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Binding of urokinase to plasminogen activator inhibitor type-1 mediates cell adhesion and spreading

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SUMMARY

Urokinase plasminogen activator and its receptor are both found at the surface of the cell membrane in many cell types. The plasminogen activator inhibitor type-1 (PAI-1) is often associated with the extracellular matrix. The spatial localization of these three molecules could account for their involvement in cell adhesion and/or migration. We have shown previously that the urokinase receptor mediates mechanical force transmission across the cell surface to the cytoskeleton. Here we investigated whether immobilized plasminogen activator inhibitor type 1 (PAI-1) could regulate cell spreading and cytoskeleton reorganization. Serum deprived human myogenic cells were plated in serum free medium onto bacteriologic dishes precoated with different extracellular matrix ligands (fibronectin, vitronectin, or type 1 collagen) or PAI-1 at increasing concentrations. The number of adherent cells and their projected area were quantitated after 3 hours of plating. PAI-1 promoted cell adhesion and spreading in a dose dependent manner. Addition of antibodies to PAI-1 inhibited the adhesion on PAI-1 coated dishes in a dose dependent way. The PAI-1 mediated cell adhesion required the presence of urokinase at the cell surface. Removal of the glycosylphosphatidylinositol (GPI)-linked proteins abolished cell adhesion on PAI-1 dish, suggesting its dependence on the presence of the urokinase receptor, a GPI-linked receptor. Furthermore, addition of antibodies against αvβ3 integrin completely inhibited cell adhesion on PAI-1, suggesting that αvβ3 might be the transmembrane molecule that physically connects the complex of PAI-1, urokinase, and urokinase receptor to the cytoskeleton. Visualization of spread cells stained for filamentous actin with confocal microscopy showed a dose-dependent increase of filopodia on PAI-1 coated dishes and cytoskeletal reorganization, suggesting a migratory profile. These data indicate that PAI-1 plays a direct role in dynamic cell adhesion particularly at the leading edge, where increased levels of urokinase plasminogen activator (uPA) and its receptor (uPAR) are localized in migrating cells. Immobilized PAI-1 could therefore serve to bridge the cell surface with the extracellular matrix via the formation of a multimolecular complex that includes αvβ3 integrins in myogenic cells.

Key words: Cell migration, Extracellular matrix, Mechanical signaling

INTRODUCTION

Extracellular matrix (ECM) is critical for regulation of cell growth (Ingber and Folkman, 1989; Ingber, 1990), gene expression and differentiation (Bissell et al., 1982; Ben-Ze'ev et al., 1988; Opas, 1989; Mooney et al., 1992), and cell migration (Lauffenburger and Horwitz, 1996). The urokinase type plasminogen activation complex is one of the major regulators of ECM remodeling. This enzymatic complex consists of the urokinase receptor, urokinase, and plasminogen activator inhibitors: type-1 (PAI-1) or type-2 (PAI-2). The urokinase activates plasminogen into plasmin which plays both a direct role in ECM degradation and an indirect role by activating other matrix metalloproteinases (Dano et al., 1985; Vassalli et al., 1991; Liotta, 1992). PAI-1, a molecule found in the ECM, prevents excessive matrix destruction by inhibiting the catalytic activity of uPA and thus plasminogen activation (reviewed by Pepper and Montesano, 1992). Although the proteolytic role of uPA has been the focus of research for years (for review, see Vassalli et al., 1991), recently a new function for uPAR/uPA has begun to emerge: uPAR/uPA appears to be involved in cell adhesion, migration and differentiation independent of urokinase's catalytic activity (Nusrat and Chapman, 1991; Quax et al., 1992; Waltz et al., 1993; Gyetko et al., 1994). However, the direct role of matrix-bound PAI-1 and its dependence on uPA and uPAR for cell adhesion and spreading have not been explored. Previously it was shown that normal human myogenic cells express uPAR, uPA, and PAI-1 (Quax et al., 1992). To test whether the complex formed by uPAR, uPA, and PAI-1 is directly involved in cell adhesion, we chose to use first a few passages of these cells as a model system since cultured normal myogenic cells adhere and spread well in the absence of serum.

We have recently shown that uPAR can mediate mechanical stress transfer across the surface of these myogenic cells to the cytoskeleton (CSK), thus suggesting a direct mechanical role for uPAR in cell anchorage and migration. Interfering with the uPAR/uPA/PAI-1 complex by addition of the amino-terminal fragment of uPA (ATF), antibodies against uPA, or antibodies against PAI-1 also altered mechanical coupling between integrins and the CSK and/or the CSK mechanics, suggesting a role for the uPAR/uPA/PAI-1 complex in regulating cell adhesion (Wang et al., 1995). In this study we show that PAI-1 forms one of the molecular bridges between the cell surface and the ECM and thus acts directly to regulate cell adhesion.

MATERIALS AND METHODS

Materials

Culture medium, glutamine, albumin bovine fraction V, pronase, calf serum, antibiotics, cell dissociation solution, glutaraldehyde, Crystal Violet, glycerol, and salts were from Sigma Co. (St Louis, MO). 96-well plates (Immulon II) were from Dynatech Laboratories, Inc. (Indianapolis, IN). Recombinant active human PAI-1 and active urokinase were obtained from American Diagnostica Inc. (Greenwich, CT). Human fibronectin (FN) was from Cappel (Malvern, PA). Human vitronectin (VN) was from Sigma Chemical Co. (St Louis, MO). Type I collagen was from Celtrix Labs (Vitrogen 50).

