Title: Plasmin-induced migration requires signaling through protease-activated receptor 1 and integrin α9β1.

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Abbreviations: ADAMs, a disintegrin and metalloprotease; BAE, bovine artery endothelial; CHO, Chinese hamster ovary; CPAE, calf pulmonary artery endothelial; EACA, ε-aminocaproic acid; EPCR, endothelial cell protein C receptor; GPCR, G protein-coupled receptors; mAb, monoclonal antibody; PAR, protease-activated receptor; VCAM-1, vascular cell adhesion molecule-1;
ABSTRACT

Plasmin is a major extracellular protease that elicits intracellular signals to mediate platelet aggregation, chemotaxis of peripheral blood monocytes, and release of arachidonate and leukotriene from several cell types in a G protein-dependent manner. Angiostatin, a fragment of plasmin(ogen), is a ligand and an antagonist for integrin \( \alpha_9 \beta_1 \). Here we report that plasmin specifically interacts with \( \alpha_9 \beta_1 \), and that plasmin induces migration of cells expressing recombinant \( \alpha_9 \beta_1 \) (\( \alpha_9 \)-CHO cells). Migration was dependent on an interaction of the kringle domains of plasmin with \( \alpha_9 \beta_1 \), as well as the catalytic activity of plasmin. Angiostatin, representing the kringle domains of plasmin, alone did not induce migration of \( \alpha_9 \)-CHO cells, but simultaneous activation of the G-protein coupled protease-activated receptor (PAR)-1 with an agonist peptide induced migration on angiostatin, while PAR-2 or PAR-4 agonist peptides were without effect. Furthermore, a small chemical inhibitor of PAR-1 (RWJ 58259) and a palmitoylated PAR-1-blocking peptide (pal1) inhibited plasmin-induced migration of \( \alpha_9 \)-CHO cells. These results suggest that plasmin induces migration by kringle-mediated binding to \( \alpha_9 \beta_1 \) and simultaneous proteolytic activation of PAR-1.
INTRODUCTION

Integrins are a family of heterodimeric cell adhesion receptors that regulate many critical cellular processes including cell migration, adhesion, signal transduction, and gene expression. By controlling such cellular behaviors, they play a role in mediating important biological functions such as embryonic development, differentiation, and wound healing, as well as pathological processes such as inflammation, angiogenesis and tumor progression. Integrin $\alpha_9\beta_1$ is constitutively expressed in liver, smooth and skeletal muscle, as well as in squamous and airway epithelium (1-3). $\alpha_9\beta_1$ is also expressed on neutrophils and is up-regulated after neutrophil activation (4,5). Its role in activated neutrophils may involve binding to vascular cell adhesion molecule (VCAM-1) on endothelial cells resulting in chemotaxis across endothelial monolayers by interaction with VCAM-1 during inflammation (5,6). $\alpha 9\beta 1$ recognizes many other ligands such as tenascin-C (7) and osteopontin (8), both extracellular matrix ligands, and ADAMs (a disintegrin and metalloproteases) (9,10). Thus, this integrin may potentially play a crucial role in inflammatory responses and metastasis as well as during development and wound healing.

Some coagulation cascade proteases play a role in wound healing, tissue remodeling, angiogenesis and metastasis, both by extracellular matrix degradation and by regulating cell migration and proliferation (11). One such protease, plasmin, is generated from its precursor plasminogen. Plasminogen is first converted to the two-chain serine protease plasmin by cleavage of a single Arg561-Val 562 peptide bond by urokinase-type plasminogen activator (uPA), and plasmin serves as both the substrate and enzyme for the generation of angiostatin (12). Angiostatin contains either the first three or first four kringle domains of plasminogen (13,14). After plasminogen is cleaved by uPA, the kringle domain of plasmin is still attached to the catalytic domain by inter-chain disulfide linkage.

