Vein wall remodeling after deep vein thrombosis involves matrix metalloproteinases and late fibrosis in a mouse model

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Hypothesis: **Deep venous thrombosis (DVT) confers vein wall injury associated with fibrosis and extracellular matrix (ECM) turnover, likely mediated by matrix proteases. This study investigated the expression of proteases and collagen involved in early vein wall remodeling.**

Methods: **In the mouse, DVT was produced by ligation of the infrarenal inferior vena cava (IVC) or sham operation, and tissue was harvested at 4, 8, and 12 days. The vein wall tissue was processed for real-time reverse transcriptase-polymerase chain reaction (6 to 8 per time point), Western immunoblotting (5 per time point), and gelatin zymography (5 per time point). Analysis of variance was used for multiple comparisons, and a $P < .05$ was significant.**

Results: **Thrombus resolution was documented by a 38% decrease in the thrombosed IVC weight from day 4 to day 12 ($P = .007$). Total vein wall collagen increased over time, with a corresponding increase in procollagen I and III, and expression peaked at 12 days (24-fold and 6.1-fold, respectively, $P < .02$). Matrix metalloproteinase-2 (MMP-2) gene expression was 23-fold greater at 12 days after thrombus formation compared with sham or 4 days after thrombosis ($P < .05$). Total MMP-2 activity was also significantly elevated at 12 days compared with sham ($P < .05$). MMP-9 expression was 19-fold and 27-fold higher at days 4 and 8, respectively, relative to sham ($P < .05$), with no difference in activity. MMP-14 expression was twofold to 3.6-fold greater at day 12 compared with earlier time points and shams ($P < .001$), but no differences in protein levels were found. Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) protein levels were not significantly different from sham over time; however, the ratio of uPA to PAI-1 was decreased through 8 days.**

Conclusions: **Vein wall remodeling after DVT is similar to wound healing and is associated with increased procollagen gene expression and total collagen. It is also associated with increased early MMP-9 expression, followed by MMP-2 expression and activity after DVT resolution. (J Vasc Surg 2005;42:140-8.)**

Clinical Relevance: **Deep vein thrombosis is an often neglected problem that long term is associated with the postphlebitic syndrome of limb swelling, pain, and often ulceration. The basic mechanisms of the vein wall damage that results have not been delineated. The following study describes the vein wall matrix metalloproteinase gene and activity response induced over time in the vein wall after DVT. Additionally, the corresponding collagen upregulation and proximate plasmin system mediators are determined. With this knowledge, potential therapies to reduce vein wall injury directly might be possible.**

Deep venous thrombosis (DVT) is an ongoing clinical problem for hospitalized patients, affecting nearly 250,000 patients each year.[1,2] In addition to the feared complication of pulmonary embolism, DVT is associated with other sequelae such as chronic venous insufficiency (CVI). A young, active group of patients is typically affected by CVI, resulting in significant morbidity (chronic venous stasis ulcers, venous claudication) to an otherwise healthy population.[3,4] CVI has previously been associated with thickened, fibrotic, and noncompliant vein walls and incompetent valvular function.[5]

Further, it has been shown that DVT formation and resolution are both strongly associated with inflammation.[6,7] The subacute inflammatory reaction to DVT creates an environment with the release of proinflammatory cytokines and leukocyte influx of neutrophils, followed later by monocytes and macrophages, that promotes remodeling of the vessel wall. In this sense, DVT resolution is akin to wound healing.

The matrix metalloproteinases (MMPs) are a family of highly conserved, highly regulated enzymes that mediate the remodeling of the extracellular matrix (ECM). MMPs are regulated at the level of transcription, secretion, and activation and are further regulated in vivo by the presence of tissue inhibitors of metalloproteinases (TIMPs).[8-10] MMPs are known to be active in wound healing and tissue regeneration as well as in diseases that feature prominent

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vascular remodeling, notably atherosclerosis and aneurysmal disease.[9,11] Further, it has been shown that MMP activity is modulated in the presence of serine proteases, notably, plasmin. This may be especially important in DVT resolution, as plasmin is the prime mediator of fibrinolysis.[12,13]

Models of arterial vascular remodeling suggest both a proteolytic and a fibroproliferative response. For example, models of aneurysmal disease show elevated levels of matrix metalloproteinases (especially MMP-2 and MMP-9) that may contribute to a loss of wall elasticity and matrix strength.[14] Models of arterial occlusive disease demonstrate increased numbers of activated vascular smooth muscle cells and fibroblasts in atherosclerotic plaques and areas of neointimal hyperplasia.[15,16] Other studies have demonstrated the accumulation of extracellular matrix proteins (tropoelastin and fibronectin) and collagen deposition in remodeling vessels, associated with localized activation of MMPs.[17] Additionally, some studies of arterial injury have demonstrated an early accumulation of glycosaminoglycan (GAG) after vessel injury, which has been postulated to relate to the inflammatory response and cellular influx.[18,19]

Given the physiologic similarities between arteries and veins, the aim of this study was to characterize the relation of MMPs to DVT resolution, collagen metabolism, and GAG metabolism in the vein wall.

MATERIALS AND METHODS

Mouse model. The model of stasis-induced DVT after ligation of the inferior vena cava (IVC) has been described previously.[6,7,20,21] This experiment used 75 male BALB/c mice weighing approximately 18 to 25 g and between 4 to 8 weeks of age. Only male mice were used to avoid the potentially confounding effects of sex. The BALB/c strain was chosen as it has provided the background for previous experiments and has the availability of various genetic knockouts. Animal care and handling was approved by the University Committee on the Use and Care of Animals.

