uPA Binding to PAI-1 Induces Corneal Myofibroblast Differentiation on Vitronectin

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PURPOSE. Vitronectin (VN) in provisional extracellular matrix (ECM) promotes cell migration. Fibrotic ECM also includes VN and, paradoxically, strongly adherent myofibroblasts (Mfs). Because fibrotic Mfs secrete elevated amounts of urokinase plasminogen activator (uPA), we tested whether increased extracellular uPA promotes the persistence of Mfs on VN.

METHODS. Primary human corneal fibroblasts (HCFs) were cultured in supplemented serum-free medium on VN or collagen (CL) with 1 ng/mL transforming growth factor β 1 (TGF β 1). Adherent cells were quantified using crystal violet. Protein expression was measured by Western blotting and flow cytometry. Transfection of short interfering RNAs was performed by nucleofection. Mfs were identified by α -smooth muscle actin (α -SMA) stress fibers. Plasminogen activator inhibitor (PAI-1) levels were quantified by ELISA.

RESULTS. TGF β 1-treated HCFs secreted PAI-1 (0.5 μ M) that bound to VN, competing with α v β 3/ α v β 5 integrin/VN binding, thus promoting cell detachment from VN. However, addition of uPA to cells on VN increased Mf differentiation (9.7-fold), cell-adhesion (2.2-fold), and binding by the VN integrins α v β 3 and β 5 (2.2-fold). Plasmin activity was not involved in promoting these changes, as treatment with the plasmin inhibitor aprotinin had no effect. A dominant negative PAI-1 mutant (PAI-1R) that binds to VN but does not inhibit uPA prevented the increase in uPA-stimulated cell adhesion and reduced uPA-stimulated integrin α v β 3/ α v β 5 binding to VN by 73%.

CONCLUSIONS. uPA induction of TGF β 1-dependent Mf differentiation on VN supports the hypothesis that elevated secretion of uPA in fibrotic tissue may promote cell adhesion and the persistence of Mfs. By blocking uPA-stimulated cell adhesion, PAI-1R may be a useful agent in combating corneal scarring. (*Invest Ophthalmol Vis Sci.* 2012;53:4765–4775) DOI: 10.1167/iovs.12-10042

Currently there is no approved therapy for the treatment of fibrosis, or ocular scarring. Because ocular scarring leads to vision loss, preventing scarring in the eye, including in the retina, trabecular meshwork, and cornea, is of unique importance. We focused on corneal stromal scarring and its correlation with the persistence of myofibroblasts (Mfs).^{1,2} Penetrating corneal injuries induce a significant wound healing response that repairs the tissue and seals the wound with replacement connective tissue. However, because ordered collagen fibers are necessary for corneal transparency, replacement tissue that is disorganized results in corneal haze and scarring.³ This condition is compounded by the persistence of Mfs in a healing wound that secrete large amounts of extracellular matrix (ECM) and protease inhibitors that prevent matrix degradation, resulting in excessive ECM accumulation.^{1,4,5} Furthermore, although strong integrin-mediated Mf adhesion promotes wound closure through increased cell-matrix tension, the persistence of Mfs also leads to excessive contracture.⁶

Corneal stroma Mfs differentiate from fibroblasts that originate either from keratocytes (resident stromal cells) or from fibrocytes (circulating fibroblasts from bone marrow) in response to epithelium-derived transforming growth factor β (TGF β).^{7–9} Because Mfs generate locally active TGF β , they participate in a positive feedback mechanism that maintains and generates more Mf differentiation, fibrotic ECM production, and contraction.^{10,11} Although neutralizing TGF β reduces Mf differentiation, it also prevents cell repopulation and wound closure.^{12–14} Thus, new antifibrotic strategies are needed that target Mf-specific proteins to reduce the Mf population and their fibrotic contributions.^{11,15,16}

Our study focused on the ECM component vitronectin (VN) and the serine proteinase inhibitor, plasminogen activator inhibitor-1 (PAI-1) and their relationship to Mf differentiation and scarring. At a site of injury in vascularized tissues, serum-derived VN binds to proteins on exposed collagen of the tissue, thus promoting integration of VN into the provisional wounding matrix.^{17,18} VN also binds to the surface of activated platelets. Platelet-derived PAI-1 binds to matrix-bound VN. In the avascular cornea after wounding, VN and PAI-1 from tears and epithelium have access to the stroma.^{19–22}

The conformation of VN is dependent upon context: soluble VN is detected in a closed conformation, whereas the RGD sequence and somatomedin B domain in VN bound to matrix are exposed. The RGD sequence in matrix-bound VN