It has been demonstrated in various systems that plasmin can activate signaling leading to protein phosphorylation, $Ca^{2+}$ mobilization, and activation of phospholipase C and protein kinase C (15-18). Plasmin has been shown to increase arachidonate release in bovine artery endothelial (BAE) cells and to trigger leukotriene B4 release in peripheral monocytes in a pertussis toxin-sensitive manner (15,18). A few recent reports suggest that plasmin can induce cell migration, for example
in human peripheral blood monocytes, and other processes important in angiogenesis (19).
However, very little is known about the mechanisms and molecules participating in plasmin signaling. Recent studies suggest that plasmin may also mediate its effects through activation of a G-protein coupled receptor-mediated signaling pathway (20,21). This is supported by the fact that some effects of plasmin are sensitive to pertussis toxin, which inhibits G_{i/o} family proteins, suggesting involvement of such a G protein (15,18,22). However, the signal transduction mechanism downstream of plasmin activation remains largely unknown.

Protease-activated receptors (PARs) are seven transmembrane-spanning, G protein-coupled receptors (GPCRs) that are activated by N-terminal cleavage to expose a tethered ligand (23,24). GPCRs are known to induce stimulation of heterotrimeric G proteins and can regulate various signaling cascades leading to cellular responses such as gene expression, mitogenesis and cell motility. Thrombin, the major protease in the coagulation cascade, elicits its responses through activation of PARs. PAR-1 (thrombin receptor) and -4 are expressed on human platelets and their activation mediates platelet aggregation and plays a role in hemostasis and thrombosis. PARs also have been shown to play a role in cell invasion during breast carcinoma metastasis and placental implantation (25). The role of PARs in metastasis and invasion relies on its ability to regulate chemotaxis, mitogenesis and adhesion by PAR-1 signaling in an \( \alpha_v\beta_5 \)-dependent manner (26). Recent reports indicate that plasmin may signal through PAR-1 (22). It is somewhat surprising that PAR-1, which is an ideal substrate for thrombin, also serves as the signaling receptor for plasmin that has very different substrate specificity. However, the concept is emerging that co-receptors play important roles in regulating the activation of PARs. PAR-1 has been shown to be activated efficiently by receptor-targeted proteases in the initiation of coagulation (by the tissue factor-factor VIIa-factor Xa complex (27) and of the anticoagulant pathway (by activated protein C bound to the endothelial cell protein C receptor EPCR (28). Whether integrins play similar roles as co-receptors in plasmin signaling through PARs has not been explored.

In the present study we demonstrate that plasmin, a serine protease and the parent molecule of angiostatin, specifically binds to integrin \( \alpha_9\beta_1 \) through its kringle domains to induce signaling. The pro-migratory activity of plasmin requires \( \alpha_9\beta_1 \) and the catalytic activity of plasmin. We
show that PAR-1 is involved in plasmin-induced cell migration using PAR agonist peptides and small-molecule PAR-1 inhibitors. Thus, both $\alpha_9\beta_1$ and PAR-1 are critical for plasmin-induced cell migration.
MATERIALS AND METHODS

Materials. GRGDSP and GRGESP peptides were purchased from Advanced ChemTech (Louisville, KY). Human plasmin, ε-aminocaproic acid (EACA), purified mouse IgG, aprotinin, and fibronectin, were obtained from Sigma. Monoclonal antibody (mAb) KH72 (anti-integrin α5) was provided by K. Miyake (Tokyo University, Tokyo, Japan). Y9A2 (anti- integrin α9) (2) was provided by D. Sheppard (University of California San Francisco, CA). P1F6 (anti-αvβ5) was a kind gift from D. Cheresh (The Scripps Research Institute). Angiostatin (K1-3), human Glu-plasminogen, and mAb 51 are kindly provided by L. Miles (The Scripps Research Institute). MAb 51 reacts with plasminogen K1-3 and plasminogen K4 (29). The PAR-1 inhibitor RWJ58259 was kindly provided by P. Andrade-Gordon (Johnson and Johnson).

Cells. D. Sheppard kindly provided Chinese hamster ovary (CHO) cells expressing human α9β1 (designated α9-CHO cells). CHO cells expressing αvβ3 (hamster β3/human αv hybrid) (designated β3-CHO cells) have been described (30). As a control, CHO cells were transfected only with vector (pBJ-1) together with the neomycin gene and selected for G418 resistance (designated mock-CHO cells).