The mice were anesthetized with isoflurane inhalational anesthesia and underwent laparotomy, with exposure of the infrarenal IVC. The IVC was then dissected from the adjacent section of aorta, and all visible side and back branches distal to the renal veins and proximal to the iliac bifurcation were ligated. The IVC was then ligated with a single 8-0 Prolene suture (Ethicon, Somerville, NJ) immediately distal to the renal veins. The sham group of mice received the same preparation, along with dissection and mobilization of the IVC, but a ligature was not placed on the IVC nor were any side branches ligated.

The mice tolerated the procedure well and were recovered in the laboratory before being returned to the animal housing facility. All operative deaths occurred in the immediate perioperative period and were related to anaesthetic administration. No remote deaths secondary to infection occurred, and the mice continued to feed well.

The mice were sacrificed at day 4, 8, or 12, and the infrarenal IVC to the iliac bifurcation was harvested. Thrombi were separated from the IVCs, and both were measured for total length and weighed. The samples were then immediately snap-frozen by immersion in liquid nitrogen and stored at −70°C for processing. One group of samples was processed for mRNA extraction, one group was processed for zymography and direct colorimetric assay, and one group was processed for Western blotting. For these experiments, the IVC tissue was used for analysis. Several samples from the group processed for zymography were also sectioned for analysis by immunohistochemistry.

Measurement of thrombus and IVC weight. Thrombus weights are a simple, but reliable, measure of thrombus resolution.[22] As this study was focused on the vein wall, the weights of the thrombi, IVCs, and the combined weights for each sample were measured. At the time of harvest, thrombi were separated from the IVCs. The IVC tissue was prepared for analysis in this study. The tissue was prepared as described later for the following analyses.

Total glycosaminoglycan assay and total collagen assay. The total GAG and total collagen content of the vein wall were determined by using a commercially available direct colorimetric assay (Blyscan GAG Assay and Sircol Collagen Assay, Biocolor Ltd, Belfast, Northern Ireland). Samples underwent mechanical homogenization and ultrasonic disruption in 500 μL of lysis buffer, and the assays were run according to the manufacturer's instructions. Values were standardized to the weight (mg) of IVC tissue with an assay sensitivity of 1 μg/mL. The collagen assay is a quantitative assay in which Sirius red, an anionic dye, binds to the side chains of amino acids found in collagens I to IV. In the GAG assay, 1,9-dimethylmethylene blue dye is used under conditions that produce a specific label for the sulfated polysaccharide component of proteoglycans and protein-free sulfated GAG chains, including chondroitin sulfates, dermatan sulfates, keratin sulfates, and heparan sulfates.

Real-time reverse-transcriptase polymerase chain reaction. Expression of each gene of interest was determined using a quantitative real-time polymerase chain reaction, as described by Ginzinger.[23] To extract mRNA, the tissue was subjected to mechanical homogenization in TRIzol reagent (Molecular Research Center, Inc, Cincinnati, Ohio) and combined with chloroform. Samples were centrifuged, and nucleic acids (RNA) were extracted from the aqueous layer. The RNA was subjected to reverse transcription to produce cDNA.

Genes of interest and their respective sense and antisense primers are listed in the Table. Primer sequences were derived using Primer Premier Software (Premier Biosoft Int, Palo Alto, Calif) and gene accession data available from the National Institutes of Health LocusLink index to GenBank. The expression for each gene was determined as the relative expression, or cycle threshold (Ct), of the gene of interest to the expression of β-actin, a known, constitutively expressed reference gene, by the formula:

$$\Delta Ct = 2^{[(Ct\ \beta\text{-actin})-(Ct\ \text{gene of interest})]}$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Activity of the gelatinases (MMP-2 and MMP-9, active and latent forms) was determined by

Table. Primers

Procollagen I sense
GCG AGT GCT GTG CTT TCT G
Procollagen I antisense
TCC CTC GAC TCC TAC ATC TTC
Procollagen III sense
GCC CAC AGC CTT CTA CAC
Procollagen III antisense
CCA GGG TCA CCA TTT CTC
MMP-9 sense
AGC ACA ACA GCT GAC TAC GAT AAG
MMP-9 antisense
GCG CTT CCG GCA CGC TGG AAT GAT CTA A
MMP-2 sense
GAT AAC CTG GAT GCT GTC G
MMP-2 antisense
CCA AAC TTC ACG CTC TTG
MMP-14 Sense
TTA CAA GTG ACA GGC AAG G
MMP-14 Antisense
AAC ACC ACA GCG AGG G
PAI-1 Sense
CCG ATG GGC TCG AGT ATG A
PAI-1 Antisense
TTG TCT GAT GAG TTC AGC ATC CA
β-Actin sense
TTC TTT GCA GCT CCT TCG
β-Actin antisense
TTC TGA CCC ATT CCC ACC

MMP, Matrix metalloproteinase; *PAI,* plasminogen activator inhibitor.

