Materials and Methods

Recombinant PAI-1 mutants

Recombinant human PAI-1 was expressed and purified as described.[1] These were: 1) PAI-1-14-1b (PAI-1 N150H, K154T, Q319L, M354I), which inhibits u-PA and t-PA and binds VN with wild-type (WT) activities. PAI-1-14-1b is resistant to conversion to the inactive (i.e. latent) form (half-life >140 hrs). Throughout this study PAI-1-14-1b is referred to as "PAI-1-WT." 2) PAI-1-R (T333R, A335R), a reactive center loop mutant that binds VN normally, but has no detectable anti-proteolytic activity and cannot assume a latent conformation.[2] 3) PAI-1-AK (PAI-1 N150H, K154T, Q319L, M354I, R101A, Q123K), an active, stable mutant with no detectable VN binding.[3] 4) PAI-1-E (PAI-1 N150H, K154T, Q319L, M354I, R76E), an active stable mutant with markedly reduced binding affinity for LRP1 and other LDL receptor family members, but normal VN binding affinity.[4] 5) PAI-1-AKE (PAI-1 N150H, K154T, Q319L, M354I, R101A, Q123K, R76E), an active, stable mutant with markedly reduced binding to LRP1 and other LDL receptor family members and no detectable binding to VN.

Reagents

Collagen type I was from Sigma. Growth-factor-reduced BD Matrigel Matrix (BD Biosciences) and anti-$\alpha_v\beta_3$ integrin antibody were from Chemicon International. Human VN was from Promega. Human fibronectin and antibodies to focal adhesion kinase (FAK), β_3 integrin, platelet/endothelial cell adhesion molecule-1 (PECAM-1), and PAI-1 were from Santa Cruz Biotechnology. Mouse anti-human early endosome antigen 1 (EEA1) antibody was from Abcam. Antibodies to phosphorylated and non-phosphorylated p44/42, phosphorylated VEGFR-2 (Tyr 1175), and total VEGFR-2 were from Cell Signaling Technology. Antibody against very low density lipoprotein receptor (VLDLR), which specifically blocks ligand binding to this receptor (clone 1H10), was from Molecular Innovations.[5] Rat anti-mouse VN monoclonal antibody was from R&D Systems. Anti-smooth muscle α-actin antibody was from Sigma. Anti-β actin polyclonal antibody was from Cell Signaling Technology. Recombinant VEGF-A was from R&D Systems. The latent form of human PAI-1 and receptor-associated protein (RAP) were from Molecular Innovations. Anti-VEGF monoclonal antibody 2C3 was from ATCC. Peptide D2A-Ala (IQEGAAGRPKDDR), derived from domain 2 of uPAR, and which blocks uPAR binding to $\alpha_V\beta_3$,[6] as well as a control (scrambled) peptide (DEIGQDKERPGRE) were from NeoBioLab. PAI-039, a pharmacological inhibitor of PAI-1, was from Pfizer.

Animals

C57BL/6J mice were from Jackson Labs. C57BL/6J-congenic VN-deficient (*Vn*$^{-/-}$) mice were a gift from Dr. David Ginsburg, University of Michigan.[7] All animal care and experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Cell culture

Human umbilical vein endothelial cells (**HUVECs, Cascade** Biologics) and human dermal microvascular endothelial cells (**Cascade** Biologics) were grown in Medium 200 (**Cascade** Biologics) containing low-serum growth supplement. Cells used were passaged 3-7 times.

Immunoassays

To screen for potential binding of PAI-1 to VEGF, microtiter plate wells were coated with VEGF. After washing and blocking, PAI-1-WT (0.01-10 µg/mL) was added and incubated at room temperature for 1hr, after which biotinylated anti-PAI-1 antibody and streptavidin-HRP conjugate were sequentially added. After washing wells, HRP substrate was added and OD450 was measured. To screen for potential inhibition of binding of VEGF to VEGFR-2 by PAI-1, 96-well

plates were coated with recombinant VEGFR-2/Fc (R&D Systems). After washing and blocking wells, VEGF (25 ng/mL) was added in the presence or absence of PAI-1-WT (0.01-10µg/mL) and incubated for 1h. Bound VEGF was detected by sequential addition of biotinylated anti-human VEGF antibody, streptavidin-HRP conjugate, and HRP substrate and measurement of OD450.

