*, 1993; Wei *et al.*, 1994). Morphological examination of M24met melanoma cells showed that although uPA could mediate cell attachment to VN in the absence of divalent cations, uPA/uPAR complexes were unable to support cell spreading. Thus, the GPI-linked uPAR can immobilize cells on a VN matrix, but fails to organize the cytoskeleton. In the presence of bivalent cations, however, cell surface uPA has been found in HT 1080 fibrosarcoma cells adhering to VN to be focally localized (Ciambrone and McKeown-Longo, 1992) and to colocalize with the cytoskeletal protein vinculin (Pollanen *et al.*, 1988). Such observations suggest that cell adhesion to VN may involve a direct physical contact between uPAR and integrins that may connect the GPI-linked uPAR to the cytoskeleton. In fact, a direct interaction between uPAR and β_2 integrins has been implicated in the adhesion of monocytic cells to fibrinogen (Sitrin *et al.*, 1996). Moreover, it has been shown that in order to mediate VN adhesion, uPAR can interact with and modify the function of β_1 or β_2 integrins on different cell types (Wei *et al.*, 1996). We show here that M24met cell adhesion to a VN matrix, in the absence of bivalent cations, solely depends on uPA/uPAR. In the presence of functional integrin VN receptors, cell adhesion is a function of both uPA/uPAR and integrins, with the cytoplasmic portion of the integrins engaging the cytoskeleton and organizing focal contacts.

