The role of urokinase plasminogen activator and plasmin activator inhibitor-1 on vein wall remodeling in experimental deep vein thrombosis


Objective: Deep vein thrombosis (DVT) resolution instigates an inflammatory response, resulting in vessel wall damage and scarring. Urokinase-plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1), are integral components of the fibrinolytic system, essential for venous thrombosis (VT) resolution. This study determined the vein wall response when exposed to increased and decreased plasmin activity.

Methods: A mouse inferior vena cava (IVC) ligation model in uPA+/− and PAI-1+/− and their genetic wild types (B6/SvEv and C57/BL6, respectively) was used to create stasis thrombi, with tissue harvest at either 8 or 21 days. Tissue analysis included gene expression of vascular smooth muscle cells (alpha smooth muscle actin [αSMA], SM22) and endothelial marker (CD31), by real-time polymerase chain reaction, enzyme-linked immunosorbent assay, matrix metalloproteinase (MMP)-2 and -9 activity by zymography, and vein wall collagen by picro-Sirius red histologic analysis.

A P < .05 was considered significant.

Results: Thrombi were significantly larger in both 8-day and 21-day uPA+/− as compared with wild type (WT) and were significantly smaller in both 8-day and 21-day PAI-1−/− as compared with WT. Correspondingly, 8-day plasmin levels were reduced in half in uPA+/− and increased three-fold in PAI-1−/− when compared with respective WT thrombi (P < .05; n = 5-6). The endothelial marker CD31 was elevated two-fold in PAI-1−/− mice at 8 days, but reduced 2.5-fold at 21 days in uPA+/− as compared with WT (P = .02; n = 5-6), suggesting less endothelial preservation. Vein wall vascular smooth muscle cell (VSMC) gene expression showed that 8-day and 21-day PAI-1−/− mice had 2.3- and 3.8-fold more SM22 and 1.8- and 2.3-fold more αSMA expression than respective WT (P < .05; n = 5-7), as well as 1.8-fold increased αSMA (+) cells (P < .05; n = 3-5). No significant difference in MMP-2 or -9 activity was found in the PAI-1−/− mice compared with WT, while 5.4-fold more MMP-9 was present in 21-day WT than 21-day uPA+/− (P = .03; n = 5). Lastly, collagen was two-fold greater at 8 days in PAI-1−/− IVC as compared with WT (P = .03; n = 6) with no differences observed in uPA+/− mice.

Conclusions: In stasis DVT, plasmin activity is critical for thrombus resolution. Divergent vein wall responses occur with gain or loss of plasmin activity, and despite smaller VT, greater vein wall fibrosis was associated with lack of PAI-1.

(J Vasc Surg 2012;56:1089-97.)

Clinical Relevance: DVT resolution is dependent on many factors. Clinical studies suggest that slowly resolving extensive DVTs are associated with greater likelihood of post-thrombotic syndrome, and this is experimentally modeled by vein wall fibrosis and inflammation. The current study suggests a more complex relationship in that the plasmin-mediated events are central to thrombus resolution as well as to how the vessel wall responds. Surprisingly, a larger thrombus from a stasis model is not associated with greater vein wall damage. Thus, novel modalities such as PAI-1 inhibition may require supplemental anticoagulants for full efficacy.

Deep vein thrombosis (DVT) is estimated to affect more than 300,000 people in North America and 460,000 people in the European Union each year. Approximately one-third of patients with DVT will develop post-thrombotic syndrome (PTS), which impairs quality of life and productivity. The rate at which DVT resolves has been found to correlate with the development of PTS. Little is known about what occurs at the vein wall level after DVT in relation to PTS. Current management is limited to elastic compression stockings, and other approaches such as pneumatic compression units and short-term use of venoactive agents are being explored. DVT resolution is an inflammatory process that affects vessel wall remodeling. Leukocytes, chemokines, and hemostatic regulators play a role in both thrombosis and vein wall inflammation. Vein wall remodeling in the rodent after venous thrombosis (VT) involves profibrotic growth factors, collagen deposition, a change in the vein wall cell milieu, and matrix metalloproteinase (MMP) expression and activation. Currently, both the mechanism of throm-
bus formation and the length of contact time between the thrombus and the vein wall are thought to influence the vessel wall response. After vascular injury, there is denudation of the endothelial lining, deposition of new extracellular matrix, increased vascular smooth muscle cell (VSMC) proliferation and migration, and a switch of VSMC to a cellular synthetic state. While all of these changes have been characterized in the arterial vasculature, the vessel wall undergoing DVT in the presence of increased or decreased plasmin has not been similarly characterized.