Adhesion assays. Adhesion assays were performed as previously described (31). Briefly, we coated wells in 96-well Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA) with 100 µl of PBS (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4) containing substrates at a concentration of 50-500 nM and incubated 1 h at 37°C. We blocked remaining protein binding sites by incubating with 0.2% BSA (Calbiochem, San Diego, CA) for 1 h at room temperature. Cells (10^5 cells/well) in 100 µl of Hepes-Tyrode buffer (10 mM Hepes, 150 mM NaCl, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 2.5 mM KCl, 0.1% glucose, 0.02% BSA) supplemented with 2 mM MgCl₂ were added to the wells and incubated at 37°C for 1 h, unless stated otherwise. After non-bound cells were removed by rinsing the wells with the same buffer, bound cells were quantified by measuring endogenous phosphatase activity (32). For inhibition assays, cells were preincubated with mAbs and peptides on ice for 10 min before cells were added to plasmin-coated wells.
Migration assays. Cell migration was analyzed using tissue culture-treated 24-well Transwell plates (Costar, Cambridge, MA) with polycarbonate membranes of pore size 8 μm. The lower side of the filter was coated with various concentrations of substrates and blocked with 0.5% BSA. Coated filters were placed into serum-free migration buffer (Dulbecco's modified Eagle's medium, 10 mM Hepes, 0.5% BSA, and 1X penicillin-streptomycin), and cells (100 μl) suspended in the same buffer (2 x 10^5 cells/ml) were added to the upper chamber. The cells were incubated at 37°C in 5% CO₂ for 20 h. Cells on the upper surface of the membrane were removed by gently wiping with a cotton swab and cells that migrated to the lower surface of the filters were fixed and stained with 0.5% crystal violet in 2% ethanol, and counted using high magnification microscopy. The result is the mean cell number of four randomly selected, high magnification microscopic fields from duplicate wells in two or three separate experiments. In order to verify quantification of mean number of migrated cells, we took a picture of the stained membrane and determined the number of cells on the entire lower surface of the membrane using ImageJ software (http://rsb.info.nih.gov/ij/). We obtained essentially identical results using either method. Anti-integrin mAbs (10 μg/ml), angiostatin (500 nM) or other inhibitory peptides or mAbs were pre-incubated with cells for 15 min at 37°C prior to the assay. When the effect of aprotinin on plasmin-induced cell migration was tested, aprotinin at designated concentrations was incubated with immobilized plasmin. Free aprotinin was removed before starting migration assays.

Staining stress fibers. Stress fiber staining were carried out as described previously (34). Briefly, visualization of cell spreading and morphology on substrates and actin fiber staining was carried out in cells that were incubated in 0.5% serum for 18 h and resuspended in Hepes-Tyrode buffer with 2 mM MgCl₂ at 2 x 10⁴ cells/coverlip before plating on poly-L-lysine, fibronectin-, plasmin-, or angiostatin-coated coverslips. After allowing cells to spread for 90 min at 37°C, they were fixed in 3% paraformaldehyde, and visualized by phase-contrast microscopy. For actin fiber staining, the fixed cells were permeabilized with 0.5% Triton X-100. The actin fibers of these fixed cells were then stained with FITC-conjugated phalloidin. Actin fiber morphology was visualized using confocal microscopy.
Immunoblotting of PAR-1 in CHO cells. Membrane proteins of CHO cells were prepared essentially as described (33). Briefly, confluent CHO cells were harvested and resuspended in 0.25 ml of extraction buffer (10 mM Tris/HCl buffer, pH 7.4 containing 10 mM NaCl, 25 mM NaF, 1 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors leupeptin, aprotinin, pepstatin, and bestatin), and lysed by sonication. The lysate was centrifuged at 15000 x g for 20 min, and the supernatant was further centrifuged at 100000 x g for 1 h. The pellet was resuspended in 100 µl of the extraction buffer containing 1.0 % Nonidet P-40, rocked on ice for 1 h, and centrifuged at 15000 x g for 15 min. The supernatant (containing membrane proteins) was analyzed by SDS-PAGE using 10% acrylamide gels (15 µg protein per lane) and transferred to Immobilon-P membrane (Millipore) for immunoblotting. The membrane was blocked with 5% non-fat dry milk in PBS, and incubated with a rabbit polyclonal antibody against PAR-1 (H-111, Santa Cruz Biotecnology, Santa Cruz, CA), or with control rabbit serum, at 4°C overnight. Bound IgG was detected using horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) and ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK).
RESULTS