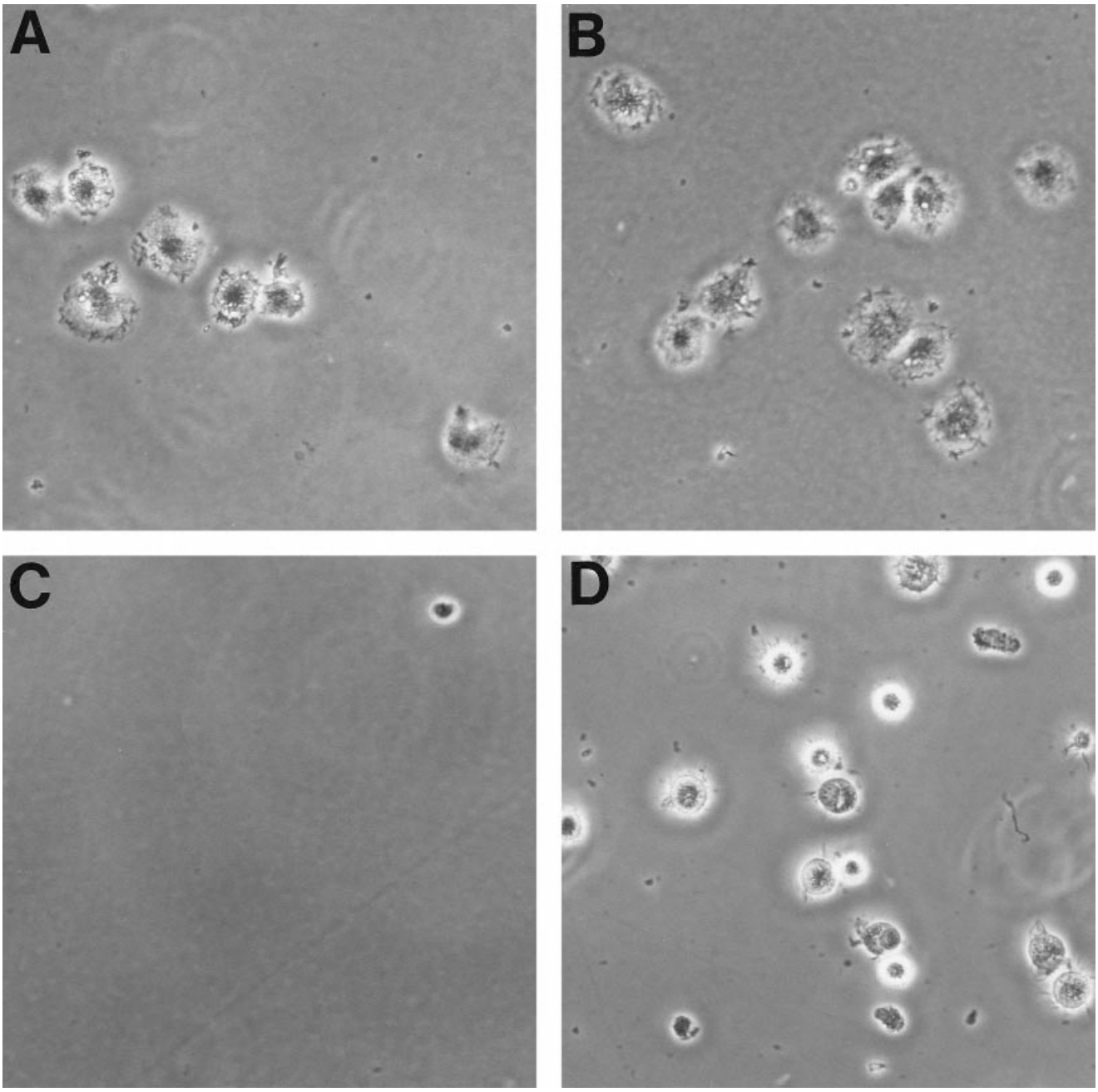

FIGURE 2 – Cell spreading of M24met cells in the presence or absence of divalent cations. M24met cells were resuspended either in RPMI or in RPMI with 3 mM EDTA, and 10^5 cells were placed in VN-coated six-well plates. After 1 hr incubation, wells were gently rinsed with the respective media. The morphology of untreated cells *(a, c)* or cells in the presence of 25 nM uPA *(b, d)* in RPMI *(a, b)* or in RPMI/3 mM EDTA *(c, d)* was determined by phase-contrast microscopy (×60 magnification).

The saturable uPA-dependent increase in cell adhesion to VN was paralleled by a decrease in chemotactic migration in the presence of a high and uniform concentration of VN. The inhibition of MDCM on VN was, like the uPA effect on VN adhesion, mediated by uPAR and specific for VN. Therefore, we suggest that the uPA-induced increase in cellular adhesiveness to VN is directly related to the reduced cell motility observed in the MDCM assay. This hypothesis is consistent with a model for cell migration that DiMilla *et al.* (1991) deduced from mathematical considerations, as well as from experimental data (DiMilla *et al.*, 1993). In this model, initial cell-substratum attachment strength is a central variable governing cell migration speed. Migration speed depends in a biphasic manner on attachment strength, with maximal migration at an intermediate level of cell adhesiveness. This implies that a significant increase in cell-substratum attachment strength would lead to a drastic reduction of migration speed at a given level of motile force. The mechanical strength of protein-protein bonds is logarithmically related to their biochemical affinities (Kuo and Lauffenburger, 1993). The affinity of uPA/uPAR for VN has been determined to be 30 nM (Wei *et al.*, 1994) and is thus equal to or higher than the affinity of integrin receptors for matrix components, which have been reported to range from 1 nM (Hu *et al.*, 1995) to approximately 1 μM (Akiyama and Yamada, 1985). Thus, engaging uPA/uPAR as an additional adhesion

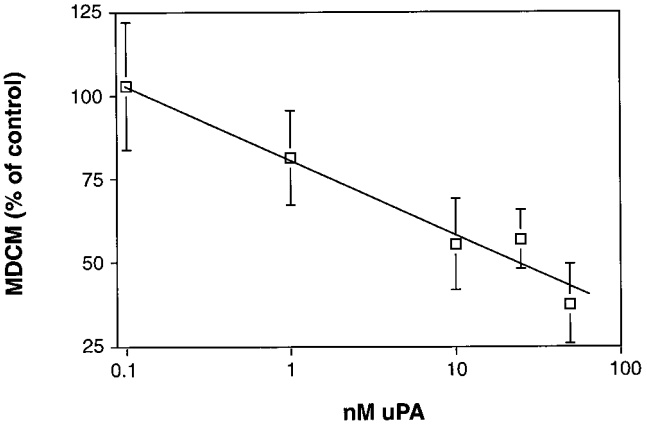

FIGURE 3 – Effect of uPA on the MDCM on a VN matrix. ^{3}H-thymidine-labeled M24met cells migrated overnight on a VN matrix in the presence of the indicated concentrations of uPA toward FBS as chemo-attractant. Cell migration was quantified, as described. Data points represent the mean of quadruplicate determinations (± SD) and are expressed as percentage of control.