Plasmin, a serine protease formed by the cleavage of its proenzyme plasminogen, is the primary fibrinolytic enzyme responsible for DVT resolution. Plasminogen activators are serine proteases that activate plasminogen to the enzyme plasmin. The major plasminogen activator found in circulation is urokinase-plasminogen activator (uPA), and the major inhibitor of plasminogen activators is plasminogen activator inhibitor-1 (PAI-1). While the studies on the role of PAI-1 and risk of DVT have been inconsistent, impaired thrombus resolution has been observed in uPA −/− mice. Moreover, the role of PAI-1 in vascular response depends on the model, genetic background, and timing of analysis. By manipulating uPA and PAI-1 through genetically deleted mice, corresponding larger and smaller VTs can also be indirectly examined to assess the vein wall effect.

In this study, we hypothesize that greater plasmin activity (PAI-1 −/−) would be associated with less vein wall fibrosis, and less plasmin activity (uPA −/−) would be associated with greater fibrosis in a stasis model.

METHODS

Animal model. Male mice from B6/SvEv wild type (WT), B6/SvEv uPA −/− (kindly donated by Drs D. Collen and P. Carmiliet), C57BL6 WT and C57BL6 PAI-1 −/− (kindly donated by Dr D. Lawrence) were used for all studies. Because of genetic background differences, each genetic deletion group was only compared with their respective genetic background controls. All animal studies were done with University of Michigan Animal Use Committee approval. For all surgical procedures, the mice underwent general anesthesia with isoflurane/O2. Stasis venous thrombosis was induced by inferior vena cava (IVC) ligation. A laparotomy with ligation of the IVC below the renal veins and division of all visible side branches was done. At sacrifice, at 8 and 21 days postligation, the thrombosed IVC was carefully dissected and removed for histologic and biochemical studies. The thrombus is easy to remove at or before 8 days. After this, the thrombus and vein wall form a residual fibrosed remnant through 21 days. Thus, at 8 days, the thrombus and vein wall were able to be assessed separately, while at 21 days, these were examined together. This time frame was chosen to correlate with mid to late vein wall remodeling after DVT, and when PTS develops.

Histology/immunohistochemical/collagen staining. Tissue samples were formalin fixed, paraffin embedded, and cut into 5-µm sections as described. Nonspecific sites were blocked with normal serum, and sections were incubated with primary antibodies to Mac2 (1:200; Cedarlane Laboratories, Burlington, NC), alpha smooth muscle actin (αSMA; 1:500; Santa Cruz Biotechnology, Santa Cruz, Calif), and fibroblast specific protein-1 (FSP-1; 1:500; Santa Cruz Biotechnology). A species-specific avidin-biotin complex (ABC) peroxidase kit for either rabbit or rat (Vector Laboratories Inc, Burlingame, Calif) was used according to the manufacturer’s instructions for the corresponding secondary antibody and subsequent steps. Color development was performed with diaminobenzidine (DAB). The slides were counterstained with hematoxylin. In a blinded fashion, positive cells in five high-power fields (1000x) radially around the IVC were counted and totaled.

Picrosirius red staining to quantify collagen content was performed as described. These sections were then analyzed in crossed-plane polarized light from a monochromatic source to assess crosslinked collagen. Two images for each were obtained using a Zeiss Axio M1 scope and Zeiss AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) at 0 and 90 degrees to the plane of polarization, in order to capture the birefringence of fibers extinguished in one direction. The images were analyzed blindly utilizing National Institutes of Health ImageJ software. The area corresponding to the vein wall was selected as a region of interest, and then the image underwent threshold segmentation to differentiate collagen from other (mainly cellular and empty space) components of the vein wall. A vein wall collagen score was assigned by the formula [(% birefringent area) × (measured vein wall area)]/(total specimen area).