Plasmin binding to integrin \( \alpha_9\beta_1 \)

Since \( \alpha_9\beta_1 \) on endothelial cells binds to angiostatin, but not to plasminogen (35) it is possible that proteolytic activation of plasminogen to plasmin produces a similar conformational change to expose the integrin-binding sites located in the kringle domains. We found that plasmin supported adhesion of \( \alpha_9 \)-CHO cells to a greater extent than it did of mock-transfected CHO cells (Fig. 1A and (36)). Anti-\( \alpha_9 \) mAb Y9A2 significantly blocked adhesion of \( \alpha_9 \)-CHO cells to plasmin, but control anti-\( \alpha_5 \) (KH72) or anti-\( \alpha_v\beta_5 \) mAb (P1F6) did not (Fig. 1B). RGD peptides did not completely block adhesion of \( \alpha_9 \)-CHO cells to plasmin (consistent with other reported \( \alpha_9\beta_1 \)-ligand interactions). This also indicates that the integrin-binding sites within the kringle domain of plasminogen are exposed by proteolytic activation of plasminogen to plasmin.

The kringle domains of plasminogen have multiple Lys-binding sites (37). We have shown that a Lys-analogue (\( \epsilon \)-aminocaproic acid, EACA) almost completely blocked plasmin binding to \( \alpha_9 \)-1. Also, a mAb against plasminogen kringles (mAb 51) blocked interaction between plasmin and this integrin (Fig. 1B). These results suggest that plasmin specifically binds to \( \alpha_9 \)-1 through the kringle domains as in the case of angiostatin.

We previously found that angiostatin did not induce cell spreading or stress fiber formation in \( \alpha_v\beta_3 \)-expressing \( \beta_3 \)-CHO cells (35). Here, we find that \( \alpha_9 \)-CHO cells also did not spread or form stress fibers on angiostatin (Fig. 2). In contrast, plasmin induced cell spreading and stress fiber formation in \( \alpha_9 \)-CHO cells (Fig. 2), suggesting that plasmin transduces signals that cause cytoskeletal reorganization upon binding to integrins. Control experiments with mock-CHO (vector-transfected) on these substrates demonstrate that these cells do not spread on poly-L-lysine, angiostatin or plasmin (data not shown). Thus, plasmin specifically signals through integrin \( \alpha_9\beta_1 \) (Fig. 2) and integrin \( \alpha_v\beta_3 \) (35) to induce cell spreading, while plasmin does not signal through endogenous integrins on mock-transfected CHO cells (e.g., \( \alpha_5\beta_1 \), \( \alpha_v\beta_5 \), or \( \alpha_v\beta_1 \)).
Plasmin induces haptotaxis of α9-CHO cells in an integrin- and catalytic activity-dependent manner, and angiostatin (K1-3) blocks the plasmin-induced migration.

It has been reported that plasmin is a potent chemoattractant for human peripheral monocytes (19). We studied whether plasmin induces migration in an integrin-dependent manner using a modified Boyden chamber assay. Notably, plasmin induced haptotaxis of α9-CHO cells, but not significantly of mock-transfected CHO cells (Fig. 3A). α9-CHO cells migrated at a much higher rate than mock-transfected CHO cells. This is consistent with the previous observation that α9β1 integrin significantly enhances cell migration (38). An anti-α9 mAb Y9A2 effectively blocked plasmin-induced haptotaxis of α9-CHO cells, while the anti-αvβ5 mAb P1F6 did not block migration (Fig. 3B), suggesting that α9β1 integrin in particular is critical for plasmin-induced migration. Notably, we found that soluble angiostatin effectively blocked plasmin-induced haptotaxis of α9-CHO cells in a dose-dependent manner (Fig. 3C).

Since angiostatin does not induce migration of these cells (35), the serine protease activity of plasmin may be important for plasmin-induced migration. Consistently, aprotinin, a serine protease inhibitor, effectively blocked the haptotaxis of these cells on plasmin (Fig. 3D). Mock-transfected CHO cells do not migrate to a significant extent on plasmin and are not affected by aprotinin under the conditions used. These results suggest that the catalytic activity of plasmin is critical for plasmin-mediated migration, and that blocking plasmin-induced migration of endothelial cells is a potential mechanism by which angiostatin can exert its effects.