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binds to the extracellular domain of integrins $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$, and its adjacent somatomedin B domain binds to PAI-1.²³ Together these integrins and PAI-1 have a coordinated role in promoting cell migration on the provisional matrix, which includes VN in two ways. First, because the PAI-1 binding site on VN (somatomedin B domain) is adjacent to the integrin binding site on VN (RGD domain), PAI-1 binding to VN sterically hinders integrin binding to VN, thus reducing strong cell adhesion.²⁴ Second, although PAI-1 binds to VN with high affinity, when PAI-1 binds to urokinase plasminogen activator (uPA), it dissociates from VN.²⁵ In addition, PAI-1 also detaches cells from VN by binding to and stimulating internalization of a complex that includes uPA, its receptor, uPAR, and integrins through the LRP receptor.^{26,27} The cycle of integrin internalization leading to the disruption of integrin/matrix adhesion and recycling of integrins back to the cell surface fosters directional movement on VN.²⁸ Therefore, it is paradoxical that in fibrotic disease, persistent Mfs are strongly adherent while surrounded by ECM containing VN and PAI-1.²⁹ Why, under normal wounding conditions, these factors promote wound closure and regenerative healing, whereas in fibrotic healing the overexpression of these factors strongly correlates with the persistence of adherent Mfs that induce scarring is unknown.

We focused on the contribution of increasing amounts of extracellular uPA on cell adhesion and Mf differentiation, because the amount of secreted uPA is significantly elevated in Mfs derived from fibrotic tissue.^{30,31} (Note that uPA protease activity is decreased because of TGF β -induced overexpression of PAI-1.) We hypothesized that the increase in secreted uPA (not bound to uPAR) can alter PAI-1 binding to matrix VN, leading to an increase in the mediation of cell adhesion on VN by the integrins $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$. Increased cell adhesion generates the mechanical tension needed to incorporate α -smooth muscle actin (α -SMA) into stress fibers, a marker that identifies Mfs. Therefore, we proposed that the uPA-mediated increase in cell adhesion generates Mfs. Our data support this hypothesis and suggest that by blocking $\alpha\text{v}\beta 3$ - and $\alpha\text{v}\beta 5$ -mediated cell adhesion to VN, we may be able to reduce the persistence of Mfs in fibrotic tissue.

MATERIALS AND METHODS

Antibodies and Reagents

Western blot antibodies for integrins $\beta 5$ (ab15459) and $\beta 3$ (ab75872) and for flow cytometry (ab7167) and antibodies for uPA (Western blot; ab24121), PAI-1 (ab20562), VN (ab11591), and GAPDH (ab36845) were from Abcam. α -SMA antibody (04-1094) was from Millipore (Billerica, MA). Integrin $\beta 5$ antibody for flow cytometry (MAB2528) was from R&D Systems (Minneapolis, MN). Horseradish-peroxidase-labeled, fluorescein isothiocyanate (FITC)-labeled, or Cy3-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). VN protein was from BD Biosciences (San Jose, CA) and was used at 2 $\mu\text{g}/\text{mL}$ for plate coating. Bovine collagen type I (CL) was from Advanced Biomatrix, Inc. (San Diego, CA) and was used at 10 $\mu\text{g}/\text{mL}$ for plate coating. uPA protein was from Fisher Scientific (Pittsburgh, PA) and was used at a concentration of 50 units/mL. Aprotinin was from MP Biomedicals (Santa Ana, CA) and was used at a concentration of 20 $\mu\text{g}/\text{mL}$. PAI-1R protein (nonprotease-inhibiting PAI-1)³² was applied at a concentration of 100 nM.

Cells and Medium

Human primary corneal fibroblasts (HCFs) were derived from stroma of human corneas that were not suitable for transplantation (obtained from National Disease Research Interchange, Philadelphia, PA). Stromal fibroblasts were isolated as previously described³³ and maintained in

complete medium (Dulbecco's modified Eagle's medium [DMEM]-F12 medium [Invitrogen Life Technologies, Grand Island, NY]) with 10% fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA) with ABAM (antibiotic-antimycotic) and gentamicin (Sigma-Aldrich Corp., St. Louis, MO). For experiments, cells were plated on 2 $\mu\text{g}/\text{mL}$ VN or 10 $\mu\text{g}/\text{mL}$ CL. Identical results were observed for all CL concentrations tested, 1 to 100 $\mu\text{g}/\text{mL}$, and all VN concentrations tested, 2 to 10 $\mu\text{g}/\text{mL}$. Cells were seeded in supplemented serum-free medium consisting of DMEM-F12 plus 1 $\times$ RPMI-1640 vitamin mixture, 1 $\times$ insulin transferring selenium liquid medium supplement, 1 $\mu\text{g}/\text{mL}$ glutathione (all from Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acids (Invitrogen) with ABAM and gentamicin. For experiments where α -SMA stress fibers were detected, cells were cultured for 72 hours with 1 ng/mL TGF $\beta 1$ prior to the addition of 50 units/mL uPA (with) or PBS (without) for 3 hours. In all other experiments, HCFs were treated for 24 hours with 1 ng/mL TGF $\beta 1$ prior to addition of 50 units/mL uPA (with) or PBS (without) for 3 hours.