Cell culture

Human muscle satellite cells were cultured according to a previously described method with few modifications (Quax et al., 1992). Briefly, muscle biopsies obtained from orthopedic surgery were digested in 0.15% pronase in PBS (phosphate buffered saline without calcium and magnesium) for 1 hour at 37°C. After centrifugation and washing, the cells were seeded at 6×10^4 cells per 25 cm^2 flask in Ham's F12 medium supplemented with 20% calf serum, 2 mM glutamine, 100 i.u./ml penicillin, and 100 μg/ml streptomycin. The culture medium was routinely changed every 4 days. The expression of desmin was tested as a specific marker of the myogenic lineage using a standard immunoperoxidase. For all experiments passage 1-3 cells were used. The cells were deprived of serum and maintained in tissue Ham's F12 medium with 1% BSA at least 24 hours prior being harvested with a cell dissociation solution containing EDTA, glycerol and sodium citrate in PBS (a non enzymatic dissociation solution; Sigma). In some experiments cells were preincubated with 10 μg/ml cycloheximide to inhibit new protein synthesis one hour before harvesting and during the incubation time of the adhesion assay.

Adhesion assay

96-well bacteriologic plates were coated with different ligands: human fibronectin, human vitronectin, type I collagen or recombinant pure PAI-1 (No 1092, American Diagnostica), at an increasing concentration (0, 5, 50, 100, 500 μg/ml, 45 μg per well, corresponding to 0, 0.225, 2.25, 4.5, 22.5 μg per well) in PBS at 37°C for 3 hours. These wells could not support cell attachment without precoating with proteins or serum (Dynatech, Immulon II). After 3 hours of coating the wells were washed twice with PBS and incubated in serum free medium supplemented with 1% BSA for 30 minutes at 37°C to block nonspecific binding. Then the cells were plated at $0.5\text{-}1\times10^4$ cells/well in serum free medium with 1% BSA for 3 hours at 37°C. At the end of the incubation period, wells were rinsed twice with warm PBS to remove non adherent cells, and the attached cells were fixed with 1% glutaraldehyde in PBS for 10 minutes at room temperature, and subsequently stained with 1% Crystal Violet in water for 10 minutes. Cell numbers were quantitated with two independent methods: (1) by reading the associated optical density at 570 nm with a microplate reader (SOFTmax). This method was calibrated with a standard curve after cells were plated from 500 cells to 10,000 cells per well for 3 hours in the same medium and counted indirectly using the Crystal Violet method. Some wells incubated in parallel in 1% BSA medium, but without cells, were stained, washed and served as background. The background was subtracted out for the determination of optical density; (2) by direct counting with a Nikon TMSF microscope. Cells were counted field by field (one field equals 0.5 mm^2). At least 40 fields were counted per well corresponding to about 63% of the well surface area. Projected areas of adherent cells were determined using a computerized image analysis system (Zeiss interactive digital analysis system) (Ingber, 1990). A total of 60 cells within randomly selected areas from three different wells were measured for each cell area determination.

Confocal microscope analysis for cell area and perimeter measurements

Small round coverslides (5 mm in diameter) were precoated with PAI-1 or FN at 50 μg/ml for 3 hours at 37°C and placed into the 96-wells. Then the wells with the coverslides were rinsed twice with PBS and incubated for 30 minutes in serum-free medium with 1% BSA to block nonspecific binding. The cells were plated at a density of 5,000 cells/well in serum-free medium for 3 hours at 37°C (under the same conditions as described in the cell adhesion assay). At the end of this incubation, the wells were rinsed twice with warm cytoskeleton buffer (CSK buffer: 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes, pH 6.8) that maintains the integrity of the CSK, as previously described (Plopper and Ingber, 1993). Then the adherent cells were fixed in methanol at –20°C for 6 minutes. Methanol was then removed and the samples were air dried. Rhodaminated phalloidin (100 nM, final concentration) in CSK buffer was added to each well for 45 minutes in a dark humid chamber at room temperature. After this incubation, the coverslides were rinsed twice in CSK buffer for 5 minutes each, with a final rinse in distilled water. The coverslides were then mounted on a slide with the cell side facing down in a 1:1 solution of water and glycerol, and the edges of the coverslides were sealed with black nail polish. The samples were stored at 4°C overnight before the analysis with a laser confocal microscope. Confocal microscopy was performed using a Sarastro 2000 (Molecular Dynamics, Inc.) laser scanning microscope fitted with a 25 mW argon-ion laser and an upright microscope (Optiphot-2; Nikon, Inc.). Single channel fluorescence imaging was used to record cells labeled with rhodaminated phalloidin. Optical bench settings for the confocal microscope were: excitation, 514 nm; emission, >535 nm long pass filter; laser power, 18.7 mW; 10% laser transmission, and photo multiplier voltage of 539 mV. Fields of adherent cells were randomly selected, brought into focus using a ×60/1.4 numeric aperture Plan Apo objective under brightfield conditions and briefly examined. A cross sectional image (*x*-*z*) was recorded under confocal conditions and used to establish a plane of focus 0.2-0.5 μm above the glass surface. Thus these optical sections (*x*-*y*) were recorded to reveal the basal features of adherent cells.