The role of protease activated receptors (PAR) in plasmin signaling.

It has recently been reported that plasmin transduces signals through activating PAR-1 (22), but it is unclear whether integrins are required for this signaling process. We have tested whether PARs are involved in plasmin-induced migration of α9-CHO cells. Tethered ligands (PAR-agonist peptides; TFLLRN for PAR-1, SLIGRL for PAR-2, and GYPGQV for PAR-4) activate PARs without proteolysis. We tested whether PAR-agonist peptides can mimic the possible plasmin activation of PARs and stimulate cell migration. We used angiostatin that binds to α9β1, but does not by itself induce migration of α9-CHO cells as a haptotactic substrate. We found that PAR-1 agonist, but not PAR-2 or PAR-4 agonist, significantly induced migration of α9-CHO
cells on angiostatin (Fig. 4). These results suggest that PAR-1 is a likely candidate that mediates plasmin-induced signaling.

RWJ 58259 is a synthetic PAR-1 antagonist that efficiently blocks PAR-1 mediated signaling (39). We found that RWJ 58259 inhibited plasmin-induced migration of a9-CHO cells (Fig. 5). These results support the notion that PAR-1 is primarily responsible for plasmin-induced migration of a9-CHO cells. We also found that a palmitoylated peptide, which specifically recognizes the third intracellular loop of PAR-1 and blocks G protein activation (pal 1, also known as Pepducin) (40), can significantly block PAR-1 agonist peptide-induced migration of a9-CHO cells, while a similar palmitoylated inhibitory peptide for PAR-4 (pal 4) did not (Fig. 6A). Mock-transfected CHO cells did not respond to stimulatory PAR-1 and PAR-4 agonist peptides, or inhibitory pal 1 and pal 4 peptides. This suggests that PAR-1 signaling specifically cooperates with a9b1. Plasmin-induced migration of a9-CHO cells is also significantly inhibited by pal 1, but not by pal 4 (Fig. 6B). These experiments further support a role for PAR-1 in plasmin-induced migration of a9-CHO cells. We have confirmed the expression of PAR-1 in CHO and a9-CHO cells by immunoblotting (Fig. 6C), which is consistent with the previous report that CHO cells express functional hamster PAR-1 (27). These results demonstrate that integrins a9b1 serves as a migration stimulating co-signaling receptor for plasmin and displays specificity to PAR-1.

DISCUSSION

We have previously shown that angiostatin binds to integrin a9b1, but plasminogen does not (35). The present results establish that plasmin, like angiostatin, binds to integrin a9b1 through the kringle domains. Our results suggest that the a9b1 binding sites in the kringle domains are exposed by proteolytic cleavage of plasminogen. It should be noted that the kringle domain of plasmin is still attached to the catalytic domain by inter-chain disulfide linkage. The present study demonstrates that plasmin binding to a9b1 is required for cell migration and that the catalytic activity of plasmin is also essential for this process. a9-CHO cells showed much more pronounced migration than mock-transfected CHO cells, which is consistent with the previous observation (38), and plasmin further enhanced the migration of a9-CHO cells.
\( \alpha_9\beta_1 \) expressing CHO cells did not form stress fibers on poly-L-lysine or angiostatin (K1-3), but did spread and form stress fibers on plasmin. Control experiments with mock-CHO cells on these substrates demonstrate that these cells do not spread well on poly-L-lysine, angiostatin, or plasmin. These results suggest that plasmin signaling requires \( \alpha_9\beta_1 \). Mock-CHO cells, however, do show a low level of binding and migration, which may be due to binding of other integrins (e.g. \( \alpha_5\beta_1 \), \( \alpha_v\beta_5 \), and \( \alpha_v\beta_1 \)) that are endogenous in CHO cells. Such endogenous receptors may also be the cause of the partial inhibition of adhesion of \( \alpha_9 \)-CHO cells seen on plasmin by the RGD peptide and the anti-\( \alpha_9 \) mAb.

We also provide direct evidence that PAR-1 is involved in plasmin-induced signaling in \( \alpha_9 \)-CHO cells using PAR-agonist peptides that substitutes for the catalytic activity of plasmin and induces migration of \( \alpha_9 \)-CHO cells on angiostatin. Consistently, we also found that inhibition of PAR-1 activation by treatment with a small molecule inhibitor, RWJ 58259, or a palmitoylated inhibitory peptide against PAR-1 (pal 1) is effective in blocking plasmin-induced migration of \( \alpha_9 \)-CHO cells. Taken together, the present evidence suggests that PAR-1 is critical for plasmin-mediated migration of \( \alpha_9 \)-CHO cells.