Western Blots

Protein was separated on 10% bis-Tris precast gels (Life Technologies Corp., Grand Island, NY) and transferred to polyvinylidene fluoride membranes. Primary antibody was added to 5% BSA or milk in tris-buffered saline (TBS), and secondary antibody was added to 1% milk in TBS with 1% Tween 20. Bands were visualized with ECL reagent (Thermo Fisher Scientific, Rockford, IL).

Immunoprecipitation

HCFs were seeded on VN and treated for 72 hours with 1 ng/mL TGF $\beta 1$. After 3-hour treatment with or without 50 units/mL uPA, HCFs were detached with enzyme-free cell dissociation buffer (product code S-014-B; Millipore) with protease inhibitor cocktail (Roche, New York, NY) and 1 mM phenylmethanesulfonyl fluoride PMSF and removed by gentle scraping. Matrix was solubilized with 1% Triton buffer (1% Triton X-100, 140 mM NaCl, 10 mM Tris, pH 7.6) with protease inhibitor cocktail and 1 mM PMSF and the matrix was collected by scraping. For each immunoprecipitation, 5 μg of VN antibody was added to 0.5 mg of total protein and incubated overnight at 4°C. Eluted proteins were separated under reducing conditions. Western blots were processed in 5% BSA in TBS and incubated with anti-PAI-1 antibody. Bands were visualized with ECL reagent (Thermo Fisher Scientific).

Immunocytochemistry

HCFs were plated on VN or CL and incubated for 72 hours with 1 ng/mL TGF $\beta 1$. After 3-hour treatment with or without 50 units/mL uPA, cells were fixed with 3% *p*-formaldehyde (Fisher Scientific) and permeabilized with 0.1% Triton X-100 (Sigma). After being blocked with 3% normal mouse serum (Jackson Immuno Research), cells were incubated with α -SMA antibody and then secondary antibody labeled with Cy3. Coverslips were viewed with a Zeiss AxioScope microscope (Carl Zeiss Microscopy, Thornwood, NY), and images were captured using a Zeiss AxioScope with a SPOT-2 charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) and processed using Photoshop software (Adobe Systems, Inc., San Jose, CA).

Adhesion Assay

HCFs (2.5×10^4 cells) were seeded on VN-coated 96-well plates with 1 ng/mL TGF $\beta 1$. After 24 hours, cells were treated with or without 50 units/mL uPA, 100 nM PAI-1R, or 20 $\mu\text{g}/\text{mL}$ aprotinin. Cells were fixed with 100% methanol for 30 minutes at room temperature and washed with water. Then the cells were stained with 100 μL of filtered 0.1% crystal violet for 60 minutes at room temperature. After the crystal violet was aspirated and wells were washed three times with 400 μL of water, the dye was solubilized in 100 μL of 10% (v/v) acetic acid and incubated for 5 minutes at room temperature. Absorbance was

measured at 570 nm, using a plate reader (Synergy 2; BioTek, Winooski, VT).

Cross-Linking Assay

HCFs were plated on VN and incubated for 24 hours with 1 ng/mL TGF β 1. After 3-hour treatment with or without 50 units/mL uPA, cells and matrix were cross-linked with 1 mM bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (Thermo Fisher Scientific) for 10 minutes and then quenched twice with a buffer containing (50 mM Tris-HCl [pH 7.2] and 100 mM NaCl). Cells were then removed by lysis with 0.1% SDS and protease inhibitor cocktail, PMSF, and phosphatase inhibitor (Thermo Scientific), and plates were washed three times with PBS. After washing, plates were incubated in 50 mM NaHCO₃ (pH 11.6) and 0.1% SDS with protease inhibitor, PMSF, and phosphatase inhibitor at 37°C with shaking for 2 hours to reverse cross-linking. The soluble fraction was concentrated by using a centrifugal filter (Amicon Ultra; Millipore) and stored at -80°C for Western blotting. A lack of GAPDH on Western blots confirmed that cells were not present in the protein fraction that was released from the matrix.