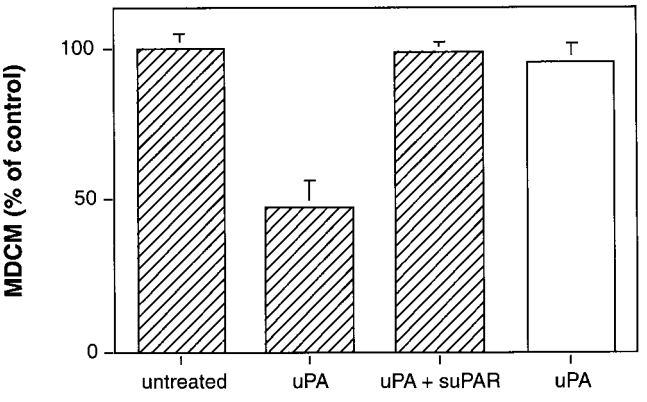

FIGURE 4 – Specificity of the uPA effect on MDCM. MDCM of ^{3}H-thymidine-labeled M24met cells was determined, as described. MDCM of untreated cells on VN *(a);* of cells in the presence of 25 nM uPA on VN *(b);* of cells in the presence of 25 nM uPA and 100 nM soluble recombinant uPAR *(c);* of cells in the presence of 25 nM uPA on collagen-type I. Data points represent the mean of quadruplicate determinations (± SD) and are expressed as percentage of control.

receptor should have a marked effect on the overall adhesion strength to VN and could therefore account for a pronounced inhibition of migration on this matrix component. Both predictions are in accordance with our findings that incubation of cells with uPA leads to a >3-fold increase in VN adhesion and to a 50% inhibition of MDCM on this substrate.

The plasminogen activation system has been previously implicated in cell motility. Polarization of cell surface uPAR has been described toward the leading edge of migrating monocytic cells (Gyetko *et al.,* 1994) and on migrating cells at the edge of wounded monolayers of endothelial cells (Pepper *et al.,* 1987) and smooth muscle cells (Okada *et al.,* 1995). In *in vitro* invasion models, cell migration requires the degradation of occluding extracellular matrix and in such models, invasion depends on regulated cell-surface-associated uPA activity (reviewed in Mignatti and Rifkin, 1993). In contrast, several models for chemotactic or haptotactic cell migration demonstrated that binding of catalytically active or inactive uPA to uPAR enhanced cell migration (Stahl and Mueller, 1994; Odekon *et al.,* 1992; Busso *et al.,* 1994), thus implicating the GPI-linked uPAR as a signal transducing receptor. Here, we describe an *in vitro* model, MDCM on a VN substrate, in which

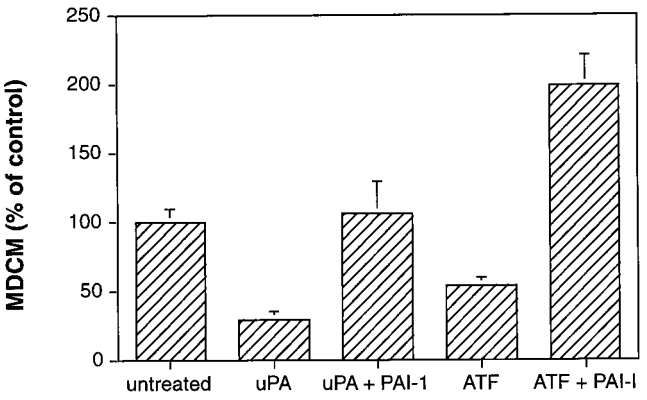

FIGURE 5 – Effects of PAI-1 on the MDCM of M24met cells on VN. MDCM on a VN matrix of ^{3}H-thymidine-labeled M24met cells was determined in the presence of 25 nM uPA, 25 nM ATF, 25 nM uPA + 25 nM PAI-1 or 25 nM ATF + 25 nM PAI-1, as described. Data points represent the mean of quadruplicate determinations (± SD) and are expressed as percentage of control.

uPA binding to uPAR inhibits cell motility by enhancing cell adhesion. It should be noted, however, that haptotactic migration of M24met cells toward VN or collagen type I, *i.e.,* migration toward a matrix gradient, rather than migration toward a chemotactic stimulus on a uniform VN matrix, was enhanced by uPA binding to uPAR (data not shown). Together, these data suggest that uPA/uPAR can modulate cell migration differentially depending on the microenvironment, *e.g.,* matrix composition and the stimuli that induce cell motility.