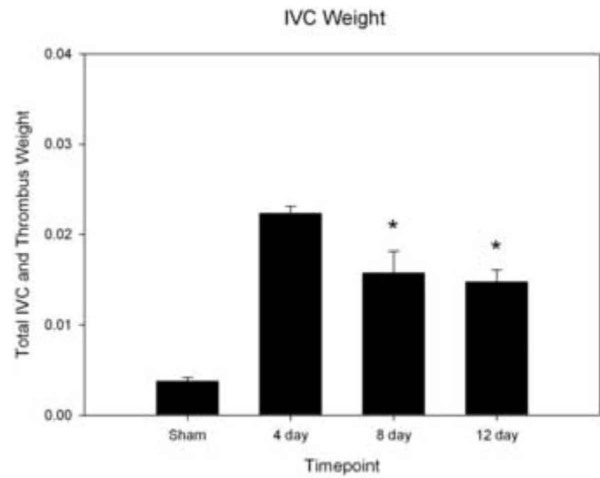

Fig 1. Thrombosed inferior vena cava *(IVC)* weight. The total weight of the IVC and thrombus decreased from 4 days (earliest time point with thrombus present) through 12 days, demonstrating thrombus resolution. The comparison here is between days 4, 8, and 12 of the experimental groups, as no thrombus was present in animals that underwent sham operation (n = 35). Data are means ± SEM, *$P < .01$ between day 4 and day 8, and between day 8 and day 12.

gelatin zymography on 10% SDS-polyacrylamide gels, as previously described.[24] Activity was visualized as light staining bands on a dark background. Images were analyzed by densitometry performed with a FOTO/Analyst CCD Camera (Fotodyne, Heartland, Wis) and Gel-Pro Analyzer Software (v 3.1) (Media Cybernetics, Silver Springs, Md) and normalized to the total amount of protein present in each sample, as determined by the total protein assay.

SDS-PAGE gelatin reverse zymography. The tissue inhibitors of metalloproteinases (TIMPs) are the main physiologic inhibitors of MMP activity in vivo.[8] A perceived increase in MMP activity can be related to increases in MMP production or decreases in the production of these natural MMP inhibitors. Activity of the TIMPs (active and latent forms of TIMP-1, TIMP-2, and TIMP-3) was determined by zymography on 10% SDS polyacrylamide gels, copolymerized with gelatin and human pro-MMP-2, which degrades gelatin and is susceptible to inhibition by all TIMPs, as previously described.[24] Activity was visualized as dark staining bands (areas of TIMP inhibition) on a light background (area of MMP-2 activity). Images were analyzed by densitometry performed with a FOTO/Analyst CCD Camera and Gel-Pro Analyzer Software (v 3) and normalized to the total amount of protein present in each sample as determined by the total protein assay.

Western immunoblot. Protein was extracted from IVC samples by incubating IVC separated from thrombus overnight in 1% SDS. Western blotting for urokinase-like plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), and MMP-14 was performed as previously described. (All buffers, gels, reaction chambers, and membranes were from Bio-Rad, Hercules, Calif.)

Primary antibodies uPA (Santa Cruz Biotechnology, Santa Cruz, Calif), PAI-1, and MMP-14 (BD Pharmingen, San Diego, Calif) were diluted in Tris-buffered saline with Tween-20 (T-TBS) to a dilution of 1:5,000 and applied to the membranes for 1 hour at room temperature. After washing, the corresponding secondary goat, rabbit, and mouse antibodies (Calbiochem, San Diego, Calif) were applied and incubated for 1 hour at room temperature. The secondary antibodies were then also washed from the gel, the labeled proteins were detected by the application of enhanced chemiluminescence agents, and the membranes were exposed to film for 10 to 45 seconds, depending on the protein. (Detection reagents and film were from Amersham Biosciences, Piscataway, NJ.) Images were detected by using a FOTO/Analyst CCD Camera. Densitometry was performed with Gel-Pro Analyzer Software (v 3) and normalized to the total amount of protein present in each sample, as determined by a commercially available total protein assay (Pierce Biochemical, Rockford, Ill).

Statistical analysis. Descriptive statistics were collected and data reported as mean ± SEM. All data were subjected to statistical analysis by one-way analysis of variance (normally distributed) or the Kruskal-Wallis (nonnormal distribution) for comparison between the various time points represented, and when significance was found ($P < .05$), *t* tests were used to compare thrombosed samples with sham controls. Analysis was performed with Sigma Stat (v 2.0), and graphic representations were cre-