Image analysis and processing

Image analysis and image processing were performed using ImageSpace software (Molecular Dynamics, Inc.). Optical sections recorded in a 1,024 × 1,024 image size were subjected to 2-D image analysis to determine area and perimeter of cells. Cell projected area was plotted against cell perimeter to determine an index of cell shape. Pixel dimensions of all micrographs were 0.17 mm, and ranged from 0-254 units of intensity. Areas not containing cells had pixel intensities from 0-5, whereas cells containing fluorescence stained actin were observed to have pixel intensities from 6-254. To determine cell area, pixel values between 6 and 254 were set to a value of 1, and pixel intensities in the range of 0-5 were set to 0. Occasionally, small areas within cells were observed to have pixel intensities between 0 and 5.

Such areas were manually set to a pixel value of 1 if the area was clearly within the cytoplasm, or represented the area occupied by the nucleus. A 2-D object count was performed on each micrograph to determine the total area for each cell. The object count criteria were set to a minimum size of 300 adjacent pixels with a value of 1. Cells touching the edge of the micrograph were excluded from the analysis. Perimeter measurements were determined by assigning a gradient filter set to detect the location where pixel values changed from 0 to 1. The output of the gradient filter was presented as a line one pixel wide that exactly followed the perimeter of each cell. The number of pixels contained within each line was then reported as a line length.

RESULTS

PAI-1-dependent cell adhesion

Myogenic cell adhesion was found to increase when the density of immobilized PAI-1 was raised on otherwise non-adhesive dishes with saturating levels observed at coating density about 5 μg/well (96-well plate) (Fig. 1A). Addition of 10 μg/ml cycloheximide for 1 hour to suspended cells before plating inhibited new protein synthesis by more than 90% but had no effect on cell adhesion on PAI-1 coated dishes (not shown). Similarly, cells plated on dishes precoated with fibronectin (FN), type 1 collagen (Col-1), or vitronectin (VN) also exhibited a dose dependent increase in cell adhesion, although they exhibited maximal levels of adhesion at a much lower coating concentration: 2.25 μg/well, 0.225 μg/well, and 0.225 μg/well, respectively (Fig. 1B,C,D). The molecular mass of PAI-1 is 43 kDa, that of FN is about 480 kDa, that of Col-1 is 60 kDa, and that of VN is about 70 kDa. Assuming they attached to the dishes equally well, when these molecules were compared on an equimolar basis, it was found that PAI-1 was 22 times less adhesive than FN, 28 times less adhesive than Col-1, and 32 times less adhesive than VN. Interestingly, FN, Col-1, and VN had similar adhesiveness on an equimolar basis. PAI-1 mediated cell adhesion was specific since addition of antibodies against PAI-1 (ab-PAI-1; Cat No: 3790A, American Diagnostica) resulted in a dose dependent decrease in cell adhesion (Fig. 2A) whereas addition of nonimmune IgG had no effect on PAI-1 adhesion (not shown). Moreover, ab-PAI-1 had no effects on VN mediated cell adhesion (Fig 2B). In contrast, although they inhibited VN mediated cell adhesion in a dose dependent manner, antibodies against VN had no effect on PAI-1 adhesion (data not shown).

PAI-1 adhesion depends on the presence of membrane-bound uPA and uPAR

Since PAI-1 is a natural ligand in the ECM that binds to uPA and inhibits uPA activity (Lawrence et al., 1994), we asked whether PAI-1 mediated cell adhesion depends on the presence of membrane-bound uPA. Mixing myogenic cells with soluble active uPA at the time of plating had no effect on FN or Col-1 coated dishes, however, it inhibited the adhesion on PAI-1 coated dishes by over 90% (Fig. 3). In order to further determine whether this inhibition effect was due to direct uPA-PAI-1 binding interactions, we added soluble uPA to PAI-1 coated dishes for 30 minutes, washed away unbound uPA, and then plated cells. A 70% decrease in adhesion was observed (Fig.