Flow Cytometry

HCFs were seeded on VN with 1 ng/mL TGF β 1 for 24 hours prior to detachment with enzyme-free cell dissociation buffer (Millipore). After washing with ice-cold PBS containing 3% BSA, 100 μ L of cells at 1×10^6 cells/mL were treated with antibody to the integrin α v β 5 (1 μ g/10⁵ cells) or antibody to the integrin α v β 3 (1 μ g/10⁵ cells) at 4°C for 30 minutes. Cells were then washed and treated with FITC-labeled secondary antibody (1:400 dilution) at 4°C for 30 minutes. After washing and staining with propidium iodide (1 μ g/mL; MP Biomedicals), immunostaining was measured using a model C6 flow cytometer (BD Accuri; BD Biosciences).

PAI-1 ELISA

HCFs were seeded on VN and cultured with 1 ng/mL TGF β 1 for 24 hours. Conditioned medium was collected, and PMSF and protease inhibitor were added before freezing at -80°C. After samples were thawed, they were diluted 1:2 and 1:5 by using the kit dilution buffer (Quantikine human serpin E1/PAI-1 immunoassay kit; R&D Systems). ELISA was performed according to the manufacturer's instructions. Absorbance was measured at 450 nm (reference, 540 nm) using a microplate reader (Synergy 2; BioTek).

Statistical Analysis

Numerical data are means $\pm$ standard error of the means for three independent experiments. *P* values were calculated using Student's *t*-test, where **P* < 0.05, ***P* value < 0.01, and ****P* < 0.001 (see Figs. 1–5 legends).

RESULTS

Adhesion and Mf Differentiation of HCFs on VN Were Less than Those on CL

TGF β released after wounding induces PAI-1 secretion. Binding of PAI-1 to VN in the provisional matrix stimulates cell migration by promoting the cycles of cell detachment and reattachment necessary for migration.²⁸ However, in vitro, 1 ng/mL TGF β ("high concentration") induces high levels of PAI-1 that promote cell detachment without reattachment.²⁴ We determined that after treatment with 1 ng/mL TGF β 1 for 24 hours, the concentration of secreted PAI-1 was 0.51 μ M with or without 0.07 (ELISA data not shown), a high concentration for PAI-1 that would induce cell detachment from VN.²⁴ Consistent with this, HCFs plated on VN and treated with 1

ng/mL TGF β 1 had 25% reduced cell adhesion after 1 hour compared to cells plated on CL (Fig. 1A). After 24 hours, the difference became more striking; VN-seeded cells were 75% less adhesive than those on CL. Because the organization of α -SMA stress fibers that characterize Mfs is dependent upon enhanced integrin-mediated cell adhesion and the effects of VN and PAI-1 promote cell detachment, it was predicted that TGF β -induced Mfs could not form on VN. As predicted when HCFs were plated on VN and treated with TGF β 1, there were significantly fewer Mfs on VN than on CL (Figs. 1B, C).

uPA-Induced Organization of α -SMA Containing Stress Fibers in HCFs on VN

Scarring and fibrosis in vivo are characterized by Mfs that secrete elevated levels of uPA.^{30,31} Thus, we asked whether elevated uPA contributes to Mf differentiation in scar tissue despite the presence of PAI-1 and VN. Although HCFs secreted some uPA with and without treatment with TGF β 1,³³ we tested whether increasing the extracellular concentration of uPA would increase Mf adhesion on VN. HCFs were seeded on VN and treated for 72 hours with 1 ng/mL TGF β 1. uPA was added to the cell culture medium 3 hours prior to fixation. Addition of uPA to these HCFs stimulated cell spread and incorporation of α -SMA into stress fibers (Fig. 2A). Eighty percent of cells treated with uPA were Mfs compared to 10% in nontreated cultures (Fig. 2B), and cell adhesion was 2.1-fold increased (see Fig. 5A, lanes 1 and 2). To investigate whether the dramatic increase in α -SMA containing stress fibers after uPA treatment was the result of an increase in total α -SMA protein, the same experiment was performed, and lysates from uPA-treated and -nontreated cells were lysed and Western blotted for α -SMA. We found that the 3-hour treatment with uPA did not increase the total levels of α -SMA (Fig. 2C). Thus, addition of uPA induced Mf differentiation in TGF β -treated HCFs on VN.

uPA Removed PAI-1 from VN

We hypothesized that treatment with uPA would alter PAI-1 binding to VN and promote integrin-mediated HCF adhesion leading to organization of α -SMA containing stress fibers on VN. This hypothesis was based on the finding that, although HCF cell adhesion was decreased on VN in the presence of TGF β -stimulated PAI-1 (Fig. 1), which binds to VN and sterically hinders integrin/VN binding,²⁴ uPA removes PAI-1 from VN.²⁵ Because cell detachment was evident by 24 hours (Fig. 1A), this time point was used in the remaining experiments to test changes in cell adhesion.