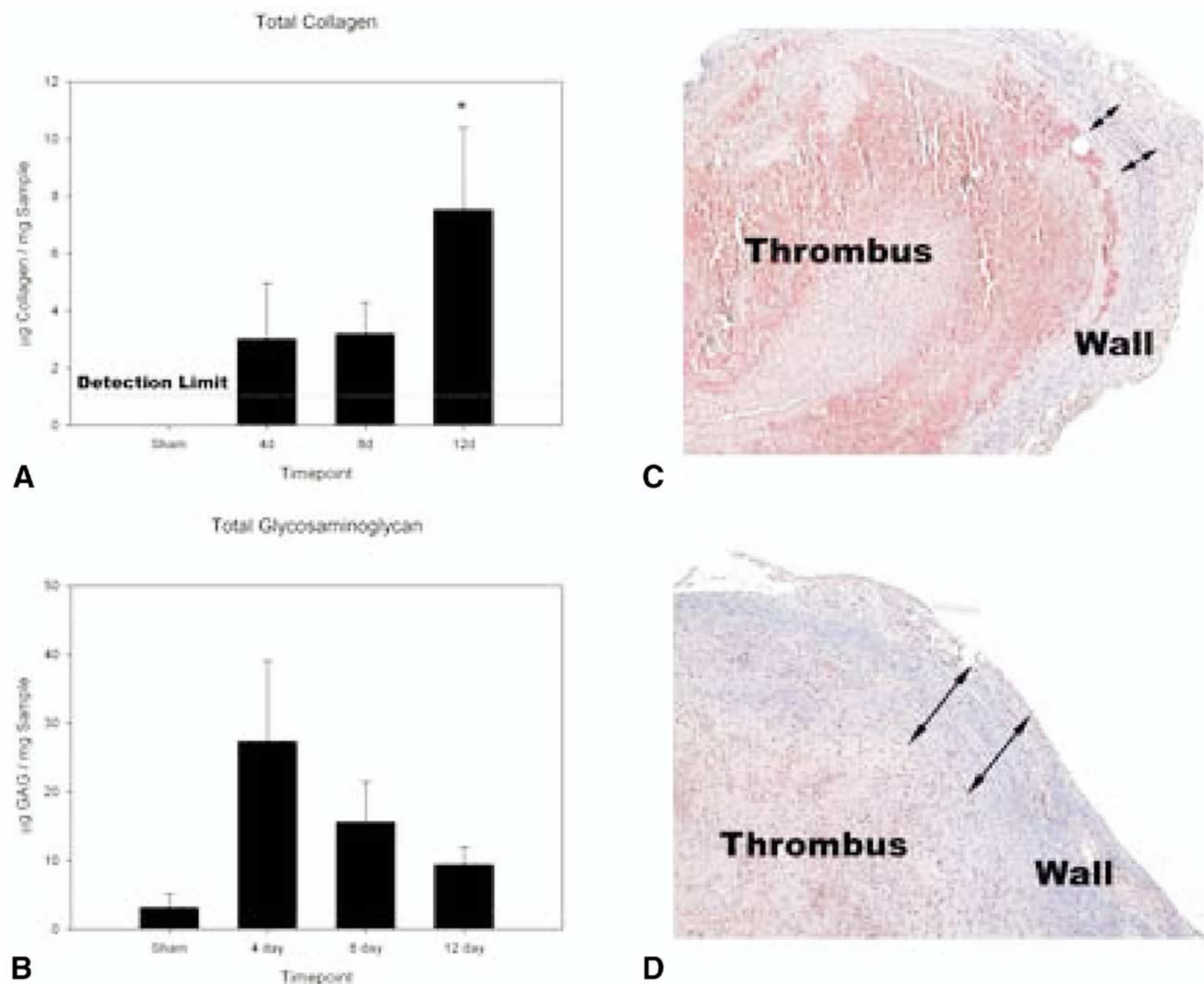

Fig 2. Total collagen and glycosaminoglycan *(GAG)* content (data are means ± SEM). **a,** Total collagen content increased significantly after thrombus formation, far above the minimal amount detectable at baseline (n = 5, *P = .015). **b,** Total GAGs, however, increased acutely but decreased during the period of thrombus resolution (day 4 to day 12). As the duration of the thrombus exposure increased from **(c)** 4 to **(d)** 12 days, Brillmeyer's trichrome stain for collagen in the vessel wall demonstrated an increased thickness *(arrows)* of the vein wall (n = 5) (×125).

ated using Sigma Plot (SPSS Inc, Chicago, Ill). $P < .05$ was assigned as significant.

RESULTS

Total IVC collagen increases during thrombus resolution. The sham IVCs were small, with a mean weight of .376 mg. The thrombosed IVC were significantly larger. The thrombosed IVC was heaviest, with a sixfold increase at 4 days, and trended toward baseline over the ensuing study period (Fig 1). Sham IVC samples demonstrated minimal measurable collagen at the detection level (1 μg/mL) of the direct assay. However, vein wall collagen increased significantly by 12 days after thrombosis compared with shams ($P < .05$). Conversely, an early increase in the amount of GAGs present in vein walls was found after acute thrombus formation, followed by a decrease in vessel wall GAG content during thrombus resolution from day 4 to day 12 (n = 4 to 5, $P < .05$). Additionally, an increase in the amount of collagen staining in the vein wall was noted in Brillmeyer's trichrome sections of the thrombosed IVC segments with the passage of time (Fig 2).

Procollagen I and III mRNA are upregulated during thrombus resolution. In addition to results just described, previous experimental work suggests collagen content of the vein wall may increase acutely after DVT.[22] Expression of procollagen I mRNA, a subunit of and precursor to type I collagen, increased 18-fold at day 8 and 24-fold at day 12 after DVT relative to sham (n = 6 to 8, $P < .05$). Procollagen III mRNA expression increased sixfold at day 12 after DVT compared with sham (n = 6 to 8, $P < .05$). Day 4 levels for procollagen I and procollagen III mRNA were increased above base-