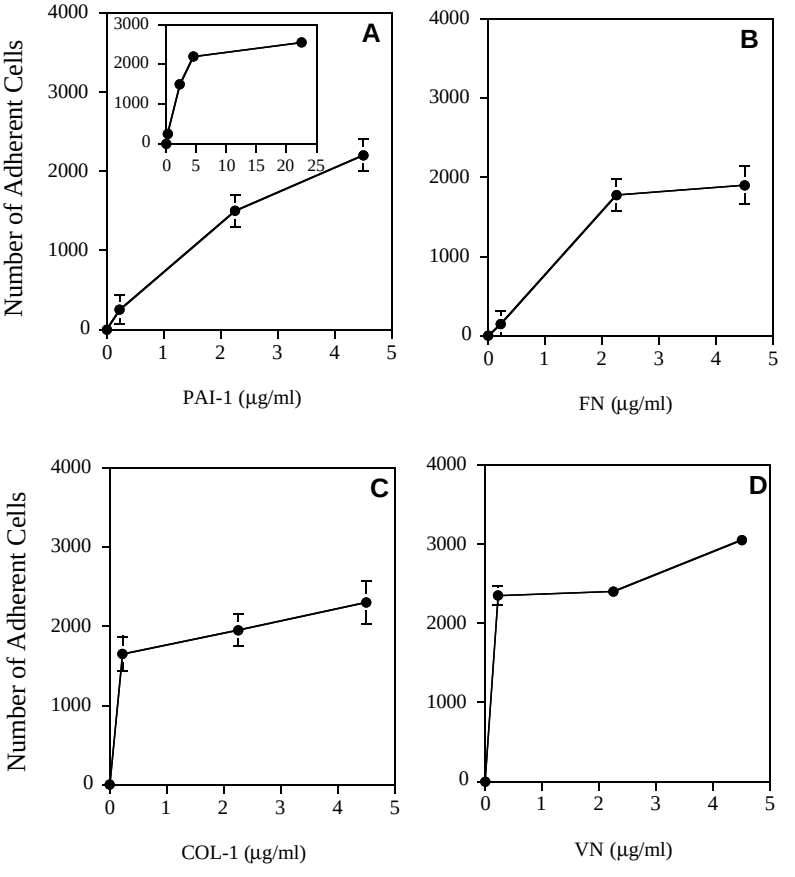

Fig. 1. Dose dependent adhesion of myogenic cells on PAI-1 and other different molecules. The wells of 96-well plates were precoated with different ligands in PBS for 3 hours. Then the cells were plated in serum-free medium with 1% BSA for 3 hours. The number of adherent cells were quantitated (A) PAI-1; (B) FN; (C) Col-1; (D) VN. In this representative experiment the values plotted are means ± s.d. (*n*=3 wells). Similar results were obtained in three other independent experiments.

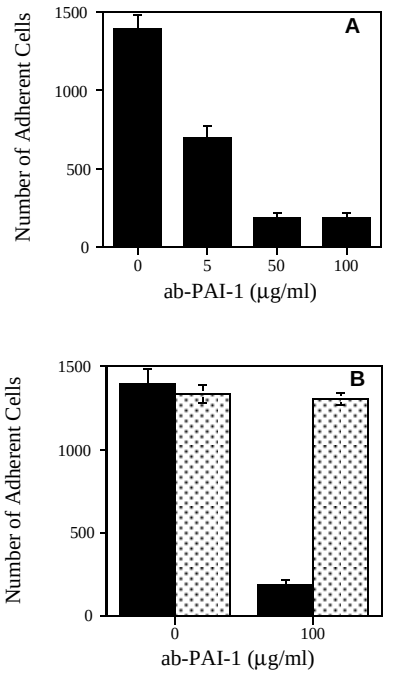

Fig. 2. Inhibition of cell adhesion on PAI-1 by addition of monoclonal antibodies against PAI-1 (ab-PAI-1). All procedures were identical to those described in the legend to Fig. 1 except that ab-PAI-1 was added at the time of cell plating. (A) Dishes coated with 2.25 μg PAI-1 per well; (B) dishes coated with 2.25 μg VN per well (stippled columns) or 2.25 μg PAI-1 per well (filled columns). Mean ± s.d. (*n*=3 wells). Similar results were obtained in three other independent experiments.

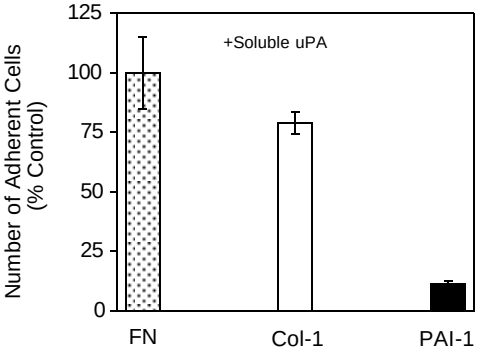

Fig. 3. Effects of soluble uPA on cell adhesion on PAI-1 coated dishes. Addition of soluble uPA (50 μg/ml; American Diagnostica) at the time of cell plating resulted in a 90% inhibition in adhesion on PAI-1 coated dishes (2.25 μg/well), but had no effect on FN or Col-1 mediated adhesion. Values plotted are means ± s.d. (*n*=3 wells). Similar results were obtained in three other independent experiments.

4A). Interestingly, when we mixed the cells in suspension with soluble uPA for 30 minutes, centrifuged cells to remove unbound uPA, resuspended the cells in serum free medium, and then plated the cells on PAI-1 coated dishes, we did not observe any decrease in cell adhesion (Fig. 4A); rather, there was a 20% increase in cell adhesion on PAI-1, probably due to the binding of added soluble uPA to some unoccupied uPAR on the cell membrane. Finally, we wanted to know if it was possible to reverse the process of PAI-1 adhesion, so we added soluble uPA

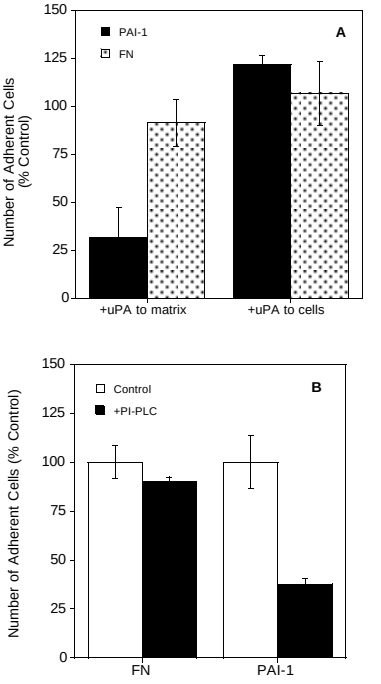

Fig. 4. (A) Modulation of cell adhesion on PAI-1 by soluble uPA (50 μg/ml), added either directly to immobilized PAI-1, or to cells, before plating. Addition of soluble uPA to immobilized PAI-1 resulted in a 70% decrease in cell adhesion whereas saturation of cell membrane by soluble uPA resulted in a small increase (20%) in cell adhesion. In contrast, soluble uPA had no effect on FN coated dishes (stippled columns). Values plotted are means ± s.d. (*n*=3 wells). Similar results were obtained in three other experiments.
(B) Inhibition of cell adhesion on PAI-1 by addition of soluble PI-PLC (5 units/ml). PI-PLC inhibited cell adhesion on PAI-1 by more than 60% whereas it had little effects on FN mediated cell adhesion.

to cells already spread on PAI-1. We found that addition of soluble uPA for 2 hours resulted in detachment of most cells (over 90%) from PAI-1 coated dishes (not shown).