To account for noncollagen vein wall changes, intimal thickness scoring was assessed from hematoxylin and cosin sections as described. A consistent midsection thrombosed IVC segment was used for all histologic analysis.

Real-time quantitative polymerase chain reaction. Expression of β-actin, αSMA, SM22, CD31, procollagen I (Col1a2), and procollagen III (Col3a) was determined as follows: RNA was isolated by treatment of IVC wall segments with TRIzol reagent and reverse transcribed by incubating with Oligo(dT) primer and M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, Calif) at 94°C for 3 minutes followed by 40°C for 70 minutes. The resultant cDNA was amplified by Taq Polymerase (Promega, Madison, Wisc) in the Rotogene quantitative polymerase chain reaction system (Qiagen Inc, Valencia, Calif). SYBR Intercalating Dye (Roche, Indianapolis, Ind) was used to monitor levels of cDNA amplification for each gene. Primer sequences were derived using validated primer sequences from Superarray (Fredrick, Md). Rotogene quantification utilizes the cycle threshold (Ct) for the gene of interest normalized to the housekeeping gene β-actin. Relative mRNA expression is calculated by the formula 2−(Ct target gene−Ct reference gene), and cycle lengths used are within the exponential phase of the polymerase chain reaction. These primers include β-actin PPM02945A Ref Pos 164 to 183; αSMA PPM04483A
Enzyme-linked immunosorbent assay. Tissue homogenate of the thrombus for plasmin and fibrinogen (Innovative Research, Novi, Mich), collagen IV (Exocell, Philadelphia, Pa), and thrombin-antithrombin complex (TAT; Enzyme Research Laboratories, South Bend, Ind) were determined using a commercial kit according to manufacturer’s instructions. Quantification of peptide mediators was normalized to total protein as described.9,13

Sodium dodecyl sulfate polyacrylamide gel electrophoresis gelatin zymography. As described,9,10 homogenate of the thrombus for plasmin and fibrinogen (Innovative Research, Novi, Mich), collagen IV (Exocell, Philadelphia, Pa), and thrombin-antithrombin complex (TAT; Enzyme Research Laboratories, South Bend, Ind) were determined using a commercial kit according to manufacturer’s instructions. Quantification of peptide mediators was normalized to total protein as described.9,13

Statistical analysis. All data are represented as mean ± SE. Two-tailed unpaired Student’s t-test was used for comparison between control and genetically deleted group (GraphPAD Prism, San Diego, Calif); *P < .05 was assigned significance.

RESULTS

Thrombus resolution is altered in uPA−/− and PAI-1 −/− mice. As measured by weight to length of the thrombosed IVC, uPA−/− mice had significantly larger thrombi than WT at both 8 days (n = 13; P = .03) and at 21 days (n = 17; P < .01; Fig 1, A). This phenotype is consistent with past studies derived from a slightly different model.17 Correspondingly, uPA−/− mice had less plasmin activity, less thrombus fibrinogen, and TAT at 8 days than WT (n = 6; P = .02; Fig 1, B-D). Conversely, PAI-1−/− mice had significantly smaller thrombi than WT at both 8 days (n = 12; P < .01) and at 21 days (n = 17; P < .01; Fig 1, E). The PAI-1−/− mice had significantly more plasmin activity at 8 days (n = 6; P = .03; Fig 1, F), but no differences in thrombus fibrinogen or TAT (data not shown). However, no significant difference in plasmin in either group was found at 21 days (data not shown). Grossly, the thrombus histomorphology...
was different with a large nonnucleated cell thrombus in uPA $−/−$ mice and a small cellular thrombus in PAI-1 $−/−$ at 21 days (Fig 2, A-D). While the early 2-day time point was not a focus of this study, we found the thrombi were larger in uPA $−/−$ as compared with WT ($4.0 \pm 0.17$ vs $3.1 \pm 0.2$ mg/mm; $n = 4-6; P = .009$). Conversely, PAI-1 $−/−$ were not significantly different at the 2-day time point ($3.0 \pm 0.24$ vs $2.6 \pm 0.10$ mg/mm; $n = 7-8; P = .24$).