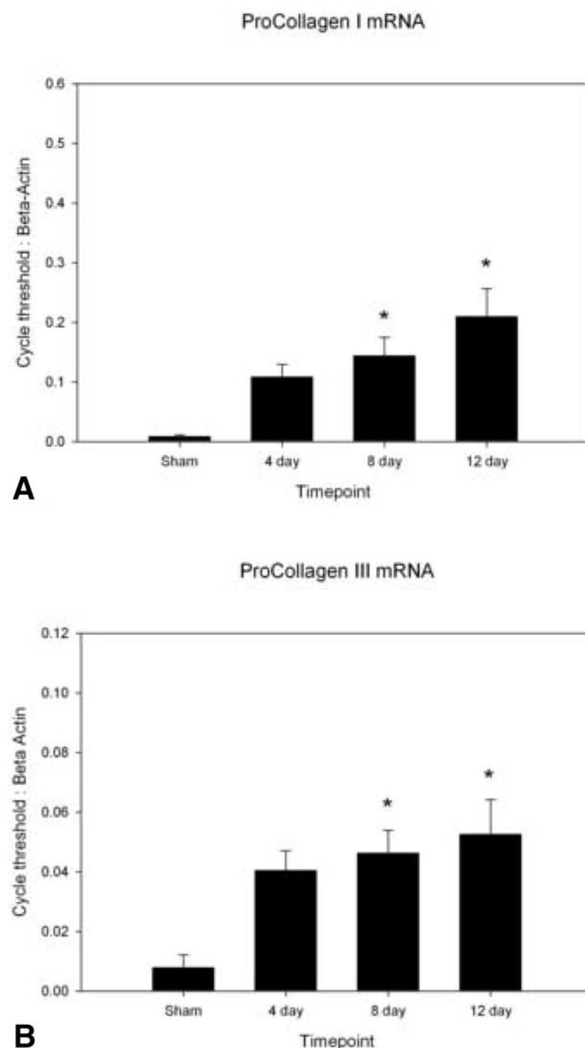

Fig 3. Procollagen I and III expression (data are means ± SEM). **a,** Procollagen I expression was increased 18-fold by day 8 and 24-fold by day 12 after deep vein thrombosis, both relative to sham (n = 6 to 8; **P* = .06). **b,** Procollagen III expression increased sixfold by day 12 (n = 6 to 8, **P* = .017).

line but were not statistically different from the levels in the sham animals (Fig 3).

MMP-9 expression and activity are increased early in the IVC during DVT resolution. During the course of DVT resolution, MMP-9 mRNA increased by 20-fold at day 4 and 28-fold at day 8 relative to sham (n = 6 to 8, $P <$ .05). By day 12, the level of MMP-9 mRNA was no different from shams. MMP-9 activity by zymographic assessment was increased relative to sham controls. Most of this increase occurred early, as there was a fivefold increase in total activity between sham and day 4 (n = 5, *P* = .007). Total MMP-9 activity remained increased through day 12, although there was a slight (insignificant) decline in activity between day 4 and day 12 (Fig 4).

MMP-2 expression and activity are increased later in the IVC during DVT resolution. MMP-2 mRNA expression increased significantly during thrombus resolution and peaked at day 12. There was a 10-fold increase in expression at day 12 relative to sham tissue (n = 6 to 8, $P <$ .05). Additionally, total MMP-2 activity (the activity of both pro- and active forms of the protease) increased during thrombus resolution. Total activity was 2.8-fold greater at day 12 than in sham tissue (n = 5, $P < .05$) A 1.5-fold increase was present by day 8, but this did not reach statistical significance. Additionally, the amount of MMP-2 detectable as the active form of the enzyme was also increased by 2.6-fold at day 12 (n = 5, $P < .03$). In a similar result to the total amount of MMP-2 activity, there was a 1.6-fold increase by day 8, but again, this did not reach statistical significance (Fig 5).

MMP-14 is a membrane-type (membrane associated) metalloproteinase that is known to be a proximal activator of other metalloproteinases, notably MMP-2.[8,25] In the present study, MMP-14 mRNA expression was increased by 6.4-fold on day 12 relative to the sham group (n = 6 to 8, $P <$ .05). Despite the increased mRNA expression of MMP-14, however, no statistically significant change in the protein level was detectable by Western immunoblot by day 12.

No change in IVC TIMP levels occurred during DVT resolution. Reverse zymography for TIMP inhibition of MMP activity demonstrated no statistically significant alterations in TIMP-1 (20.6 kDa TIMP) levels at any time point (n = 4 to 5, *P* = .48; data not shown).

Protein levels of uPA and PAI-1 in the IVC trend in opposite directions during DVT resolution. The MMPs are known to be affected by serine proteases, notably, the plasmin system.[8,26] Therefore, we sought to establish variations in the plasminogen-plasmin axis—the process and mediators involved in the activation of plasminogen to form plasmin—that might play a role, directly or indirectly, in the activation of MMPs. PAI-1 is the primary native inhibitor of plasmin activation. By Western immunoblotting, the level of PAI-1 protein trended upward and peaked at day 8 (sham optical density [OD], 0.212/mg protein vs 8 day OD, 0.328/mg protein), whereas uPA declined from sham controls to day 12, with most of the decrease occurring by day 8 (sham OD, 2.1/mg protein vs 8 day OD, 0.81/mg protein).

To reflect the relative balance of uPA (profibrinolytic) to PAI-1 (antifibrinolytic), we assessed the ratio of these antigen levels.[27,28] Although the change in the absolute amounts of uPA and PAI-1 did not change significantly, there was a significant change in the ratio of these proteins in the IVC. The ratio of uPA to PAI-1 was 5.7 times higher in the sham group than at the 8-day time point, a drop of 83% (*P* = .044) (Fig 6).