To test our hypothesis that uPA was removing HCF-secreted PAI-1, we evaluated whether adding uPA would decrease the amount of PAI-1 bound to VN matrix. HCFs were seeded on VN for 24 hours in the presence of TGF β 1 prior to treatment with or without uPA for 3 hours. HCFs were then detached with an EDTA-containing buffer, and the matrix was lysed and immunoprecipitated for VN and Western blotted to detect PAI-1. After uPA treatment, we could not detect PAI-1 bound to VN, demonstrating that uPA removed HCF-secreted PAI-1 from VN matrix (Fig. 3A, top). Western blotting of total cell lysate confirmed the presence of PAI-1 in each sample (Fig. 3A, bottom cellular PAI-1).

Next, we tested whether increasing endogenously secreted uPA would reduce PAI-1 binding to VN. One way to increase free uPA is to reduce its binding to uPAR, its receptor on the cell surface. To reduce the amount of uPAR that could bind uPA, HCFs were transfected with uPAR-targeted short interfering RNA (siRNA) or control siRNA and were seeded on VN in the presence of TGF β 1 for 24 hours. Conditioned medium (secreted uPA) and lysates of detached HCFs (cellular uPAR)

FIGURE 1. Adhesion and Mf differentiation of HCFs on VN was less than that on CL. **(A)** HCF adhesion on CL and VN. HCFs were seeded on CL- or VN-coated 96-well plates for 1 hour or 24 hours in the presence of TGF β 1 prior to fixation and detection with crystal violet. Adhesion on VN compared to CL was reduced at each time point. **(B)** HCFs were seeded on either CL or VN and treated with TGF β 1 for 72 hours prior to fixation and immunodetection for α -SMA containing stress fibers (red, arrow). The nucleus is stained with 4',6-diamidino-2-phenylindole (blue). Bar = 100 μ m. **(C)** The percentage of Mfs (HCFs with α -SMA containing stress fibers) on CL and VN were counted and graphed as percents of total cells. Mf formation on VN is significantly reduced. Standard errors of the means between experiments are shown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$. $N = 3$ for each experiment.

and matrix PAI-1 were evaluated. We found that reducing uPAR expression increased secreted uPA and reduced PAI-1 bound to matrix VN (Fig. 3B). Together these data confirmed previous results that uPA removes PAI-1 from VN and extended these findings by demonstrating that increased endogenously secreted uPA correlates with decreased PAI-1 bound to VN.

uPA Treatment Induced Integrin α v β 3 and α v β 5 Binding to VN

Because organization of α -SMA containing stress fibers is induced by increased integrin-mediated binding to ECM, we asked whether VN-binding integrins (α v β 3 and α v β 5) had greater association with VN after uPA treatment. In Figure 4A, HCFs were seeded on VN for 24 hours with TGF β 1 and then incubated with or without uPA for 3 hours, after which they were treated with a cleavable cross-linking reagent. After cross-linking, a buffer containing 0.1% SDS removed the cells, leaving behind cell membrane proteins that were cross-linked to matrix. The cross-linked proteins were released from the matrix, and equal amounts of protein in each sample were analyzed for β 3 and β 5 content by Western blotting. (Absence of detectable GAPDH on Western blots confirmed that cells were not present in the protein fraction

that was released from the matrix.) We found that both the integrin β 3 and integrin β 5 binding to VN was 2.3-fold increased after treatment with uPA (Fig. 4A). Western blotting for total integrin confirmed the fact that, as expected, this 3-hour treatment with uPA did not increase the total amount of the integrins (Fig. 4B). Finally, using flow cytometry, we demonstrated that addition of uPA does not change cell surface levels of β 3 and β 5 (Fig. 4C). These data demonstrate that uPA induced α v β 3 and α v β 5 binding to matrix VN.

uPA-Promoted Cell Adhesion on VN Is Plasmin-Independent

Addition of uPA to TGF β -treated HCFs on VN showed 2.1-fold increased cell adhesion (Fig. 5, lanes 1 and 2). Next, to determine whether the effect of uPA was dependent on its activation of plasmin, we tested whether blocking plasmin activity with the general serine protease inhibitor aprotinin would decrease uPA-induced cell adhesion. Statistically similar differences between samples with and those without the addition of uPA treated with or without aprotinin (Fig. 5, compare lanes 1 and 2 with lanes 5 and 6) suggest that the plasmin activity is not primarily involved in cell adhesion on VN.