Vein wall endothelial gene expression in uPA $−/−$ and PAI-1 $−/−$ mice. Denudation of the endothelium is associated with the mechanical stretch and inflammatory injury caused by thrombi.10,22,25 There was no difference in 8-day CD31 gene expression, an endothelial marker, in uPA $−/−$ as compared with WT ($0.20 \pm .14$ vs $0.8 \pm .4; n = 6; P = .48$) However, there was $\sim$2.5-fold greater CD31 gene expression in 21-day WT than in 21-day uPA $−/−$ mice ($n = 5-6; P = .02$) (Fig 3, A). Conversely, PAI-1 $−/−$ mice had two-fold greater CD31 gene expression at 8 days compared with WT ($n = 5-7; P = .03$; Fig 3, B).

As a measure of the noncollagen component of the vein wall, the intimal thickness score was used.20 A loss of intimal thickness was found at 8 days in uPA $−/−$ mice compared with WT (Fig 3, C-E). No significant difference was observed in the uPA $−/−$ intimal thickness as compared with WT at 21 days. No difference in intimal thickness was found in the PAI-1 $−/−$ as compared with WT at 8 or 21 days (data not shown).

Vein wall fibrosis is increased in PAI-1 $−/−$ after VT. Vein walls become thickened and fibrotic in stasis VT in both mice and in humans.10,12,27 We found that procollagen I and III gene expression was elevated three-fold in PAI-1 $−/−$ as compared with WT ($P < .05$; Fig 4, A and B). No differences in these gene expressions in procollagen I or III gene expression in uPA $−/−$ as compared with WT were found (not shown). Consistently, vein wall collagen content was elevated two-fold in 8-day PAI-1 $−/−$ as compared with WT ($2.2 \pm .45$ vs $0.9 \pm .16$ AU; $n = 6; P = .02$), and a trend at 21 days ($6.2 \pm 1.8$ vs $3.1 \pm 1$ AU, $n = 5-6; P = .13$; Fig 4, C-E). No significant differences in vein wall collagen content were found at 8 or 21 days in uPA $−/−$ as compared with WT.

Vein wall cellular and proteinase response in uPA $−/−$ and PAI-1 $−/−$ mice after VT. To assess if there were differences in matrix VSMC, we assessed for gene expression and for antigen expression by immunohistochemistry. PAI-1 $−/−$ mice had significantly more vein...
wall SM22 gene expression, at both 8 days \(n = 5-7; P = .03\) and 21 days \(n = 5-7; P = .03; \text{Fig} 5, A\), than WT. In addition, PAI-1 \(--/--\) mice also had significantly more \(\alpha\)-SMA expression at both 8 days \(n = 5-7; P = .01\) and 21 days \(n = 5-7; P = .03; \text{Fig} 5, B\). We found a corresponding 1.8-fold increase in \(\alpha\)-SMA (+) cells at 8 days in PAI-1 \(--/--\) as compared with WT \(n = 3-5; P = .02; \text{Fig} 5, C-E\). No difference in fibroblast type cells were found \(\text{FSP-1} + \) cells: \(3.7 \pm 0.3 \text{ vs} 3.4 \pm 7; n = 3-5; P = .8\) at 8 days in PAI-1 \(--/--\) compared with WT. No significant difference was found in uPA \(--/--\) vein wall VSMC marker gene expression as compared with WT at 8 or 21 days.

Monocytes direct vascular response to injury, including the vein wall response. At 8 days, no significant difference in monocyte \(\text{Mac-2}^+\) vein wall counts was observed in any of the groups. However, at 21 days, increased monocytes were found in both the PAI-1 \(--/--\) \(19 \pm 5 \text{ vs} 8 \pm 1; n = 5; P = .02\) and uPA \(--/--\) \(55 \pm 8 \text{ vs} 5 \pm 2; n = 5; P = .01\) vein wall as compared with WT controls.