Western blotting

HUVECs cell lysates were prepared as described.[8] Supernatants (25µg total protein) were subjected to SDS-PAGE (using either 7.5% homogeneous or 4-20% gradient acrylamide gels) and Western transfer. After blocking, membranes were incubated with rabbit or mouse IgG raised against human PAI-1, FAK, p44/42, or phosphorylated and non-phosphorylated forms of VEGFR-2. Secondary antibody was horseradish-peroxidase (HRP)-conjugated goat IgG raised against rabbit or mouse IgG (Santa Cruz Biotechnology). Blots were developed with ECL substrate (Pierce).

VEGFR-2 internalization

Internalization of VEGFR-2 was studied as described.[9] HUVECs surface membrane proteins were biotinylated using Cell Surface Isolation Kit (Thermo Scientific). Cells were incubated with PAI-1 (10 µg/mL) or vehicle control for 1 hr, then stimulated with VEGF (50 ng/mL) or vehicle control for 10min. Cell surface biotin was removed with glutathione, after which cell lysates were prepared and incubated with NeutrAvidin Agarose (Thermo Scientific). Captured proteins were subjected to SDS-PAGE and Western blotting with anti-VEGFR-2 antibody. Internalization of VEGFR-2 was also studied by immunofluorescence microscopy, as described,[10] with minor modification. Cell surface VEGFR-2 was labeled by incubating HUVECs grown on VN-coated coverslips with rabbit anti-human-VEGFR-2 antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit-IgG (Invitrogen). Cells were incubated with PAI-1-WT (10 µg/mL) or vehicle control for 4 hr, followed by VEGF (50 ng/mL) or vehicle control for 30 min. Cells were washed with ice-cold phosphate-buffered saline (PBS, pH 2.5) to remove remaining cell-surface labeled proteins, fixed with 4% paraformaldehyde, and permeabilized by treatment with 2% BSA,5% goat serum, and 0.1% Triton X-100 for 30 min. Cells were incubated with mouse anti-human EEA1 antibody followed by Alexa Fluor 647-conjugated goat anti-mouse IgG (Invitrogen). Nuclei were stained with DAPI, after which immunofluorescent staining was observed with an LSM 510 2-photon confocal microscope (Zeiss).

Silencing of PAI-1 gene expression

HUVECs were transfected with siRNA duplexes (5nM) directed against human PAI-1(target sequence TTCACGAGTCTTTCAGACCAA) or negative control siRNA (Qiagen) using HiPerFect reagent (Qiagen).

VEGFR-2 internalization

Internalization of biotinylated VEGFR-2 was studied as described.[9] HUVECs were incubated with PAI-1 (10 µg/mL) or vehicle control for 1 hr, then stimulated with VEGF (50 ng/mL) or vehicle control for 10min, after which internalization of VEGFR-2 was assessed by Western blotting of cell lysates. Internalization of VEGFR-2 was also studied by immunofluorescence microscopy, as described,[10] with minor modification. Additional details of the biotinylation and immunofluorescence methods can be found in the on-line supplement.

Co-immunoprecipitation

HUVECs ($2x10^5$) were incubated 4 hr in wells pre-coated with VN (10 µg/mL). PAI-1 (10 µg/mL) was added. One hr later cells were stimulated with VEGF (50 ng/mL) for 10min, then lysed in RIPA buffer (Cell Signaling Technology). Protein co-immunoprecipitation was performed with

Co-IP kit (Pierce). Anti-VEGFR-2 antibody was immobilized on coupling resin and incubated with lysates that were pre-cleared with control agarose resin. After extensively washing anti-VEGFR-2-bearing resin, bound proteins were eluted, resolved by SDS-PAGE, and analyzed by Western blotting. VEGFR-2 and β_3 integrin subunit were detected with appropriate antibodies. Secondary antibody was HRP-conjugated goat IgG raised against rabbit IgG (Santa Cruz Biotechnology).

Cell adhesion, migration, and tubule formation assays

HUVECs adhesion was studied with InnoCyte™ ECM Cell Adhesion Assay kit (Calbiochem) using 96-well plates coated with VN, fibronectin, or collagen. Cells (1×10^4) were added to wells, incubated 1 hr at 37°C, treated with recombinant PAI-1-WT (10 µg/mL) or vehicle control for 30 min, followed by addition of VEGF (50 ng/mL). HUVEC migration was studied using Transwell migration chambers (Life Technologies) with bottom membranes containing 8 µm pores. Membranes were coated with VN, fibronectin, or collagen, after which cells ($2x10^4$) were added to the upper chamber and treated with PAI-1-WT (10 µg/mL) or vehicle control for 30 min, followed by VEGF (50 ng/mL). After 24 hr at 37°C membranes were excised and stained. Cells that migrated to the lower-chamber were counted. Formation of tubules by HUVECs was studied as described,[11] using 24-well plates coated with growth-factor-reduced Matrigel. For some experiments VN (10 µg/mL) was added to Matrigel. A suspension of cells in culture medium was added into each coated well in the presence or absence of PAI-1 (10 µg/mL) 30 min before addition of VEGF (50 ng/mL). Cells were incubated 24 hr at 37°C, washed, fixed, and viewed through an IX70 inverted microscope (Olympus). Total tube length was measured in 5 fields by computer-assisted image analysis using ImagePro Plus software.[12]