Since PAI-1 mediated cell adhesion depends on the presence of membrane-bound uPA, we reasoned that this event would also depend on the presence of uPAR, which is a glycosyl-phosphatidylinositol (GPI)-linked membrane protein. Before plating the cells, when we pretreated cells for 30 minutes with 5 units of phosphatidylinositol-phospholipase C (PI-PLC, Sigma), which is known to remove all GPI-linked membrane proteins, cell adhesion on PAI-1 coated dishes was inhibited by more than 60%, suggesting that the presence of membrane-bound uPAR is necessary for PAI-1 adhesion. In contrast, PI-PLC had no effects on cell adhesion on FN coated dishes (Fig. 4B).

Cell spreading and CSK reorganization

To further investigate the effects of immobilized PAI-1 on cell shape and CSK organization, we quantitated the projected areas of the adherent cells on increasing densities of PAI-1 immobilized on plastic wells. Increasing PAI-1 coating density increased projected cell areas (Fig. 5A) in a manner similar to FN, Col-1, and VN (Fig 5B,C,D). About 25 times more PAI-1 than other ECM proteins was required, on an equimolar basis, to obtain a similar maximal size of cell projected area. Thus, these effects on spreading closely complemented with

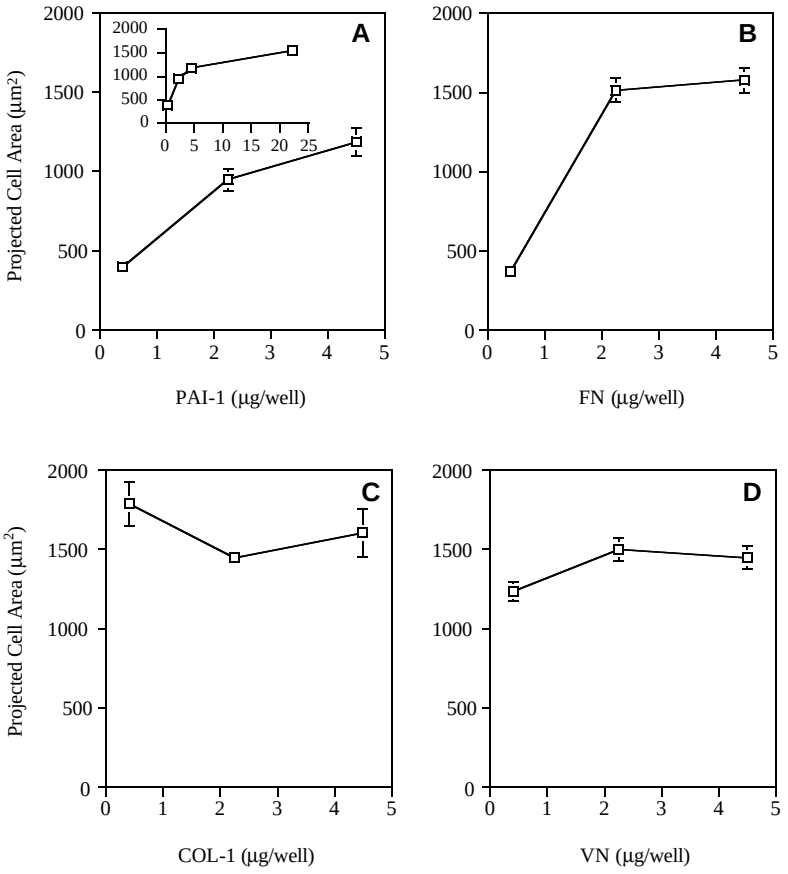

Fig. 5. Spreading of myogenic cells on PAI-1 and other substrates coated on plastic dishes. The wells of 96-well plates were precoated with different ligands in PBS for 3 hours. Then the cells were plated in serum-free medium with 1% BSA for 3 hours. The adherent cells were fixed in glutaraldehyde and stained with Crystal Violet. The projected cell areas were measured using a computarized imaging analysis system. In this representative experiment the values represented were the mean of at least 60 cells/well ± s.d. of 3 wells. Similar results were obtained in two other independant experiments.