Plasmin has about 10-times lower affinity for PAR-1 in comparison to thrombin (41). Thus, activation of PAR-1 requires a mechanism to concentrate plasmin on the cell surface. Binding of plasmin to \( \alpha_9\beta_1 \) would significantly increase the cell surface plasmin concentration. Binding of plasmin to the integrins through the kringle domain would also prevent the inactivation of plasmin because circulating abundant \( \alpha_2 \)-antiplasmin rapidly inactivates free plasmin dependent on interactions with the kringle domain of plasmin ((42) for a review). In addition, uPA and uPAR are widely expressed by proliferating tumor cells ((43) for review). Since uPA/uPAR binding can convert plasminogen to plasmin, tumor cells will generate plasmin locally and high pericellular plasmin level can be expected around tumor cells. It has recently been reported that plasmin induces PAR-1 activation leading to MAP kinase activation at very low plasmin concentrations on fibroblast (22). This is not consistent with previous reports (44) that plasmin cleaves PAR-1 only inefficiently. We suspect that plasmin may be concentrated at the cell surface of fibroblasts through binding to one or more integrins in this case as well.
The present study establishes that plasmin-induced cell migration requires both binding of plasmin to integrin \( \alpha_9 \beta_1 \) through the kringle domains and activation of PAR-1 by the catalytic activity of plasmin. We have reported that endothelial cells (BAE cells) express both \( \alpha_9 \beta_1 \) and \( \alpha_v \beta_3 \) (35), and are involved in plasmin signaling. We also found, using FACS analysis, that calf pulmonary artery endothelial (CPAE) cells, which have been widely used for angiogenesis studies (45-48), express \( \alpha_9 \beta_1 \) and \( \alpha_v \beta_3 \) (our unpublished results). These findings suggest that both \( \alpha_9 \beta_1 \) and \( \alpha_v \beta_3 \) may support plasmin-induced signals in endothelial cells and may be involved in the inhibitory effect of angiostatin.

We have previously shown that plasmin-induced haptotaxis of \( \alpha_3 \)-CHO cells required the catalytic activity of plasmin and binding to \( \alpha_v \beta_3 \) integrins, and that angiostatin effectively blocked plasmin-induced haptotaxis of \( \alpha_3 \)-CHO cells. \( \alpha_v \beta_3 \) integrin is highly expressed in angiogenic endothelial cells in tumors, wounds or inflammatory tissues (36). Although angiostatin has been shown to inhibit angiogenesis by blocking endothelial cell proliferation and migration (49,50) there was conflicting data on the underlying mechanism of action. Our study (35) provided insight into a possible mechanism by which angiostatin may inhibit angiogenesis when \( \alpha_v \beta_3 \) integrin is involved. The present findings indicate that angiostatin would simultaneously block plasmin-induced migration of cells that express \( \alpha_v \beta_3 \)- as well as \( \alpha_9 \beta_1 \)-integrins.

Angiostatin may similarly exert effects on \( \alpha_9 \beta_1 \)-dependent migration in neutrophils, hepatocytes, smooth muscle, or epithelial cells (that express \( \alpha_9 \beta_1 \)). It has been shown that the \( \alpha_9 \) cytoplasmic domain (like the highly homologous \( \alpha_4 \) cytoplasmic domain) preferentially enhances cell migration (38). It is interesting that both \( \alpha_9 \beta_1 \) and \( \alpha_4 \beta_1 \) integrins are expressed on leukocytes, which are cells that are required to migrate rapidly at sites of inflammation. However, \( \alpha_9 \beta_1 \) and \( \alpha_4 \beta_1 \) appear to regulate different signaling pathways which both lead to increased migration. The present study used cells over-expressing \( \alpha_9 \beta_1 \) to study plasmin-induced migration and signaling in a manner specific to \( \alpha_9 \beta_1 \). The use of this artificial system would be justified and essential to study \( \alpha_9 \beta_1 \)-specific plasmin signaling considering that...
endothelial cells, monocytes, neutrophils, and other natural cells express several integrins other than \( \alpha_9\beta_1 \) that interact with plasmin and angiostatin (e.g., \( \alpha_v\beta_3 \) and perhaps other integrins). Elucidating the signaling pathways downstream of \( \alpha_9\beta_1 \) and their potential inhibitory agents (e.g. angiostatin, PAR-1 inhibitory compounds, serine protease inhibitors etc.) may be relevant to understanding processes such as inflammation and wound healing in these systems.
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Figure Legends:

Fig. 1 Plasmin binding to α9-CHO cells is dose-dependent and inhibited by mAbs.

A) Adhesion of α9-CHO cells to plasmin as a function of plasmin coating concentrations. Adhesion of α9-CHO cells to plasmin was also compared to that of mock (vector only)-transfected CHO cells. Adhesion assays were carried out as described in the text. Briefly, plasmin at different concentrations was incubated in wells of 96-well microtiter plates, and the wells were then blocked with BSA. Cells were added in Tyrode-Hepes buffer, supplemented with 1 mM MgCl2. After incubating for 1 h at 37°C, bound cells were quantified as described. Data are shown as the mean ± S.D. from three separate experiments. B) The effect of anti-integrin and anti-kringle agents on adhesion of α9-CHO cells to plasmin. 100 nM plasmin was used for coating wells. Mabs, Y9A2 (anti-α9), KH72 (anti-α5), P1F6 (anti-αvβ5), and mAb 51 (anti-plasminogen kringle), are used at 4 μg/ml. EACA (β-aminocaproic acid) a Lys analogue, is used at 200 mM. RGD and RGE peptides were used at 100 μM.

Fig. 2 Plasmin induces spreading of α9-CHO cells, but not mock-CHO.

Top) α9-CHO cells were plated on glass coverslips coated with angiostatin (fragment K1-3, 500 nM) or plasmin (200 nM) in the absence of serum and allowed to spread for 2 h at 37°C, then fixed in 3.7% formaldehyde, and visualized by phase contrast microscopy to assess cell morphology. Mock-CHO cells on K1-3 or plasmin showed morphology similar to that of α9-CHO cells on K1-3 (not shown). Bottom) α9-CHO cells were plated on glass coverslips coated with poly-L-lysine (50 μg/ml), angiostatin (K1-3, 500 nM), or plasmin (200 nM) in the absence of serum, allowed to spread, and then fixed, stained for actin stress fiber formation, and their cytoskeletal morphology was visualized by confocal microscopy as described in Materials and Methods.

Fig. 3. Plasmin induces migration of α9-CHO cells, and is inhibited by mAbs, angiostatin or aprotinin. A) Migration of α9-CHO and mock-transfected CHO cells was determined as function of plasmin concentration (nM). Migration assays were performed as described in the text using modified Boyden chambers. B) Cell migration was measured on membranes coated
with 0.5% BSA (control, “no coat”) or plasmin (200 nM) in the presence of anti-integrin mAbs
(10 μg/ml) (Y9A2, anti-α9; P1F6 anti-αvβ5). C) Cell migration on plasmin (200 nM) was
measured in the presence of increasing concentrations of angiostatin (K1-3). D) The effect of a
serine protease inhibitor aprotinin on cell migration on plasmin (200 nM) was determined.
Immobilized plasmin was incubated with increased concentrations of aprotinin. Data are shown
as the mean ± S.D. from three separate experiments done in triplicate relative to the migration
level of α9-CHO cells on non-coated wells

**Fig. 4. The effect of PAR-agonist peptides on cell migration towards angiostatin.** Migration
assays with α9-CHO cells were performed as described in Materials and Methods on membranes
coated with 0.5% BSA (control, open column), angiostatin (K1-3, 500 nM, closed column), or
plasmin (200 nM, gray column). PAR-agonist peptides were preincubated with cells before
adding cells to migration chambers at the following concentrations: PAR-1 (20 μM), PAR-2
(200 μM), or PAR-4 (400 μM). Increase in migration relative to the migration level of α9-CHO
cells on K1-3-coated wells are shown as means ± S.D. from at least three separate experiments
done in triplicate.