The specific uPA inhibitor PAI-1 did inhibit the uPA/uPAR-mediated adhesion to VN and overcame the uPA/uPAR-induced inhibition of MDCM on VN. We demonstrated also that cell adhesion through uPA/uPAR was independent of uPA's proteolytic activity. Therefore, the PAI-1 effect was apparently not mediated by inhibiting uPA, but rather by interfering directly with the molecular interaction between uPA/uPAR and VN. VN is a major binding protein for PAI-1 (DeClerck *et al.,* 1988; Mimuro and Loskutoff, 1989), as well as for uPA (Moser *et al.,* 1995), and based on this, several models can be proposed to explain the interaction between uPA, uPAR, PAI-1 and VN. uPA could function as a bridge between cell and matrix by binding to VN and uPAR simultaneously. Alternatively, as proposed by Wei *et al.* (1994), uPA could induce a change in uPAR, thus stabilizing an active VN binding conformation of uPAR. PAI-1 could interfere with these interactions either by inducing a rapid clearance of uPA from the cell surface (Conese and Blasi, 1995) or by competing with uPA or uPAR for a closely related binding site on VN. The fact that PAI-1 is unable to bind to ATF or ATF/uPAR complexes, but is able to overcome the ATF-mediated inhibition of MDCM on VN, makes the first explanation seem less likely. The PAI-1 binding site on VN has been localized to the amino-terminal somatomedian B domain (Seiffert *et al.,* 1994). It has been shown that soluble uPAR also binds to the somatomedian B domain and that, in fact, PAI-1 competes directly with uPAR binding (Deng *et al.,* 1996). These findings support the idea that in our model, PAI-1 interferes with uPA/uPAR-mediated cell adhesion to VN by competing for binding to VN.

Expression of uPA and uPAR has been demonstrated for many tumor types, and correlates with tumor progression and a poor prognosis for cancer patients (Duffy, 1996). This is consistent with the hypothesis that plasminogen activation contributes to the invasive phenotype of tumor cells. Paradoxically, expression of the uPA inhibitor, PAI-1, has been shown in breast cancer (Jaenicke *et al.,* 1994) and in other tumors (Pappot *et al.,* 1995) to correlate with a poor prognosis, which has been interpreted as evidence for a requirement of controlled plasminogen activation in the malignant process. The observation that *in vitro* PAI-1 prevents uPA/uPAR-mediated adhesion of tumor cells to VN and thereby leads to the

remobilization of uPAR-expressing tumor cells on VN-containing matrices suggests a proinvasive and prometastatic role for PAI-1 that warrants further investigation in preclinical tumor models.

In conclusion, our findings indicate that the uPA/uPAR system not only regulates pericellular proteolytic degradation of the extracellular matrix, but also integrates a complex network of different signals, such as matrix composition and uPA and PAI concentrations in order to regulate and direct cellular migration and thus invasion.

ACKNOWLEDGEMENTS

We thank Drs. B. Felding-Habermann, J. Smith and L. Miles for their helpful comments on this manuscript. This work was supported in part by a Junior Faculty Research Award to B.M.M. from the American Cancer Society. Data are part of the doctoral thesis submitted by A.S. to the University of Hamburg. This is the Scripps Research Institute's manuscript 10095-IMM.

REFERENCES

AKIYAMA, S.K. and YAMADA, K.M., Synthetic peptides competitively inhibit both direct binding to fibroblasts and functional biological assays for the purified cell-binding domain of fibronectin. *J. biol. Chem.,* **260,** 10402–10405 (1985).

ALBELDA, S.M., METTE, S.A., ELDER, D.E., STEWART, M., DAMJANOVICH, L., HERLYN, M. and BUCK, C.A., Integrin distribution in malignant melanoma: association of the β_3 subunit with tumor progression. *Cancer Res.,* **50,** 6757–6764 (1990).

ASHKENAS, J., DAMSKY, C.H., BISSELL, M.J. and WERB, Z., Integrins, signaling and the remodeling of the extracellular matrix. *In:* D.A. Cheresh and R.P. Mecham (eds.), *Integrins: molecular responses to the extracellular matrix,* pp. 79–109, Academic Press, New York (1994).