FIGURE 2. uPA induced the organization of α -SMA containing stress fibers in HCFs on VN. (A) HCFs were seeded on VN and treated with TGF β 1 for 72 hours. Before fixation, cells were treated with or without 50 units/mL uPA for 3 hours and then fixed and immunodetected for α -SMA (red, arrow). The nucleus is stained with 4',6-diamidino-2-phenylindole (blue). Bar = 100 μ m. Images from two experiments are shown. Addition of uPA stimulated Mf differentiation on VN. (B) The percentage of Mfs under the two conditions were counted and graphed as a percentage of total cells. (C) α -SMA protein expression was similar in HCFs with or without uPA treatment. HCFs were treated as shown in (A) prior to lysis with radioimmunoassay radioimmunoprecipitation buffer and then Western blotted for α -SMA. GAPDH controls were used for equal loading. Addition of uPA did not affect α -SMA expression but did affect its incorporation into stress fibers. $N = 3$ for each experiment. ** $P < 0.01$.

PAI-1R Reduced Cell Adhesion on VN

Our data indicated that uPA reduced TGF β -induced PAI-1 binding to VN and, in turn, increased integrin-VN binding. To test whether uPA induced cell adhesion on VN by binding to

and “removing” PAI-1, we used a dominant negative PAI-1 mutant (PAI-1R)²⁴ that does not inhibit uPA but replaces PAI-1 on VN. We asked whether PAI-1R would prevent the increase in uPA-stimulated cell adhesion on VN. Addition of 100 nM PAI-1R along with uPA to TGF β 1-treated HCFs cultured on VN

FIGURE 3. uPA removed PAI-1 from VN. **(A)** HCFs were seeded on VN with TGF β 1 for 24 hours prior to treatment for 3 hours with or without uPA. Next, cells were detached, and, to detect PAI-1 associated with the matrix, the matrix was lysed in 1% SDS, immunoprecipitated for VN and Western blotted for PAI-1 (*top*). Detached cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and Western blotted for PAI-1 and GAPDH (*bottom*). Treatment with uPA dissociated PAI-1 from VN. **(B)** To increase secreted uPA, HCFs were transfected with uPAR-targeted siRNA (siuPAR) or control siRNA (control) and seeded on VN in the presence of TGF β 1. After 24 hours, medium was collected for the detection of secreted uPA, cells were detached and lysed in RIPA and the matrix was lysed with 1% SDS. Samples were Western blotted for uPA, uPAR, and PAI-1. The increase in secreted uPA correlates with a decrease in PAI-1 on the matrix. $N = 3$ for each experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

prevented the uPA-mediated increase in cell adhesion (Fig. 5A, lane 3). Addition of aprotinin (Fig. 5A, lane 7) did not significantly affect these results (Fig. 5A, compare lanes 3 and 7). Also, addition of PAI-1R without uPA did not further decrease cell adhesion (Fig. 5A, compare lanes 4 and 8), suggesting that, with a 3-hour incubation, PAI-1R blocked primarily uPA-stimulated integrin-matrix adhesions during formation rather than establishing adhesions. Finally, to link the reduced cell adhesion in the presence of PAI-1R to a decrease in integrin-VN binding, we added uPA along with PAI-

1R, as described in Figure 5A, and then performed the integrin cross-linking experiment, as described in Figure 4A. We found that uPA induced a 2.2-fold increase in β 3 and β 5 binding to VN, confirming the data in Figure 4A. Furthermore, we found that PAI-1R reduced uPA-stimulated β 3 and β 5 adhesion to VN by an average of 73% (Fig. 5B).

Next we tested whether uPA-stimulated Mf differentiation on VN was blocked with PAI-1R addition. Treatment with uPA generated α -SMA-containing stress fibers with and without PAI-1R; however, samples treated with PAI-1R had significantly less

FIGURE 4. uPA treatment induced the integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ binding to VN. HCFs were seeded on VN with TGF $\beta 1$ for 24 hours prior to treatment with or without uPA for 3 hours. (A) HCFs were treated with a cleavable cross-linker, and cells were removed by lysis with 0.1% SDS. Next, the cross-linking was reversed, releasing protein bound to the matrix. This fraction was concentrated, and equal amounts of protein were Western blotted for detection of the integrin subunits $\beta 5$ and $\beta 3$ and for GAPDH. The absence of GAPDH demonstrates that cells had been removed prior to releasing the cross-linked fraction. Addition of uPA increased integrin binding to VN. (B) HCFs were lysed with radioimmunoprecipitation assay buffer and Western blotted for $\beta 3$, $\beta 5$, and GAPDH. GAPDH controls were used for equal loading. (C) Cell surface levels of integrins $\beta 3$ and $\beta 5$ were measured by flow cytometry. uPA treatment did not alter total or cell surface integrin expression. $N = 3$ to 5 for each experiment. * $P < 0.05$ and ** $P < 0.01$.