**Fig. 5. The PAR-1-specific inhibitor, RWJ 58259, inhibits plasmin-induced α9-CHO cell
migration.** The effect of the PAR-1 inhibitor on α9-CHO cell migration towards plasmin (200
nM coating concentration) was determined after preincubating cells with the PAR-1 inhibitor
RWJ 58259 (5 μM) for 15 min. at 37°C before the assay. Data are the means ± S.D. from two
independent experiments performed in duplicate relative to the migration level of α9-CHO cells
on non-coated wells.

**Fig. 6. Palmitoylated inhibitory peptides inhibit agonist peptide- and plasmin-induced cell
migration.** Data are the means ± S.D. from two independent experiments performed in triplicate
relative to the migration level of α9-CHO or β3-CHO cells on non-coated wells. A) The effect of
palmitoylated, inhibitory peptides against PAR-1 and PAR-4 (pal 1 and pal 4, respectively) was
determined by preincubating cells with inhibitory and agonist PAR peptides for 15 min at 37°C
prior to the assay on Transwells coated with K1-3 (500 nM) (see Materials and Methods). B) The
effect of PAR inhibitors peptides on plasmin-induced α9-CHO cell migration was determined
by preincubation of cells with inhibitory peptides (pal1 or pal4) for 15 min at 37°C prior to the assay on non-coated or plasmin-coated wells. C) Expression of PAR-1 in CHO and a9-CHO cells. The membrane protein fractions of CHO and a9-CHO cells were analyzed by SDS-PAGE (15 µg protein per lane) and immunoblotting with a rabbit polyclonal antibody against PAR-1, or with control rabbit serum. We detected PAR-1 (55 Kd) in both CHO (lane C) and a9-CHO (lane 9). We did not detect PAR-4 under the conditions used (data not shown).
Fig. 1A

% Bound cells

| Plasmin (nM) |
|--------------|
| 0            |
| 50           |
| 100          |
| 150          |
| 200          |

CHO

9-CHO
Fig. 1B

- 9-CHO cells

% Bound cells

BSA
Plasmin
+ Y9A2
+ P1F6
+ KH72
+ RGD
+ RGE
+ mAb 51
+ EACA

Plasmin (100 nM)
Fig. 2
Fig. 3A

Cell migration (per field)

Plasmin coating concentration (nM)

9-CHO

Mock-CHO
Fig. 3B

Cell migration (fold over control)

- no coat
- Plasmin
- +Y9A2
- +P1F6

9-CHO cells

Plasmin
Fig. 3C

Cell migration (fold over control)

| Condition   | K1-3 | 0  | 4   | 40  | 400 | 4000 nM |
|-------------|------|----|-----|-----|-----|---------|
| No coat     |      |    |     |     |     |         |
| Plasmin     |      |    |     |     |     |         |

---

Fig. 3C: Graph showing cell migration (fold over control) with varying concentrations of Plasmin (K1-3) in the presence and absence of a coat.
Fig. 3D

Cell migration (fold over control)

| Aprotinin (mg/ml) | Plasmin | CHO | 9-CHO |
|------------------|---------|-----|-------|
| 0                |         | -   | -     |
| 50               |         | -   | -     |
| 0                |         | +   | -     |
| 0                |         | +   | +     |
| 1                |         | +   | +     |
| 10               |         | +   | +     |
| 20               |         | +   | +     |
| 50               |         | +   | +     |

Bar graph showing the cell migration fold over control with different concentrations of Aprotinin and Plasmin for CHO and 9-CHO cells.
Fig. 4

Cell migration (fold over control)

K1-3 + - + - + - + - -
PAR-1 PAR-2 PAR-4 Plasmin

9-CHO
Fig. 5

Cell migration (fold over control)

- control
- Plasmin
- +RWJ58259 (3)
- +RWJ58259 (5)

Plasmin
Fig. 6A

Cell migration (fold over control)

K1-3   -      +      +      +      +       -      +      +      +      +
PAR-1
PAR-1 + pal 1
PAR-4
PAR-4 + pal 4
PAR-1
PAR-1 + pal 1
PAR-4
PAR-4 + pal 4

CHO

9-CHO
Fig. 6B

Cell migration (fold over control)
Plasmin-induced migration requires signaling through protease-activated receptor 1 and integrin $\alpha_9\beta_1$

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