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PAI-1 over-expression decreases experimental post-thrombotic vein wall fibrosis by a non-vitronectin dependent mechanism

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SUMMARY

Background—Factors associated with post-thrombotic syndrome are known clinically, but the underlying cellular processes at the vein wall are not well-delineated. Prior work suggests that vein wall damage does not correlate with thrombus resolution, but rather with plasminogen activator-1 (PAI-1) and matrix metalloproteinase (MMP) activity.

Objective—We hypothesized that PAI-1 would confer post venous thrombosis (VT) vein wall protection via a Vitronectin (Vn) dependent mechanism.

Methods—A stasis model of VT was used with harvest over 2 weeks, in wild type (WT), Vn−/−, and PAI-1 overexpressing mice (PAI-1 Tg).

Results—PAI-1 Tg mice had larger VT at 6 and 14 days, compared to controls, but Vn−/−mice had no alteration of VT resolution. Gene deletion of Vn resulted in increased, rather than expected decrease in circulating PAI-1 activity. While both Vn−/− and PAI-1 Tg had attenuated intimal fibrosis, PAI-1 Tg had significantly less vein wall collagen and a compensatory increase in collagen III gene expression. Both Vn−/− and PAI-1 Tg vein wall had less monocyte chemotactic factor-1, and fewer macrophages (F4/80), with significantly less MMP-2 activity and decreased TIMP-1 antigen. *Ex vivo* assessment of TGFβ mediated fibrotic response showed that PAI-1 Tg vein walls had increased profibrotic gene expression (collagen I, III, MMP-2 and α-SMA) as compared with controls, opposite of the *in vivo* response.

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Conclusions—The absence of Vn increases circulating PAI-1, which positively modulates vein wall fibrosis in a dose-dependent manner. Translationally, PAI-1 elevation may decrease vein wall damage after DVT, perhaps by decreasing macrophage-mediated activities.

Keywords

fibrosis; PAI-1; postthrombotic syndrome; venous thrombosis; vitronectin

INTRODUCTION

Deep venous thrombosis (DVT) is a significant health care problem in the United States, with 250,000 to 900,000 patients affected yearly.[1],[2] An additional 200,000 patients are affected by the late sequelae of post thrombotic syndrome (PTS), characterized by leg pain, sensations of heaviness, limb edema, discoloration and occasionally ulceration.[3] The end result after DVT is the conversion of a compliant, thin walled vein with functional valves to a thick walled vessel, often with nonfunctioning valves.[4] The physiological result of damaged veins is pooling of blood in the dependent extremities, producing venous hypertension and pain, fluid transudation, edema, and in severe cases, ulceration.

Despite effective anticoagulant therapy treatments for DVT, there are no therapies that specifically target the PTS remodeling processes.[5] While surgical and interventional therapy may be beneficial for selected patients, treatment for PTS is mainly supportive, consisting of compression and wound care. While compression therapy may provide symptomatic relief, it does not correct the fundamental changes that occur in the venous system. Indeed, recent data from a randomized controlled trial of compression for preventing PTS did not show a benefit as compared with placebo.[6] An improved understanding of the biology of PTS, including potential therapeutic targets, is crucial in the effort to develop effective treatments.

We have recently shown the importance in plasminogen activator-1 (PAI-1) in post-DVT vein wall remodeling.[7] Gene-deletion of PAI-1 hastened venous thrombus resolution in a mouse model of stasis DVT, but resulted in a thickened and collagen-rich vessel wall. Thrombin-antithrombin levels were not elevated in the thrombi of PAI-1 null mice, suggesting that the mechanism of increased fibrosis was not due to PAI-1's thrombin inhibitory activity, but possibly its role in binding Vitronectin (Vn), an abundant plasma glycoprotein.

Vitronectin is produced by the liver and found circulating at appreciable levels in the plasma, in the α-granules of platelets and in the extracellular matrix.[8] It plays a multifaceted role in vascular biology, particularly with regards to thrombogenesis, peri-cellular proteolysis, leukocyte recruitment and cellular adhesion/migration.[8, 9] Vn has been associated with fibrosis of organ systems such as liver[10, 11] and lung[12, 13] and implicated as a mediator of neointima formation in the arterial vasculature.[14-16] To date, its effect on post thrombotic venous remodeling is unknown.

Experimental models of arterial thrombosis differ with regards to the role of Vn, with some data suggesting that it plays a key role in promoting arterial thrombogenesis[17] and

stabilization of thrombi,[18] while other data suggesting an inhibitory role in thrombogenesis.[19] However, little is understood about how or if Vn contributes to venous thrombosis (VT), with only one study to date demonstrating increased time to occlusion in Vn−/− mice undergoing photochemically induced thrombosis of the jugular vein.[17] In this study, we hypothesize: (1) Vn inhibits thrombus resolution via stabilization of PAI-1 and (2) Vn contributes to vein wall fibrosis via increased monocyte vein wall infiltration.