MMPs are present in the vein wall after VT and metabolize the extracellular matrix to allow for VSMC migration, proliferation, and vessel remodeling.\(^{12,28}\) Despite an increased plasmin activity level in PAI-1 \(--/--\) mice, no significant difference in vein wall MMP-2 or -9 zymographic activity was found in PAI-1 \(--/--\) at 8 or 21 days, when compared with WT. A significant decrease in MMP-9 activity at 21 days in uPA \(--/--\) mice as compared with WT was found \(40 \pm 15 \text{ vs} 10 \pm 5 \text{ AU/mg protein}; n = 5; P = .03\), but no significant differences in MMP-2 activity at 8 or 21 days were found in uPA \(--/--\) or PAI-1 \(--/--\) mice (data not shown).

DISCUSSION

PTS affects up to 30% of people after DVT,\(^{2,3}\) leading to a significant decrease in quality of life.\(^4\) Management of PTS pharmacologically has been limited in part by a lack of understanding the underlying pathophysiology of the disease. However, observations suggest that rapid DVT resolution is associated with lower risk of PTS,\(^5\) but no specific pharmacologic agents are available that directly address this problem.\(^5\) The main findings of this study suggest that increased or decreased plasmin results in a divergent injury response at the cellular and genetic level. While this study specifically evaluated the role of increased or decreased
plasmin on the vein wall response after VT, the indirect role of thrombus size may also have contributed to the phenotypes herein. Presumably, a larger thrombus should confer greater vein wall damage, both mechanically and due to presence of inflammatory cells and mediators at the thrombus-vein wall interface, but this was not the case.

Zhou and colleagues showed early endothelial injury cracking and denudation after stasis VT, with exposure of prothrombotic elements. We also have shown early endothelial loss with recovery at 2 weeks in a rat model of stasis VT, with low-molecular-weight heparin associated with less endothelial loss at 14 days after thrombosis. In the current study, loss of plasmin activity (uPA−/−) was not associated with an increase in fibrotic injury, but primarily affected the endothelial response, with less late CD31 gene expression. Of note, nonthrombosed sham CD31 gene expression is ~20-fold higher than thrombosed 8-day IVC expression. This is also counter to our initial hypothesis that less plasmin would be associated with worsened fibrotic injury. Our data contrast with the models of arterial injury where endothelial denudation is associated with increased neointimal fibrosis. Indeed, not only was no increase in fibrosis observed with loss of uPA, but the neointimal response was significantly less than control WT. Several factors may account for this difference, including the local shear stress, distension, and cellular elements unique in the venous thrombus. Less thrombin (as measured by TAT complex) was also observed in the uPA−/− thrombi, and thrombin has profibrotic effects. Further, less fibrinogen, per weight of thrombus, was also found in the uPA−/− thrombi, and fibrinogen may stimulate leukocyte proinflammatory and secondary profibrotic activities. Moreover, lack of uPA in an arterial model was associated with increased vascular injury, but was predominately luminal occlusion and not the vessel wall. Similar to our findings, no increase in medial fibrosis was observed in uPA−/− arterial walls after balloon injury.

These observations also suggest that the thrombosis mechanism is likely more important than the size of the thrombus itself in the vein wall injury response. For example, in the rat model, the greatest injury was observed with a stasis compared with a nonstasis model. Supporting this also is that despite larger VTs in the rat due to direct plasmin inhibition, we found less biomechanical evidence...
of vein wall injury. Our data in the late vein wall response (>8 days) contrast with the early independence of uPA on VT resolution. Specifically, early VT resolution in the uPA−/− mouse is independent of uPA, and dependent on MMP-2. These observations as well as those of others highlight the time-dependent and phasic nature of VT resolution and subsequent vein wall response.