DISCUSSION

DVT resolution is known to be an inflammatory process, with clinical sequela that includes chronic venous insufficiency, marked by thickened, noncompliant vein walls and incompetent valves. Previous studies have suggested a role for the thrombus in directing the inflammatory reaction that

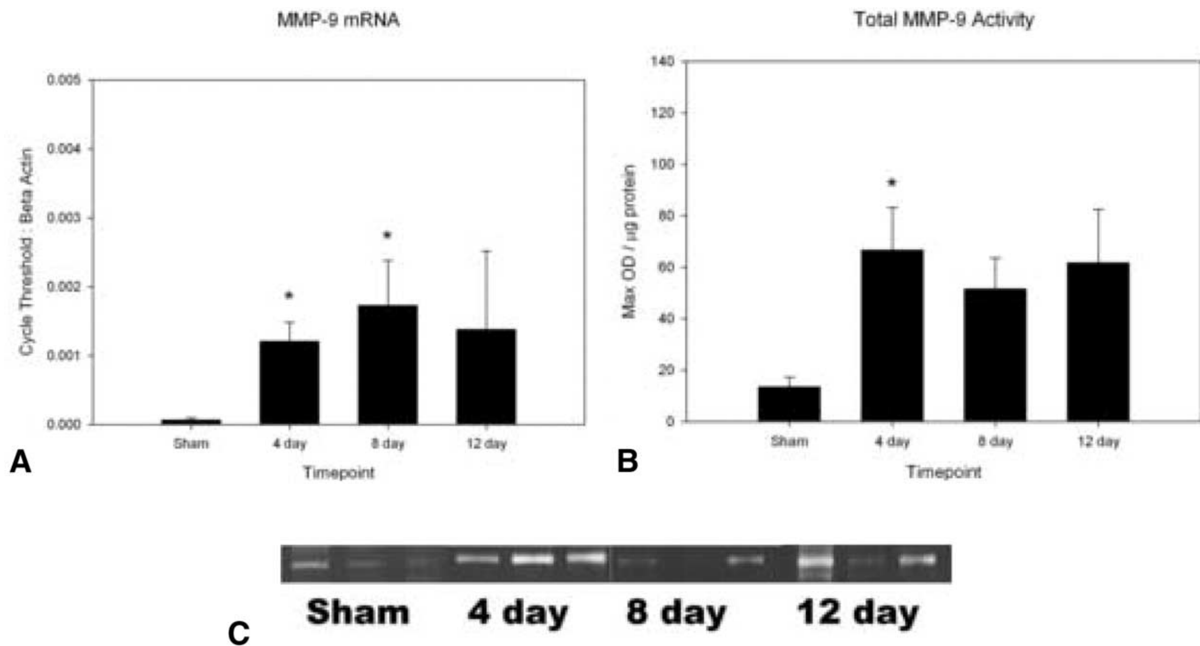

Fig 4. Matrix metalloproteinase-9 *(MMP-9)* expression and activity (data are means ± SEM). **a,** Expression of MMP-9 mRNA was increased 19.5-fold at 4 days and 27.5-fold at 8 days, both relative to sham (n = 6 to 8; **P* = .004). **b,** MMP-9 activity by zymogram peaked at day 4 (5-fold greater) compared with shams (n = 4 to 5, **P* = .011). **c,** Representative composite zymogram showing activity as a bright band on a dark background. There are four columns, each containing three separate samples from separate animals.