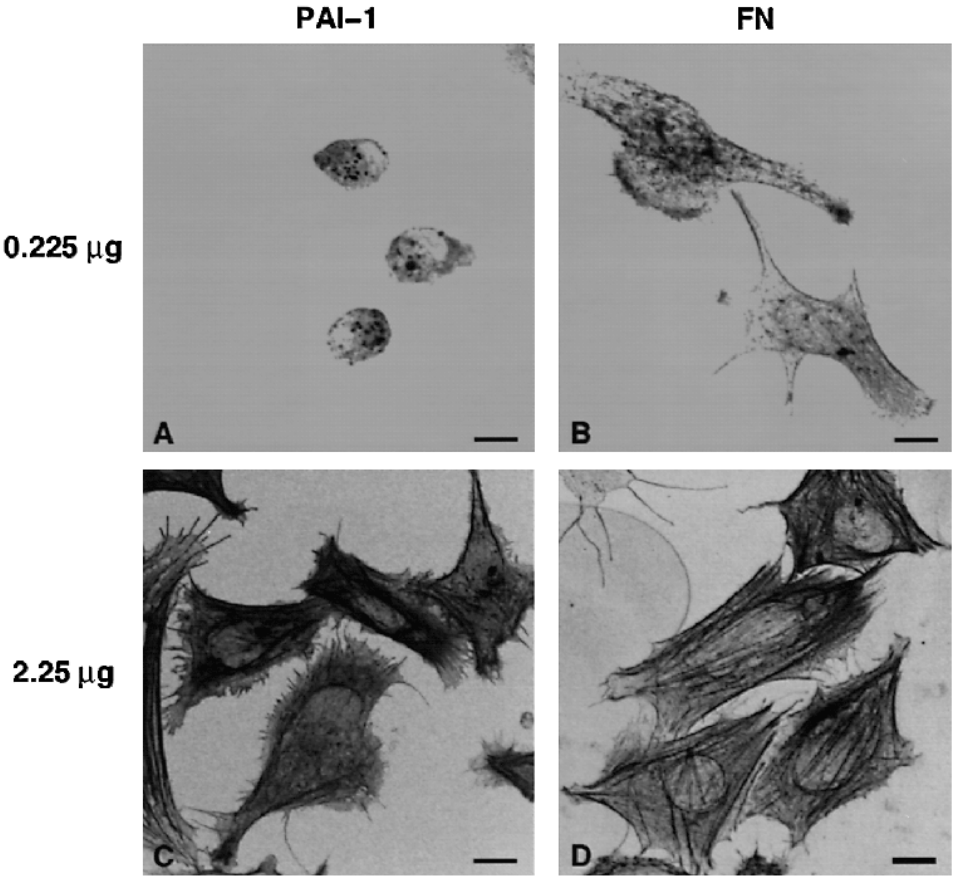

Fig. 6. Confocal microscopic images of myogenic cells plated on PAI-1 or FN for 3 hours and stained for F-actin. Compare PAI-1 coated glass slides (A: 0.225 μg/well; C: 2.25 μg/well) with FN coated glass slides (B: 0.225 μg/well; D: 2.25 μg /well). Cell spreading appeared to have more filopodia on PAI-1 (C) than on FN (D). Note that, at low concentrations of PAI-1, the cells spread less when adherent on coated glass rather than on coated plastic dish (compare A and B with results in Fig. 5A and B). Bars, 10 μm.

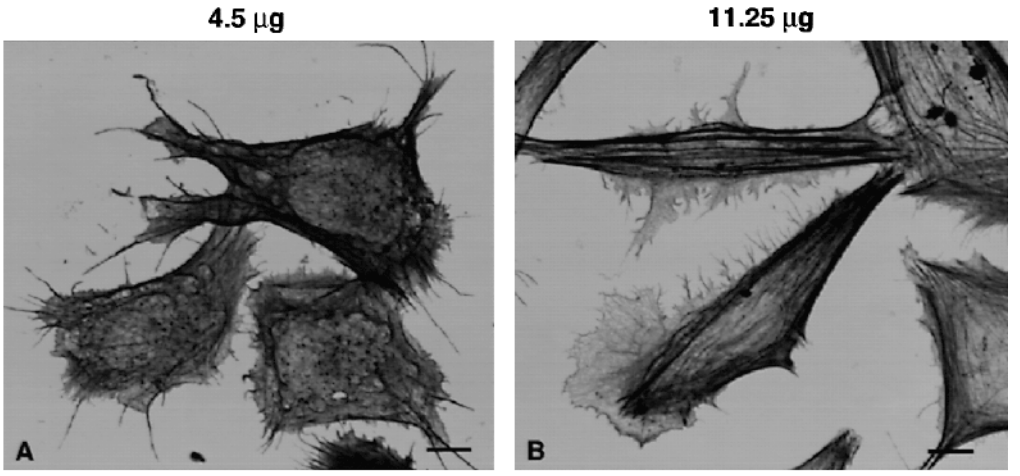

Fig. 7. Confocal microscopic images of myogenic cells adherent on increasing concentration of immobilized PAI-1 (A: 4.5 μg/well; B: 11.25 μg/well) stained for F-actin close to the basal surface of the cells. The size and the number of filopodia appeared to increase with immobilized PAI-1 concentrations. Bar, 10 μm.

the effects of these different molecules on cell adhesion (Fig. 1). In addition, analysis by confocal microscopy of spread cells labeled with rhodaminated phalloidin on PAI-1 coated glass slides revealed many thin processes stained for actin or filopodia along the cell periphery (Fig. 6C). In contrast, cells on FN appeared to have fewer filopodia (Fig. 6D). These extensions (number and length) appeared to rise as the density of immobilized PAI-1 was raised (Fig. 7). Quantitative analysis by confocal microscopy of a cell shape index (defined as the ratio of the cell perimeter to the cell projected area) revealed that cells had more cell perimeters on PAI-1 (0.34± 0.11μm/μm^2; n=43 cells) than on FN (0.23±0.06 μm/μm^2; n=49 cells) (P<0.001) regardless of cell size at the coating density of 2.25 μg per well (Fig. 8). Interestingly, a few cells adherent on PAI-1 coated dishes appeared to be very spread (Fig. 8).