Ex vivo tissue culture

Tissue culture was performed as described previously with minor modifications.[14] Briefly, thoracic aortic rings isolated from WT and *Vn*$^{-/-}$ mice were embedded between two layers of growth-factor-reduced Matrigel (250 µL/layer) in a 24-wellplate in the presence of Medium 200 (**Cascade** Biologics; 300 µL), Medium 200 containing VEGF (50 ng/mL), Medium 200 containing VEGF and recombinant PAI-1 (10 µg/mL), or Medium 200 containing only PAI-1. Medium was changed every 3 days. Aortic rings were photographed 14 days later. The number of sprouting microvessels was quantified by computer-assisted images analysis using ImagePro Plus software.[12]

In vivo angiogenesis assay

Matrigel implant experiments were performed as described,[13] with minor modifications. Growth-factor-reduced Matrigel WT (300 µL) was injected into subcutaneous tissue of WT- and *Vn*$^{-/-}$ mice. Matrigel contained heparin (60 U/mL) and one of the following 1) VEGF (250 ng/mL), 2) VEGF and PAI-1 (10 µg/mL), 3) PAI-1, or 4) vehicle control, yielding 8 experimental groups (n=6/group). After 14 days, Matrigel implants were excised, fixed, and sectioned. PECAM-1-positive infiltrating microvessels were quantified in 5 microscopic fields in each of 3 cross-sections of each implant using ImagePro Plus software.[12]

Mouse model of diet-induced PAI-1 over-expression

Obesity, hyperglycemia, and increased PAI-1 expression were induced in male C57BL/6J mice by feeding them a high-fat chow (HFC) diet (D12451; Research Diet, New Brunswick, NJ) for 14 weeks, as described previously.[14] Age-matched male mice fed a normal chow (NC) diet served as controls. Blood glucose levels were measured from tail vein blood samples using an automatic glucometer (Accu-Check; Roche Diagnostics, Mannheim, Germany).

Mouse hindlimb ischemia model

Unilateral hindlimb ischemia was induced in mice by ligation and excision of a segment of the left femoral artery, as previously described.[15] After recovering from surgery, mice were treated with PAI-039 (4 mg/kg/day; dissolved in vehicle consisting of sterile water containing 0.5% methylcellulose and 2% Tween 80), or vehicle control, administered for 14 days after surgery by twice daily oral gavage, with the first dose given 1 hour after surgery. PAI-039- and vehicle-treated mice continued to be fed HFC after surgery. Perfusion of the ischemic and non-ischemic hindlimb was measured in each mouse by laser-Doppler imaging (LDI) immediately before surgery, 1 hour after surgery, and at several subsequent time points using a moorLDI2-HIR high resolution laser Doppler imager (Moor Instruments). Mice were euthanized 14 days after surgery. Ischemic gastrocnemius muscle was excised, embedded in paraffin, and cross-sections were prepared for immunohistochemical analysis. Arterioles within gastrocnemius muscle were immunostained with anti-smooth muscle α-actin antibody and arteriole density was measured, as described previously.[16] Capillaries within gastrocnemius muscle were immunostained with anti-platelet endothelial cell adhesion molecule (PECAM-1) antibody, and capillary density was measured as described previously.[17]

Measurement of plasma PAI-1

Blood was collected into citrate anticoagulant and plasma was prepared by centrifugation. PAI-1 antigen was measured using a mouse PAI-1 total antigen assay ELISA kit (Molecular Innovations). PAI-1 activity was measured using an active mouse PAI-1 functional assay ELISA kit (Molecular Innovations).

Data analysis

Image analyses were performed in blinded fashion. Data are presented as mean ± standard error of the mean. Experimental groups were compared by the two-tailed Student's t-test or one-way analysis of variance (ANOVA).

References

1. Berkenpas MB, Lawrence DA, Ginsburg D. Molecular evolution of plasminogen activator inhibitor-1 functional stability. *EMBO J*. 1995;14:2969-2977.