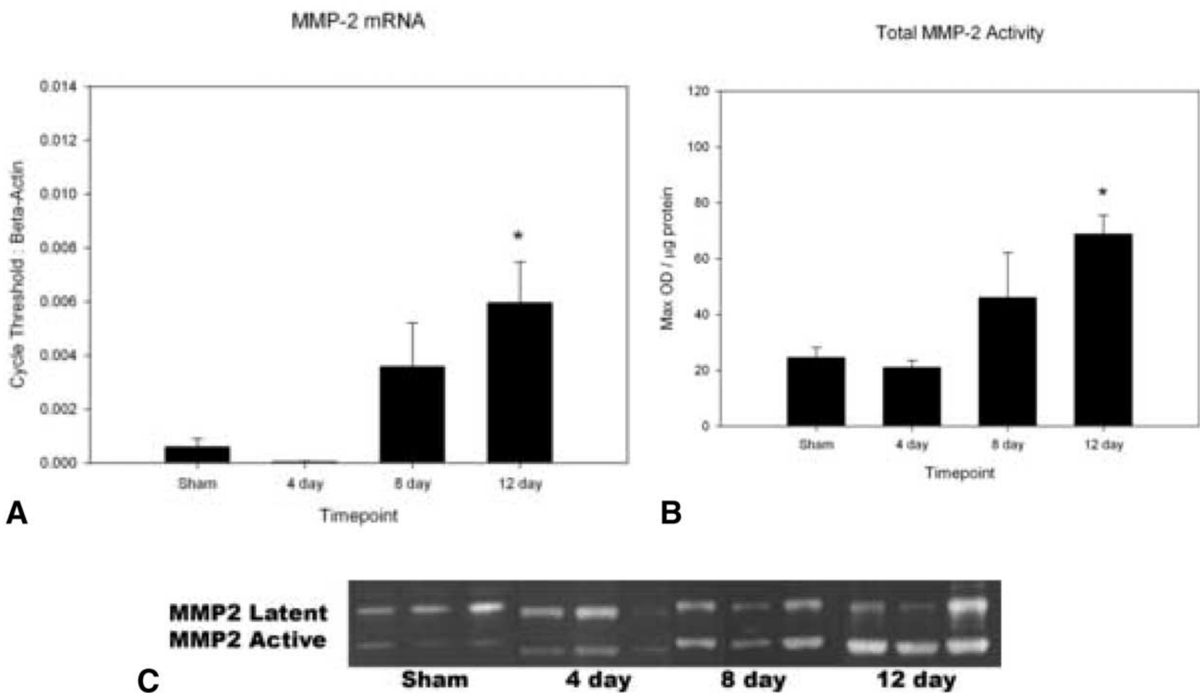

Fig 5. Matrix metalloproteinase-2 (MMP-2) expression and activity (data are means ± SEM). **a,** MMP-2 expression was 10-fold greater at day 12 than in shams (n = 6-8; **P* < .001). **b,** MMP-2 activity peaked at day 12, 2.8-fold greater than in shams (n = 4 to 5; **P* = .011) **c,** Representative composite zymogram showing activities. There are four columns, each containing three separate samples from separate animals.

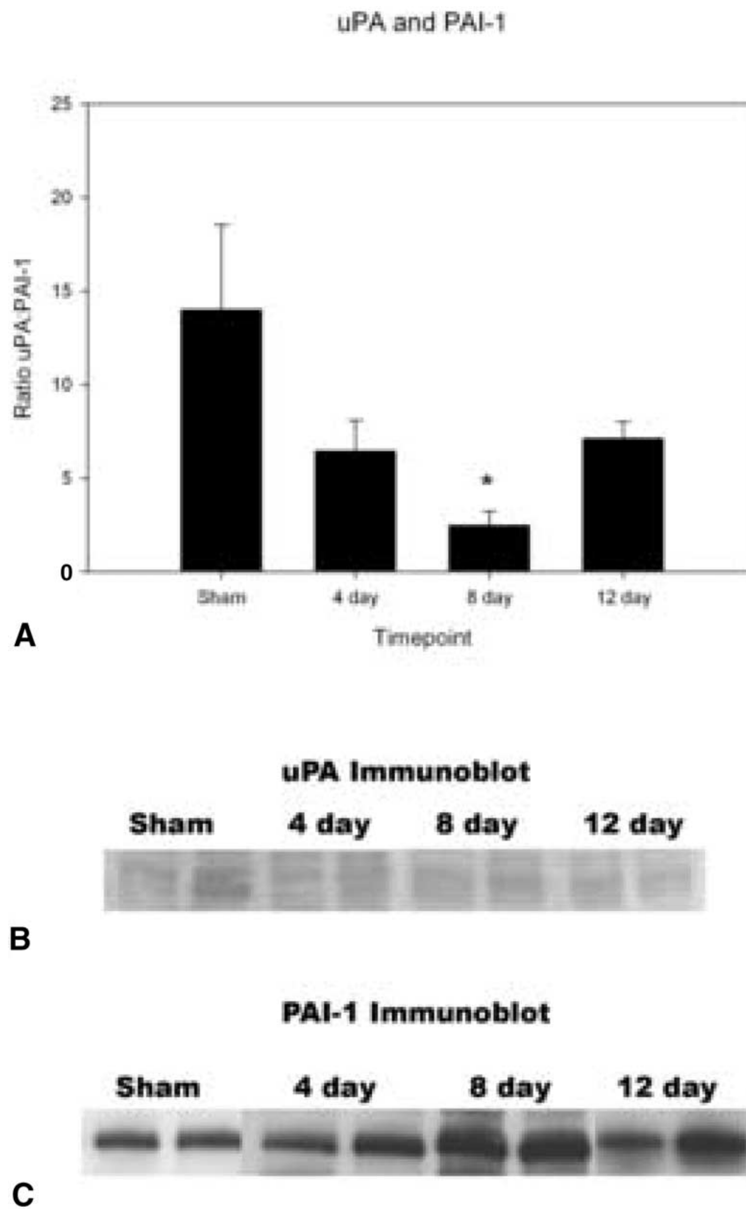

Fig 6. Urokinase-like plasminogen activator *(uPA)* and plasminogen activator inhibitor-1 *(PAI-1)* (data are means ± SEM). **a,** The ratio of uPA to PAI-1 protein levels diminished from sham controls through day 8 (n = 4 to 5; **P* = .044) **b,** and **c,** The attached blots are representative of the relative intensity of the staining for each protein. There are four columns, each containing two separate samples from separate animals.

mediates this process.[6] We sought to correlate this known nidus of inflammation temporally with changes in the vessel wall itself. We hypothesized that the inflammatory reaction that allows resolution of the thrombus would be related to the inflammation in the vein wall, as the mediators secreted at the thrombus-vessel interface should affect not only the thrombus but also the adjacent vessel wall.

The interrelation between thrombosis and inflammation has been long demonstrated.[5-7,22] Thrombus resolution is an active process of inflammation, with early neutrophil influx followed by the eventual replacement with cells of the monocyte-macrophage lineage.[6,22] This contributes to the secretion of a number of proinflammatory cytokines that may direct extracellular matrix turnover.