Although the VT were smaller in PAI-1−/− mice, the vein wall fibrotic injury at 8 days was greater than in WT controls. However, these observations are consistent with the vessel response from experimental arterial injury models. For example, in both flow restriction and FeCl₃ arterial injury models, as well as myocardial injury, PAI-1 deletion or exogenous PAI-1 inhibition were associated with increased neointimal hyperplasia. While we found no difference in intimal thickness in WT or PAI-1−/− mice after VT, a thickened and collagen-rich vein wall was found with deletion of PAI-1−/−. This phenotype was associated with increased VSMC gene expression and cell number, suggesting either proliferation or influx of VSMC. Our data do not allow us to determine which mechanism occurred, but others have shown in an arterial injury model that increased VSMC proliferation occurs with lack of PAI-1 and may be due to PAI-1’s thrombin inhibitory activity. We speculate that since TAT levels were not increased in PAI-1−/− thrombi, the role of vitronectin or other migratory processes are more important than the proliferative effect in our stasis model. Specifically, PAI-1 may be protective from fibrotic injury by both its vitronectin binding role (decreased VSMC proliferation and migration) and by its antiproteolytic activity. Moreover, this is in part driven by bone marrow-derived progenitor cells that express PAI-1, as PAI-1 competent cells rescued the neointimal hyperplastic injury phenotype. Lastly, others have shown transforming growth factor-beta may be elevated in PAI-1−/− mice after vascular injury, as this cytokine has well-documented profibrotic effects.

The differential role of the monocyte in vein wall response and thrombus resolution after stasis VT has been recently reviewed. Counterintuitively, a greater monocyte number is not necessarily associated with greater vein wall fibrotic injury.
wall damage. For example, in a rat model of stasis VT, P-selectin inhibition was associated with increased vein wall monocyte influx, but less fibrosis and stiffness. Monocyte subtypes may confer a pro- or anti-inflammatory state depending on the local environment. As this was a late response after stasis thrombus injury, and little significant difference of phenotypic injury response was observed in either genotype at 21 days, the mechanistic relevance is not clear. However, neither PAI-1 nor uPA-affected monocyte influx at 8 days when the phenotypic differences were greatest.

Despite an increase in plasmin in the PAI-1 vein wall, we did not observe an increase in either MMP-2 or MMP-9 by zymographic analysis. While plasmin can activate these MMPs, other mechanisms such as MMP-14 or MMP-3 also play a major role after vascular injury. Our findings suggest that the thrombus size itself does not affect MMP activity outside of the elevation associated with the stasis thrombus model itself. Consistent with the known action of plasmin, less MMP-9 activity was present late in the uPA mice, but this was not associated with a phenotypic difference in vein wall fibrosis. Interestingly, direct plasmin inhibition in a rat stasis model was associated with increased MMP-9 and associated with increased biomechanical injury. Although less plasmin was present in the uPA mice and no difference in late vein wall injury was found, some activity remained and may account for the difference in vein wall response between the rat and the mouse. Lastly, after day 8, our model does not allow for thrombus separation from the vein wall as they are densely adherent. The decrease in MMP-9 activity may also be due to fewer thrombus mononuclear cells, which are a prime source of MMP-9.

In humans, the longer the thrombus is in contact with the vein wall, the greater the damage. While this in vivo study shows promise for PAI-1 as a pharmacologic target for VT resolution, whether or not exogenous PAI-1 inhibition increases vein wall fibrosis awaits further study, since exogenous agents become more readily available. Moreover, our model did not allow us to assess if an increased fibrotic response affects the thrombus adherence and could lead to decreased embolization to the pulmonary circulation. Nonetheless, a potential translational therapeutic combination might be a PAI-1 inhibitor in conjunction with a thrombin specific inhibitor, such as hirudin, that abolishes the profibrotic effect of PAI-1 inhibitors.

**AUTHOR CONTRIBUTIONS**

Conception and design: JB, VS, TW, JD, DM, PH
Analysis and interpretation: JB, ME, CL, PH
Data collection: JB, VS, ME, CL, ND
Writing the article: JB, PH
Critical revision of the article: JB, PH
Final approval of the article: JB, VS, ME, CL, ND, JD, DM, PH
Statistical analysis: JB, PH
Obtained funding: PH
Overall responsibility: PH

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