BEHRENDT, N., RØNNE, E. and DANØ, K., 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 (1995).

BIANCHI, E., COHEN, R.L., DAI, A., THOR, A.T., SHUMAN, M.A. and SMITH, H.S., Immunohistochemical localization of the plasminogen activator inhibitor-1 in breast cancer. *Int. J. Cancer,* **60,** 597–603 (1995).

BIANCHI, E., COHEN, R.L., THOR, A.T., TODD, R.F. III, MIZUKAMI, I.F., LAWRENCE, D.A., LJUNG, B.M., SHUMAN, M.A. and SMITH, H.S., The urokinase receptor is expressed in invasive breast cancer but not in normal breast tissue. *Cancer Res.,* **54,** 861–866 (1994).

BUSSO, N., MASUR, S.K., LAZEGA, D., WAXMAN, S. and OSSOWSKI, L., Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *J. Cell Biol.,* **126,** 259–270 (1994).

CARRIERO, M.V., FRANCO, P., DEL VECCHIO, S., MASSA, O., BOTTI, G., D'AIUTO, G., STOPPELLI, M.P. and SALVATORE, M., Tissue distribution of soluble and receptor-bound urokinase in human breast cancer using a panel of monoclonal antibodies. *Cancer Res.,* **54,** 5445–5454 (1994).

CIAMBRONE, G.J. and MCKEOWN-LONGO, P.J., Vitronectin regulates the synthesis and localization of urokinase-type plasminogen activator in HT-1080 Cells. *J. biol. Chem.,* **267,** 13617–13622 (1992).

CONESE, M. and BLASI, F., Urokinase/urokinase receptor system: internalization/degradation of urokinase-serpin complexes: mechanism and regulation. *Biol. Chem. Hoppe-Seyler,* **376,** 143–155 (1995).

CROWLEY, C.W., COHEN, R.L., LUCAS, B.K., LIU, G., SHUMAN, M.A. and LEVINSON, A.D., Prevention of metastasis by inhibition of the urokinase receptor. *Proc. nat. Acad. Sci. (Wash.),* **90,** 5021–5025 (1993).

DANØ, K., ANDREASEN, P.A., GRONDAHL-HANSEN, J., KRISTENSEN, P., NIELSEN, L.S. and SKRIVER, L., Plasminogen activators, tissue degradation and cancer. *Adv. Cancer Res.,* **44,** 139–266 (1985).

DECLERCK, P.J., DEMOL, M., ALESSI, M.C., BAUDNER, S., PAQUES, E.P., PREISSNER, K.T., MULLER-BERGHAUS, G. and COLLEN, D., Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). *J. biol. Chem.,* **263,** 15454–15461 (1988).

DENG, G., CURRIDEN, S.A., WANG, S., ROSENBERG, S. and LOSKUTOFF, D.J., Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? *J. Cell Biol.,* **134,** 1563–1571 (1996).

DEVRIES, T., QUAX, P.H., DENIJN, M., VERRIJP, K.N., VERHEIJEN, J.H., VERSPAGET, H.W., WEIDLE, U.H., RUITER, D.J. and VAN MUIJEN, G., Plasminogen activators, their inhibitors, and urokinase receptor emerge in late stages of melanocytic tumor progression. *Amer. J. Pathol.,* **144,** 70–81 (1994).

DIMILLA, P.A., BARBEE, K. and LAUFFENBURGER, D.A., Mathematical model for the effects of adhesion and mechanics on cell migration speed. *Biophys. J.,* **60,** 15–37 (1991).

DIMILLA, P.A., STONE, J.A., QUINN, J.A., ALBELDA, S.M. and LAUFFENBURGER, D.A., Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J. Cell Biol.,* **122,** 729–737 (1993).

DUFFY, M.J., Proteases as prognostic markers in cancer. *Clin. Cancer Res.,* **2,** 613–618 (1996).

GLADSON, C.L. and CHERESH, D.A., Glioblastoma expression of vitronectin and the $\alpha V\beta 3$ integrin. Adhesion mechanism for transformed glial cells. *J. clin. Invest.,* **88,** 1924–1932 (1991).