A

B

C

D

FIGURE 5. PAI-1R reduced cell adhesion on VN. (A) HCFs were seeded on VN with TGFβ1 for 24 hours prior to treatment with combinations of uPA, PAI-1R, or aprotinin for 3 hours, as shown, and then assayed for adhesion by crystal violet staining. Addition of uPA increased cell adhesion (compare lanes 1 and 2), while PAI-1R treatment reduced uPA-mediated effects (compare lanes 1 and 3). Aprotinin did not impact adhesion (compare lanes 1–4 to lanes 5–8). (B) Cross-linking assay for HCFs with uPA or PAI-1R treatment, as described in the legend to Fig. 4A. PAI-1R reduced the uPA-mediated increase in integrin binding to VN. (C) Cells were seeded on VN and incubated with 1 ng/mL TGFβ1 for 72 hours. Before fixation, cells were treated with uPA or uPA plus PAI-1R for 3 hours and then fixed and immunodetected for α-SMA (red). The nucleus was stained with 4',6-diamidino-2-phenylindole (blue). Bar = 100 μm. (D) Cells treated as described in the legend to C were quantified α-SMA-stress fibers containing cells that were either spread or were narrow and detaching. PAI-1R reduced Mf spreading. *N* = 3 to 5 for each experiment. **P* < 0.05, ***P* < 0.01, ****P* < 0.005.

cell attachment, and fewer of those cells that were attached had spread (62% decrease). Representative images are shown in Figure 5C, and quantification of spread cells with α-SMA stress fibers are shown in Figure 5D. Together these data

support the hypothesis that excess extracellular uPA can induce cell adhesion on VN, proving a possible mechanism for the persistence of TGFβ-induced Mfs on VN in fibrotic disease. In addition, PAI-1R reduced cell adhesion even in the presence

of TGF β 1 and excess uPA, suggesting that it may be useful for combating ocular scarring.

DISCUSSION

The persistence of Mfs in wounded tissue correlates with fibrotic disease,³⁴ and thus, uncovering mechanisms that promote Mf differentiation is important to our fundamental understanding and treatment of fibrosis. Cell surface receptors such as integrins, thrombospondin, and cation-independent mannose-6-phosphate receptor with and without proteases play a role in the activation of matrix-bound latent TGF β complex into active TGF β .^{10,35–38} Although it has been established that the activation of TGF β is a key to the generation of fibrosis, the mechanisms that promote the persistence of Mfs versus Mf apoptosis or reversal of the Mf phenotype remain unclear.³⁹ Toward the goal of preventing persistent Mfs, researchers have focused on the matricellular connective tissue growth factor (CTGF/CCN2) protein that surrounds Mfs in fibrotic tissue.^{40,41} Although the precise role of CTGF in fibrosis is unknown, silencing of the CTGF gene expression has produced antifibrotic effects in many tissues, including the eye,^{41–43} and reagents that target CTGF (antibodies and RNAi) are in clinical trials for several fibrotic diseases (Fibrogen⁴⁴; RXi Pharmaceuticals [Byrne MJ, et al. *IOVS* 2012;53:ARVO E-Abstract 897]). Other strategies for reducing or preventing fibrosis include an inhibitory peptide that prevents incorporation of α -SMA into stress fibers, which reduces collagen type I synthesis in vitro and wound contraction in vivo.⁴⁵ A more general approach is to promote apoptosis of Mfs by protein kinase inhibitors, which interferes with activation of focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K)-AKT (pathways that protect Mfs from apoptosis).^{15,16} A recent study demonstrating that FGF-2 selectively induced Mf but not fibroblast apoptosis suggests that FGF-2 may be a potential antifibrotic therapy as well.⁴⁶

In this study, we focused on the expression and secretion of uPA and PAI-1 that are increased in fibrotic disease.^{29–31} How this imbalance contributes to fibrosis has been intensively studied; however, the connection between their increased expression and the generation of fibrotic disease remains elusive. PAI-1 and VN coordinate to detach cells through a disruption in integrin-mediated adhesion,²⁸ thus, it is paradoxical that Mfs persist in a matrix that contains VN and PAI-1.²⁹ Here we report that addition of uPA in the presence of TGF β -stimulated PAI-1 promoted Mf differentiation on VN. Our data support the hypothesis that uPA “removes” PAI-1 from VN, permitting integrins α v β 3 and α v β 5 access to binding VN (presumably at the RGD domain), leading to significantly increased cell adhesion and Mf differentiation. PAI-1R, a dominant negative PAI-1 mutant,³² decreased β 3/ β 5-mediated cell adhesion on VN, even in the presence of excess uPA. We hypothesize that by decreasing cell adhesion, PAI-1R could combat the persistence of Mf in healing tissue and make PAI-1R a candidate for therapy that prevents corneal scarring.