METHODS

Animals

Male C57BL/6 (WT) mice (Jackson Laboratory, Farmington, CT), Vitronectin gene-deleted (Vn−/−) mice[20] (backcrossed >10 generations on C57BL/6 mice) and PAI-1 over-expressing mice (PAI-1 Tg, backcrossed > 10 generations on C57BL/6 background)[21] were utilized in this study. Initial pilot studies comparing homozygous littermates without transgene (PAI-1 Tg littermates) to C57BL/6 controls demonstrated identical phenotype with regards to venous thrombosis (similar size and cellular morphology). Thus, in the interest of humane and responsible animal use, C57BL/6 mice were utilized as controls (rather than homozygous littermates) for all experiments. . Animals ranged from 8-10 weeks of age with average weight of 24.2g (WT), 24.4g (Vn), and 24.6g (PAI-1 Tg). All work was approved by the University of Michigan, University Committee on Use and Care of Animals and was performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Model of Deep Venous Thrombosis

A DVT model of IVC ligation, or stasis thrombosis, was performed as described.[22] Briefly, mice were anesthetized via 2% inhaled isoflurane. IVC exposure was gained via midline laparotomy and dorsal branches were interrupted with electrocautery. Side branches and the infrarenal IVC were ligated with 7-0 prolene (Ethicon, Inc., Somerville, NJ) to generate blood stasis and ultimately thrombosis. Fascial closure was performed with 5-0 Vicryl suture and skin closure with Vetbond tissue adhesive (3M Animal Care Products, St. Paul, MN). Mice were euthanized at 2, 6 and 14 days post-thrombosis with collection of plasma, vein wall, and thrombus. The vein wall and the thrombus were harvested separately at all time points.[22, 23] Prior to processing, the IVC and its associated thrombus were weighed (grams) and measured (centimeters). Thrombus weight, encompassing the thrombus and the vein wall, was used as a measure of thrombosis resolution.[22, 24]

Gelatin Zymography of MMP-2 and MMP-9

Gelatin substrate zymography was performed using pre-cast 10% SDS-polyacrylamide gels containing 1 mg/ml of gelatin (unless otherwise stated, all zymography supplies from Novex, San Diego, CA).[25-27] Vein wall tissue was homogenized in SDS, centrifuged for 10 minutes at 2000*g*. Supernatants were aliquoted equally and diluted with tris-glycine-SDS sample buffer and electrophoretically separated under non-reducing conditions. Separated proteins were re-natured in 2.7% Triton X-100 for 2 hours to induce gelatin lysis by re-natured MMPs. The gels were developed for 48 hours at 37°C in 50 mM Tris-HCL, 5 mM $CaCl_2$ and 0.2% Brij 35 followed by staining with Coomassie Blue and de-staining in 10%

acetic acid. MMP activity was evident by clear bands against a darkly stained background where the substrate has been degraded by the enzyme. Samples containing human recombinant MMP-2 and MMP-9 (Oncogene, Boston, MA) were included as standards. Concentrations of the Densitometry analysis were performed using a FOTO/Analyst CCD CAMERA (Fotodyne, Hartland, WI) and GEL-Pro Analyzer software version 3.1 (Media Cybernetics, Silver Springs, MD). Ratios of optical densities/total protein were calculated. Results were normalized to total protein using bicinchoninic acid protein assay kit, assessing colorimetric detection and quantification of total protein, read between 560 and 590 nm (Pierce, Rockford, Ill).

Antigen determination

Antigen determination via enzyme linked immunosorbent assay (ELISA) was performed on vein wall homogenate for MCP-1, Interleukin (IL)1β, and IL-13 (R&D Systems, Minneapolis, MN). Samples were aliquoted in duplicate on plates pre-coated with appropriate capture antibody and then detected by a biotinylated antibody, followed by tetramethylbenzadine (TMB) substrate according to the manufacturer's protocol. The results were read on an Elx808 plate reader (Biotek, Winooski, VT) at a 450-nm wavelength. Total protein was measured by using a bicinchoninic acid protein assay kit, as detailed in previous section. Results were normalized to total protein (mg).