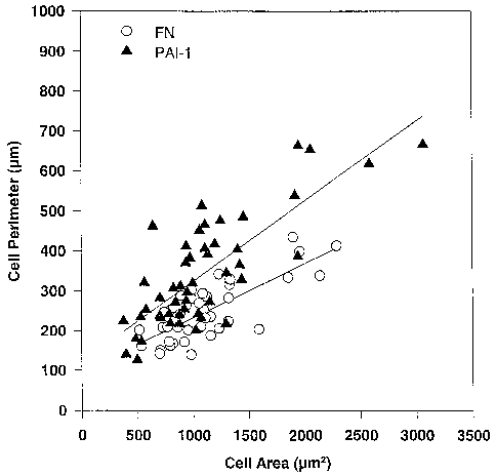

Fig. 8. Quantitation of cell perimeters and projected cell areas of myogenic cells plated on PAI-1 or FN. Plating concentrations were 2.25 μg per well for both ligands. Cell shape index was determined by plotting cell area against cell perimeter and defined as the ratio of the cell perimeter to the cell projected area. Note that for a given cell area, the mean cell perimeter is higher on PAI-1 (0.34±0.11 μm/μm^2) than on FN (0.23±0.06 μm/μm ^{2}) (P<0.001), indicating more extensions in those cells. Each symbol represents one cell chosen randomly.

PAI-1 adhesion is blocked with antibody against αvβ3 integrin

Several previous studies showed that uPAR/uPA colocalizes with focal adhesions (Pollanen et al., 1987), with vinculin (Hebert et al., 1988), and with αvβ3 in rhabdomyosarcoma cells (Myohanen et al., 1993). We hypothesized that αvβ3 integrin might be linking uPAR/uPA/PAI-1 with the CSK in myogenic cells. To test this, we treated cells with antibodies against αvβ3 (ab-αvβ3, 100 μg/ml) at the time of plating. As a result, cell adhesion was completely abolished on PAI-1 coated dishes (Fig. 9). As expected, the same dose of ab-αvβ3 inhibited cell adhesion on VN coated dishes by more than 85%. The same concentration of ab-αvβ3 did not have any inhibitory effects on cell adhesion on Col-1 coated dishes.

DISCUSSION

Several previous studies suggest a possible role for uPA/uPAR in cell adhesion independent of the enzymatic activity of the uPA (Nusrat and Chapman, 1991; Waltz et al., 1993; Wei et al., 1994). However, the direct role of matrix-bound PAI-1 in cell adhesion has not been explored. In the present study, we have demonstrated that PAI-1 can mediate cell adhesion and spreading of cultured normal human myogenic cells in the absence of serum. In addition, the PAI-1 dependent cell adhesion and spreading depends on the presence of membrane-bound uPA and uPAR. Cells on PAI-1 exhibit a different cell profile and have more filopodia than on FN, VN, or Col-1. Interestingly, αvβ3 integrins appear to play an important role in PAI-1 mediated cell adhesion and spreading. Thus our data suggest a potential role for PAI-1 in mediating cell adhesion through the binding of uPA.

PAI-1, a molecule found in the ECM, has been the focus of research over the last several years on its role in inhibiting the enzymatic activities of uPA and tPA and regulating fibrinolysis in vivo (see review by Reilly et al., 1994). In vivo PAI-1 is immobilized in the ECM by vitronectin and binding of PAI-1 to vitronectin is specific (Seiffert and Loskutoff, 1991; Mimuro et al., 1993; Lawrence et al., 1994; Van Meijer et al., 1994; Sigurdardottir and Wiman, 1994). Since PAI-1 and VN are closely related, we wondered if the observed PAI-1 adhesion was due to contamination of PAI-1 with VN. We have several

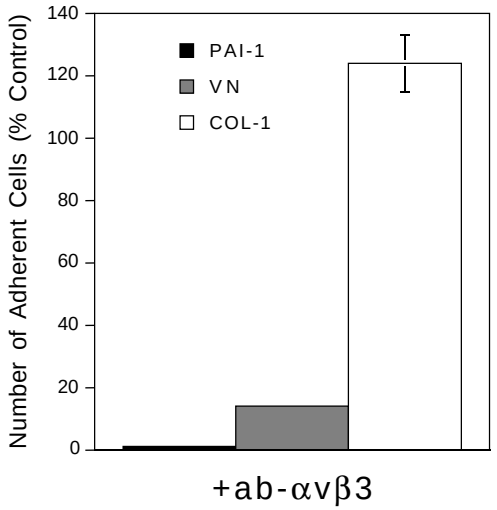

Fig. 9. Inhibition of cell adhesion on PAI-1 coated dishes by the addition of soluble antibodies against αvβ3 (ab-αvβ3). Ab-αvβ3 (100 μg/ml, Chemicon) was added at the time of cell plating and it completely inhibited cell adhesion on PAI-1 coated dishes. It also inhibited cell adhesion on VN coated dishes by 85% as expected whereas it had no inihibitory effects on cell adhesion on Col-1 coated dishes. Values are means ± s.d. (*n*=3 wells). Similar results were obtained in three other experiments.

lines of evidence that this is very unlikely: (1) ab-VN that blocked VN adhesion had no inhibitory effects on PAI-1 adhesion; (2) ab-PAI-1 that blocked PAI-1 adhesion had no effects on VN adhesion; (3) PAI-1 used in the present study migrated as a single band at about 40 kDa in a 10% SDS-polyacrylamide gel. All these suggest that adhesion on PAI-1 is specific and different from VN mediated cell adhesion.