2. Stefansson S, Su EJ, Ishigami S, Cale JM, Gao Y, Gorlatova N, Lawrence DA. The contributions of integrin affinity and integrin-cytoskeletal engagement in endothelial and smooth muscle cell adhesion to vitronectin. *J Biol Chem*. 2007;282:15679-15689.

3. Li SH, Gorlatova NV, Lawrence DA, Schwartz BS. Structural differences between active forms of plasminogen activator inhibitor type 1 revealed by conformationally sensitive ligands. *J Biol Chem*. 2008;283:18147-18157.

4. Stefansson S, Muhammad S, Cheng XF, Battey FD, Strickland DK, Lawrence DA. Plasminogen activator inhibitor-1 contains a cryptic high affinity binding site for the low density lipoprotein receptor-related protein. *J Biol Chem.* 1998; 273:6358-6366.

5. Ruiz J, Kouiavskaia D, Migliorini M, Robinson S, Saenko EL, Gorlatova N, Li D, Lawrence D, Hyman BT, Weisgraber KH, Strickland DK. The apoE isoform binding properties of the VLDL receptor reveal marked differences from LRP and the LDL receptor. *J Lipid Res*. 2005;46:1721-1731.

6. Degryse B, Resnati M, Czekay RP, Loskutoff DJ, Blasi F. Domain 2 of the urokinase receptor contains an integrin-interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor. *J Biol Chem.* 2005;280:24792-24803.

7. Zheng X, Saunders TL, Camper SA, Samuelson LC, Ginsburg D. Vitronectin is not essential for normal mammalian development and fertility. *Proc Natl Acad Sci U S A*. 1995;92:12426-12430.

8. Wu J, Stevenson MJ, Brown JM, Grunz EA, Strawn TL, Fay WP. C-reactive protein enhances tissue factor expression by vascular smooth muscle cells: mechanisms and in vivo significance. *Arterioscler Thromb Vasc Biol.* 2009;29:1565-1570.

9. Park SY, Shi X, Pang J, Yan C, Berk BC. Thioredoxin-interacting protein mediates sustained VEGFR2 signaling in endothelial cells required for angiogenesis. *Arterioscler Thromb Vasc Biol.* 2013; 33:737-743.

10. Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, Simons M. VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. *Dev Cell.* 2010;18:713-724.

11. Savani RC, Cao G, Pooler PM, Zaman A, Zhou Z, DeLisser HM. Differential involvement of the hyaluronan (HA) receptors CD44 and receptor for HA-mediated motility in endothelial cell function and angiogenesis. *J Biol Chem.* 2001;276:36770-36778.

12. Blatt RJ, Clark AN, Courtney J, Tully C, Tucker AL. Automated quantitative analysis of angiogenesis in the rat aorta model using Image-Pro Plus 4.1. *Comput Methods Programs Biomed*. 2004;75:75-79.

13. Nakano M, Satoh K, Fukumoto Y, Ito Y, Kagaya Y, Ishii N, Sugamura K, Shimokawa H. Important role of erythropoietin receptor to promote VEGF expression and angiogenesis in peripheral ischemia in mice. *Circ Res*. 2007;100:662-669.

14. Hazarika S, Dokun AO, Li Y, Popel AS, Kontos CD, Annex BH. Impaired angiogenesis after hindlimb ischemia in type 2 diabetes mellitus: differential regulation of vascular endothelial growth factor receptor 1 and soluble vascular endothelial growth factor receptor 1. *Circ Res.* 2007;101:948-956.

15. Kim HW, Lin A, Guldberg RE, Ushio-Fukai M, Fukai T. Essential role of extracellular SOD in reparative neovascularization induced by hindlimb ischemia. *Circ Res.* 2007;101: 409-419.

16. Waeckel L, Bignon J, Liu JM, Markovits D, Ebrahimian TG, Vilar J, Mees B, Blanc-Brude O, Barateau V, Le Ricousse-Roussanne S, Duriez M, TobelemG,Wdzieczak-Bakala J, Lévy BI, Silvestre JS. Tetrapeptide AcSDKP induces postischemic neovascularization through monocyte chemoattractant protein-1 signaling. *Arterioscler Thromb Vasc Biol.* 2006;26:773-779.

17. Tashiro Y, Nishida C, Sato-Kusubata K, Ohki-Koizumi M, Ishihara M, Sato A, Gritli I, Komiyama H, Sato Y, Dan T, Miyata T, Okumura K, Tomiki Y, Sakamoto K, Nakauchi H, Heissig B, Hattori K. Inhibition of PAI-1 induces neutrophil-driven neoangiogenesis and

promotes tissue regeneration via production of angiocrine factors in mice. *Blood.* 2012;119:6382-6393.