GLADSON, C.L. and CHERESH, D.A., The αV integrins. *In:* Y. Takada (ed.), *Integrins: the biological problem,* pp. 83–99, CRC Press, Boca Raton (1994).

GYETKO, M.R., TODD, R. III, WILKINSON, C.C. and SITRIN, R.G., The urokinase receptor is required for human monocyte chemotaxis *in vitro. J. clin. Invest.,* **93,** 1380–1387 (1994).

HEARING, V.J., LAW, L.W., CORTI, A., APPELLA, E. and BLASI, F., Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. *Cancer Res.,* **48,** 1270–1278 (1988).

HU, D.D., LIN, E.C., KOVACH, N.L., HOYER, J.R. and SMITH, J.W., A biochemical characterization of the binding of osteopontin to integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$. *J. biol. Chem.,* **270,** 26232–26238 (1995).

JAENICKE, F., PACHE, L., SCHMITT, M., ULM, K., THOMSSEN, C., PRECHTL, A. and GRAEFF, H., Both the cytosols and detergent extracts in breast cancer tissues are suited to evaluate the prognostic impact of the urokinase-type plasminogen activator and its inhibitor, plasminogen activator type I. *Cancer Res.,* **54,** 2527–2530 (1994).

KOOK, Y.H., ADAMSKI, J., ZELENT, A. and OSSOWSKI, L., The effect of antisense inhibition of urokinase receptor in human squamous cell carcinoma on malignancy. *EMBO J.,* **13,** 3983–3991 (1994).

KUO, S.C. and LAUFFENBURGER, D.A., Relationship between receptor/ligand binding affinity and adhesion strength. *Biophys. J.,* **65,** 2191–2200 (1993).

LOFTUS, J.C., SMITH, J.W. and GINSBERG, M.H., Integrin-mediated cell adhesion: the extracellular face. *J. biol. Chem.,* **269,** 25235–25238 (1994).

MIGNATTI, P. and RIFKIN, D.B. Biology and biochemistry of proteinases in tumor invasion. *Physiol. Rev.,* **73,** 161–195 (1993).

MIMURO, J. and LOSKUTOFF, D.J., Binding of type 1 plasminogen activator inhibitor to the extracellular matrix of cultured bovine endothelial cells. *J. biol. Chem.,* **264,** 5058–5063 (1989).

MIN, H.Y., DOYLE, L.V., VITT, C.R., ZANDONELLA, C.L., STRATTON-THOMAS, J., SHUHMAN, M.A. and ROSENBERG, S., Urokinase receptor antagonists inhibit angiogenesis and primary tumor growth in syngeneic mice. *Cancer Res.,* **56,** 2428–2433 (1996).

MOSER, T.L., ENGHILD, J.J., PIZZO, S.V. and STACK, M.S., Specific binding of urinary-type plasminogen activator (u-PA) to vitronectin and its role in mediating u-PA-dependent adhesion of U937 cells. *Biochem. J.,* **307,** 867–873 (1995).

MUELLER, B.M., YU, Y.B. and LAUG, W.E., Overexpression of plasminogen activator inhibitor 2 in human melanoma cells inhibits spontaneous metastasis in *scid/scid* mice. *Proc. nat. Acad. Sci. (Wash.),* **92,** 205–209 (1995).

NIP, J., RABBANI, S.A., SHIBATA, H.R. and BRODT, P., Coordinated expression of the vitronectin receptor and the urokinase-type plasminogen activator receptor in metastatic melanoma cells. *J. clin. Invest.,* **95,** 2096–2103 (1995).

ODEKON, L.E., SATO, Y. and RIFKIN, D.B., Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. *J. cell. Physiol.,* **150,** 258–263 (1992).

OKADA, S.S., TOMASZEWSKI, J.E. and BARNATHAN, E.S., Migrating vascular smooth muscle cells polarize cell surface urokinase receptors after injury *in vitro. Exp. Cell Res.,* **217,** 180–187 (1995).

OSSOWSKI, L. and REICH, E., Antibodies to plasminogen activator inhibit human tumor metastasis. *Cell,* **35,** 611–619 (1983).