Importantly, although cell adhesion on PAI-1 is similar to that on FN, cell shape appears to be very different. It is interesting to note that cells on PAI-1 have more thin long extensions reminiscent of filopodia than on FN (Figs 6, 7, 8). This difference in cell shape appears to persist regardless of cell size. Therefore, the kinetics of cell spreading on PAI-1 and its associated CSK reorganization appears to be very different from that of the observed cell spreading on FN.

Cell adhesion and spreading are the result of a cooperative action among adhesive systems, CSK, and the generation of forces across the cell surface (Ingber et al., 1994; Gumbiner, 1996). We have recently shown that uPAR complexed with uPA coated beads can transfer mechanical forces to the CSK (Wang et al., 1995). In this study we have shown that the presence of uPAR and membrane-bound uPA is required to mediate PAI-1 dependent adhesion. The complex of uPAR/uPA/PAI-1 is known to have a rapid turnover rate (Cubellis et al., 1990). All three components of the complex have been shown to be present in our cells (Quax et al., 1992) and many other cell types. We suggest that the uPAR/uPA/PAI-1 complex could assist in the cycle of cytoskeletal-mediated cell migration processes: extension, formation of adhesive contacts at the leading edge of the cell and break of these contacts followed by cytoskeletal retraction (Sheetz, 1994; Lauffenburger and Horwitz, 1996). Therefore we speculate that a possible role of the uPAR/uPA/PAI-1 complex might be in repeated sequences of adhesion/de-adhesion, i.e. migration.

The mechanical and biochemical signaling mechanisms of GPI-linked proteins are poorly understood (Jacobson et al., 1995). Since uPAR is a GPI-linked membrane protein and does not have a cytoplasmic tail (Ploug et al., 1991), it has to be physically connected with some other transmembrane molecule(s) that is coupled with the CSK in order to mediate specific mechanical signaling. Our present data suggest that αvβ3 might be a candidate that mediates mechanical coupling between GPI-linked uPAR and the CSK in myogenic cells. It is interesting to note that it has been observed that N-cadherin uses FGF receptors for chemical signaling (Williams et al., 1994) whereas our present data and previous results (Wang et al., 1995) suggest that a GPI-linked enzyme receptor (uPAR) might use an adhesion receptor (αvβ3 integrin) for mechanical signaling. Our results are consistent with previous studies that uPAR/uPA colocalizes with focal adhesions (Pollanen et al., 1987), with vinculin (Hebert et al., 1988), and with αvβ3 in rhabdomyosarcoma cells (Myohanen et al., 1993). A recent study in human monocytes showed that uPAR, β2 integrins, and Src-kinases are within a single receptor complex (Bohuslav et al., 1995). This complex formation of uPAR with β2 (CR3) was also shown in the involvement of neutrophil chemotaxis (Gyetko et al., 1995). Thus it appears that different cell types might couple different integrins with the uPA complex. However, how the uPAR/uPA/PAI-1 complex interacts with αvβ3 in myogenic cells and/or other transmembrane molecules is still unknown.

There has been a lot of interest in the study of the focal adhesion complex, a solid state microdomain in the cytoplasmic part of the membrane. The focal adhesion complex integrates mechanical as well as biochemical signals from the integrin to the CSK (Plopper and Ingber, 1993; Wang et al., 1993; Schwartz and Ingber, 1994; Plopper et al., 1995). Our present study suggests the existence of an extracellular multimolecular complex on the outside of the adhesion site (extracellular focal adhesion complex or EFAC) that consists of uPAR, αvβ3, uPA, PAI-1, and possibly VN or other molecules in myogenic cells. ECM remodeling, cell migration as well as cell adhesion may be all sensitive to specific modulations of one of these molecules. Recently, integrin-mediated adhesion has been shown to be regulated by the transfected uPAR that forms a stable complex with β1 integrin in human embryonic kidney 293 cells (Wei et al., 1996). Soluble PAI-1 has recently been shown to inhibit smooth muscle cell migration by blocking integrin αvβ3 binding to VN (Stefansson and Lawrence, 1996).

Our present findings of PAI-1 mediated cell adhesion and spreading are consistent with previous results showing increased amounts of PAI-1, uPA, and uPAR in normal migrating cells or metastasizing tumor cells (Sumiyoshi et al., 1991; Pepper et al., 1992, 1993; Shetty et al., 1995; Liu et al., 1994). Importantly, recent results that antibodies against PAI-1 resulted in inhibition of cultured cancer cell invasion (Liu et al., 1994) cannot be explained by the conventional function of PAI-1 (i.e. solely inhibiting enzymatic activity of uPA) but could be interpreted as follows: when PAI-1 dependent cell adhesion was blocked with the addition of antibodies, cell invasion was inhibited because cell migration had been inhibited. Thus our data could help to understand the dual function of PAI-1: to control the enzymatic activity of uPA and to promote cell migration as well as cell adhesion. Finally, our study could shed light on the participation of the uPAR/uPA/PAI-1 complex in

muscle regeneration that depends on the migration and fusion of myogenic cells (Quax et al., 1992).

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