uPA in Wounded Tissue and Fibrotic Tissue

Because ECM accumulation, a key marker of fibrosis, is in part a result of inhibition of uPA activity, a primary focus in fibrotic research has been the inhibition of uPA activity by PAI-1. Fewer studies have focused on the plasmin-independent functions of uPA in fibrosis or have considered the idea that upregulation of uPA is part of the healing program but that continued significant uPA secretion plays a role in generating fibrosis. For instance, upregulation of uPA at both mRNA and protein levels was

observed in the leading edge of corneal cells migrating into a wound as part of a normal healing program.^{47–49} However, under fibrotic conditions such as in gingival granulation tissue-derived fibroblasts, compared to normal fibroblasts from the same tissue, uPA synthesis was significantly elevated,³⁰ and in patients with liver cirrhosis, serum concentrations of soluble uPA were also significantly increased.⁵⁰ In our study, to mimic the enhanced accumulation of uPA under fibrotic conditions, uPA was added to normal HCFs. Mf differentiation (Fig. 2) and higher cell adhesion (Fig. 5) were observed after uPA treatment, even with aprotinin, supporting the hypothesis that the nonproteolytic actions of soluble uPA contribute to Mf adhesion.

uPA/uPAR Binding Also Influences Cell Adhesion

Correlating with the upregulation of uPA in fibrotic tissue is our finding that uPAR is downregulated on TGF β 1-treated human corneal Mfs after 7 days in culture, whereas Mf differentiation can be prevented by maintaining uPA bound to uPAR on the cell surface.³³ More recently, we extended these findings by demonstrating that uPA/uPAR controls Mf differentiation in part through the control of integrin α v β 5 cell surface levels. We found that silencing either uPA or uPAR resulted in an inhibition of the integrin β 5 degradation pathway. This led to the accumulation of α v β 5 on the cell surface and Mf-like differentiation (increased cell area and organization of α -SMA-containing stress fibers).⁵¹ Interestingly, a mouse model with abrogated uPA/uPAR binding had fibrin deposition and chronic inflammation, which often precedes fibrotic disease.⁵² Together, these studies with our current data suggest that the net increase in uPA secretion in fibrotic disease (resulting from increased secretion and/or altered uPAR binding) leads to an increase in Mf cell adhesion by increasing total integrin levels and integrin-matrix binding. The α v integrins (α v β 3, α v β 5, and α v β 6) also locally activate TGF β generating a feed-forward mechanism that increases persistent Mfs.^{35,36} Correspondingly, blocking α v β 6 integrin has shown promise in reducing fibrosis in animal models⁵³; inhibitors of the α v β 5 integrin have yet to be fully developed.⁵⁴

Targeting PAI-1

Because PAI-1-mediated inhibition of uPA's protease activity contributes to fibrosis, many studies have investigated the effects of silencing PAI-1. However, silencing PAI-1 has yielded complex and mixed results. Lack of PAI-1 caused cardiac-selective fibrosis⁵⁵ and an increase in Mf differentiation in PAI-1 null mouse embryonic fibroblasts⁵⁶ but protected lungs, liver, and kidney fibrosis (reviewed in ref. 57). An alternative to silencing PAI-1 is to alter the protein binding and antiprotease properties of endogenous PAI-1. The mutant version, PAI-1R, competes with endogenous PAI-1 for binding to VN to decreasing integrin-mediated binding (Fig. 5). Because PAI-1R does not inhibit uPA,³² there is a net increase in free uPA activity. This is seen in a mouse model of kidney fibrosis, in which injection of PAI-1R decreased ECM accumulation in fibrotic kidneys, which was correlated with an increase in uPA-mediated plasmin activity.^{58,59} Unfortunately its effect on Mfs was not evaluated. Our data suggest that even in the presence of TGF β 1 and elevated secreted uPA (“fibrotic conditions”), PAI-1R could reduce Mf adhesion, potentially leading to Mf apoptosis in vivo. Several other PAI-1 inhibitors have been tested for efficacy in limiting PAI-1 activity, such as PAZ-417, PAI-039, and PAI-1-749,^{60–62} and many other anti-PAI-1 agents are under development to inhibit fibrosis.^{63,64}

CONCLUSIONS

In summary, we have elucidated a mechanism by which excess uPA leads to the progression of fibrotic disease. We found that excess uPA promotes $\alpha v\beta 3/\alpha v\beta 5$ -mediated Mf differentiation on VN and that PAI-1R, the PAI-1 inhibitor that binds to VN but does not inhibit uPA, blocks this adhesion. With a net increase in uPA activity that reduces ECM accumulation coupled with a decrease in Mf adhesion, PAI-1R may be useful for preventing or reducing scarring in the eye.

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