PAPPOT, H., GARDSVOLL, H., ROMER, J., PEDERSEN, A.N., GRONDAHL-HANSEN, J., PYKE, C. and BRUNNER, N., Plasminogen activator inhibitor type 1 in cancer: therapeutic and prognostic implications. *Biol. Chem. Hoppe-Seyler,* **376,** 259–267 (1995).

PEPPER, M.S., VASSALLI, J.D., MONTESANTO, R. and ORCI, L., Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J. Cell Biol.,* **105,** 2535–2541 (1987).

POLLANEN, J., HEDMAN, K., NIELSEN, L.S., DANØ, K. and VAHERI, A., Ultrastructural localization of plasma membrane-associated urokinase-type plasminogen activator at focal contacts. *J. Cell Biol.,* **106,** 87–95 (1988).

PREISSNER, K.T., Structure and biological role of vitronectin. *Ann. Rev. Cell Biol.,* **7,** 275–310 (1991).

REINARTZ, J., SCHAFER, B., BATRLA, R., KLEIN, C.E. and KRAMER, M.D., Plasmin abrogates $\alpha_v\beta_5$-mediated adhesion of a human keratinocyte cell line (HaCaT) to vitronectin. *Exp. Cell Res.,* **220,** 274–282 (1995).

RUOSLAHTI, E., Control of cell motility and tumor invasion by extracellular matrix interactions. *Brit. J. Cancer,* **66,** 239–242 (1992).

SCHWARTZ, M.A., SCHALLER, M.D. and GINSBERG, M.H., Integrins: emerging paradigms of signal transduction. *Ann. Rev. Cell Dev. Biol.,* **11,** 549–599 (1995).

SEIFFERT, D., CIAMBRONE, G., WAGNER, N.V., BINDER, B.R. and LOSKUTOFF, D.J., 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 (1994).

SITRIN, R.G., TODD, R.F. III, PETTY, H.R., BROCK, T.G., SHOLLENBERGER, S.B., ALBRECHT, E. and GYETKO, M.R., The urokinase receptor (CD87) facilitates CD11b/CD18-mediated adhesion of human monocytes. *J. clin. Invest.,* **97,** 1942–1951 (1996).

SOFF, G.A., SANDEROWITZ, J., GATELY, S., VERRUSIO, E., WEISS, I., BREM, S. and KWAAN, H.C., Expression of plasminogen activator inhibitor type 1 by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis, and metastasis to lung and liver in an athymic mouse model. *J. clin. Invest.,* **96,** 2593–2600 (1995).

STAHL, A. and MUELLER, B.M., Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells *in vitro. Cancer Res.,* **54,** 3066–3071 (1994).

VASSALLI, J.D., SAPPINO, A.P. and BELIN, D., The plasminogen activator/plasmin system. *J. clin. Invest.,* **88,** 1067–1072 (1991).

WALTZ, D.A. and CHAPMAN, H.A., Reversible cellular adhesion to vitronectin linked to urokinase receptor occupancy. *J. biol. Chem.,* **269,** 14746–14750 (1994).

WALTZ, D.A., SAILOR, L.Z. and CHAPMAN, H.A., Cytokines induce urokinase-dependent adhesion of human myeloid cells. A regulatory role for plasminogen activator inhibitors. *J. clin. Invest.,* **91,** 1541–1552 (1993).

WEI, Y., LUKASHEV, M., SIMON, D.I., BODARY, S.C., ROSENBERG, S., DOYLE, M.V. and CHAPMAN, H.A., Regulation of integrin function by the urokinase receptor. *Science,* **273,** 1551–1555 (1996).

WEI, Y., WALTZ, D.A., RAO, N., DRUMMOND, R.J., ROSENBERG, S. and CHAPMAN, H.A., Identification of the urokinase receptor as an adhesion receptor for vitronectin. *J. biol. Chem.,* **269,** 32380–32388 (1994).

YATAHOGO, T., IZUMI, M., KASHIWAGI, H. and HAYASHI, M., Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell. Struct. Funct.,* **13,** 281–292 (1988).

YEBRA, M., PARRY, G.C.N., STROMBLAD, S., MACKMAN, N., ROSENBERG, S., MUELLER, B.M. and CHERESH, D.A., Requirement of receptor-bound urokinase-type plasminogen activator for integrin $\alpha_v\beta_5$-directed cell migration. *J. biol. Chem.,* **271,** 29393–29399 (1996).