Prior experimental study has suggested that a late fibrotic response, similar to a healing wound, occurs in vein walls after the development of a DVT.[5] This involves the progression of the normally thin and compliant vein wall to a relatively thick and fibrotic state, with the deposition and accumulation of collagen and the loss of normal vessel

ECM. Some of these studies have indicated a role for MMPs in the resolution of the thrombus, but patterns of expression for these proteinases, as well as the proximal effectors in the MMP system, have not been well characterized in the vein wall.[22]

This study demonstrates that MMP gene expression and activity is differentially induced in the vein wall after the formation of a DVT; that is, whereas multiple MMPs may be activated during the process of DVT resolution, the most significant long-term alterations in function occur in MMP-2. Put alternatively, although changes occur in the activity of other MMPs such as MMP-7 and MMP-9, these changes lose significance >4 days, and MMP-2 activation at a later time point seems to suggest that it is the gelatinase with the most significant impact on chronic remodeling of the ECM. Furthermore, this correlates with increased procollagen gene expression and total collagen levels.

Neutrophils are known to be the primary source of both secreted and membrane-associated MMP-9, which has been shown to play a role in cell invasion and migration through normal matrix.[29] Consistent with this notion is the observed early increase in IVC MMP-9 activity. Persistent elevation of MMP-9 gene expression and activity suggests cells other than neutrophils may later modulate MMP-9 production as the thrombus resolves. It is likely this protease is involved with early ECM turnover, probably released from the infiltrating neutrophils that occurs after DVT.[5,20]

Though MMP-9 was prominent early, MMP-2 had greater and more persistent elevation of gene expression and activity in the vessel wall at later time points. MMP-2 is expressed by smooth muscle cells and fibroblasts and is activated during the vessel response to injury.[15,30,31] This protease degrades elastin and collagens, and its relatively late upregulation suggests that it may be most important in the remodeling of the ECM deposited as part of the scar matrix rather than in the degradation of the normal ECM or in the actual lysis of the thrombus itself. Interestingly, MT-1 MMP (MMP-14), known to be an activator of MMP-2, showed an increase in amount early after DVT. Whether this is integrally related to MMP-2 activation has not been evaluated in the current study.[8,25] Thus, activation of MMP-2 in the perithrombus vein wall may or may not be dependent on increased production of MMP-14.

Venous thrombolysis in vivo is mediated primarily by activation of the plasminogen-plasmin system.[32] Plasmin, a serine protease, is a proximal activator of MMP activity.[26] It is perhaps not surprising that in a resolving thrombus, MMP activation may occur as the result of activation by the plasmin system, and this activity may be present in the vein wall as well. Additionally, PAI-1 is known to be involved in interleukin-1–mediated matrix remodeling by macrophages.[33-35] Given that uPA is a central activator of fibrinolysis, the observed decrease in vein wall uPA may represent its consumption as it activates plasminogen to plasmin during the acute phase of thrombolysis and possibly by downregulation with increases in MMP-2 and -9. The changing ratio of uPA and PAI-1 demonstrates an alteration in the normal profibrinolytic to antifibrinolytic state of the vessel. However, we acknowledge and caution that the ratio comparison is subject to error propagation of the numerator and denominator. The definitive method to quantify this would be a direct activity assay, which has so far proved elusive in our model system.

In other studies, loss of uPA activity confers a relatively antifibrinolytic state, with increased fibrin deposition.[28,36] Interestingly, the decrease of this ratio in this model demonstrates a shift away from a relatively profibrinolytic state (uPA-rich) to a relatively antifibrinolytic state (PAI-1-rich). Normal endothelium maintains a relatively profibrinolytic state that prevents the formation of pathologic thrombosis and aids in the clearance of fibrin products after normal hemostasis.[36,37] Thus, we speculate that the DVT does damage to the vein wall endothelium, which may act to promote continuing thrombus deposition that is only abrogated once the vessel has re-endothelialized.

These data support the belief that normal vein wall matrix is replaced with contents similar to those that are found in a healing wound.[38] There was a marked increase in the amount of collagen present in the IVC segments late in the resolution process. Consistent with the increase in total collagen, an increase was found in procollagen I and procollagen III, which are both known to be a part of wound granulation tissue.[38,39] The profibrotic mediator transforming growth factor-β is released from thrombus during thrombolysis[40] and suggests that the *thrombus itself* may direct the vein wall response. Indeed, this has been shown in the rat model of DVT (unpublished data by Henke).

Conversely, there was an acute peak in the amount of GAGs, followed by a steep decrease from day 4 through day 12. This could represent an acute increase in the presence of heparan or the release of chondroitin sulfate in response to the immediate inflammation associated with thrombosis, followed by degradation in the process of matrix replacement.[41] Another possible explanation for the acute increase in the presence of GAGs in the sample relates to the immediate response to tissue injury. Models of arterial injury have demonstrated an acute increase in GAG components of the ECM after damage to the vessel.[18,19] In these systems, it has been suggested that these matrix components are secreted by smooth muscle cells and fibroblasts in the vascular media and facilitate the influx of inflammatory cells. A similar phenomenon may be occurring that allows the development of the thrombus-associated inflammation. In these models, there is an acute peak in GAG, allowing for the development of acute inflammation, followed by a decline over time. Although this occurs in a long-term time scale, such a phenomenon could account for our results. Alternatively, heparan sulfate proteoglycans may be released and consumed through activation of the fibrinolytic system during the process of thrombus resolution.

The current study does not differentiate the roles of influxing leukocytes from the stimulated resident vein wall cellular production of the MMPs. The role of the thrombus itself (and the timing of thrombus duration as a mechanism in directing MMP activity) is not defined here but is the subject of ongoing studies. Furthermore, whether these

proteases can be targeted selectively to decrease the destructive injury response is attractive but premature.

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