PAI-1 activity assay

To measure active murine PAI-1 concentrations in plasma samples, human uPA (rheotromb) was coupled to carboxylated beads (Luminex) and used for capture active PAI-1. A standard curve was generated using known concentrations of murine PAI-1 in PAI-1 depleted mouse plasma (Molecular Innovations, Novi, MI). The sample, 10uL PBS-1% BSA and 5000 beads in 30mL of PBS-1% BSA were incubated overnight at 4°C. The beads were mixed with biotin-labeled rabbit anti-mouse PAI-1 (Molecular Innovations) followed by addition of streptavidin-R-phycoerythrin (Molecular Probes). The beads were then read with the Luminex 100 (median setting, 100 ml sample size, 100 events/bead).

Real-time quantitative polymerase chain reaction (RT-PCR)

Immediately upon harvest, vein wall segments were preserved in TRIzol reagent. The samples underwent reverse transcription with addition of Oligo(dT) primer and dNTP at 65°C for 5 minutes followed by first strand buffer, dithiothreitol and ribonucleoside inhibitor at 37°C for 2 minutes. M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, CA) was added and RNA heated to 37°C for 50 minutes then 70°C for 20 minutes. The resultant cDNA was amplified by Taq polymerase in the Rotogene quantitative polymerase chain reaction system (Qiagen Inc., Valencia, CA). 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 $2^{-(\text{Ct target gene}-\text{Ct reference gene})}$ and cycle lengths used are within the exponential phase of the polymerase chain reaction.[28] These primers included β-actin, procollagen (Col)1a2, Col3a, MMP-2 and α-SMA.

Histology / Immunohistochemistry/ Collagen Staining

Inferior vena cava and associated thrombi were harvested *en bloc*, fixed in formalin, embedded in paraffin and cut into 5 m sections as previously described.[29, 30] Vein wall monocyte cell counts were performed following staining with F4/80 antibody (1:100; Abcam, Cambridge, MA). Briefly, non-specific binding sites were blocked with normal serum, primary antibody was added, followed by biotin-labeled secondary antibody (Rat IgG). An avidin, biotin, peroxidase complex was performed according to manufacturer's instructions (Vector Laboratories Inc., Burlingame, California) and the slides were counterstained with hematoxylin. Monocyte cell counts of five representative high power fields of both vein wall and thrombus were performed in a blinded fashion.[31]

Collagen content was quantified with Picrosirius red stain as previously described.[7, 32, 33] These sections were then analyzed in crossed-plane polarized light from a monochromatic source to assess cross linked 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 NIH Image J software. The inferior vena cava wall was outlined as a region of interest, and then the image underwent threshold segmentation to differentiate collagen from other (primarily 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 non-collagen vein wall changes, intimal thickness and fibrosis scoring assessed from H and E-sections were scored by a board-certified veterinary pathologist in a blinded manner as previously described.[7, 22, 31] Briefly, the scoring system ranges from a score of 0 (no evidence of fibrosis) to 3 (intima contains numbers fibroblasts and is irregularly thickened by large amounts of collagenous connective tissue). A consistent mid-section thrombosed IVC segment was used for all histological analysis.[34-36]

Ex vivo methods vein wall assessment

Inferior vena cava was harvested in control mice (WT and PAI-1 Tg), placed in cold Dulbecco phosphate buffered saline with 2× penicillin/streptomycin/glutamine (Gibco, Invitrogen, Carlsbad) and cleaned using a dissecting microscope.[24, 34, 37] The vein wall was divided lengthwise, weighed and each half further divided into 3-4 pieces. The vein was incubated in Dulbecco Modified Eagle Medium (Gibco) containing 5% FCS and 1% PSG with or without transforming growth factor β1 (10mcg/ml, mouse recombinant, R&D Systems, Minneapolis, MN) in a 12-well plate. [38] The tissue culture was incubated for 24 hours at 37° in 5% carbon dioxide. The wet weight of the vein wall was recorded, the samples were gently washed in DMEM, placed in Trizol and frozen at −80° for RT-PCR processing.

Statistical analyses

All statistical analyses were performed using Graph Pad Prism version 6.01 for Windows (Graph Pad Software, San Diego, CA). The statistical differences between groups were

determined by an unpaired t-test with Welch's correction, one-way ANOVA analysis with Tukey's multiple comparison test, or Dunnett's multiple comparison test as deemed appropriate. Data is reported as mean ± standard error of the mean (SEM) and differences considered significant at a p value of ≤.05. Animal numbers (n) for experiments were pre-determined based upon independent statistical review, protocols and numbers needed for significance. For an alpha=0.5 and power of 80%, n=5 is sufficient for immunological tests and n=10 sufficient for zymography.

RESULTS

Vn−/− mice have paradoxically increased circulating PAI-1 activity

Despite the recent commercial availability of Vn−/− mice and subsequent increase in use, no studies have characterized levels of circulating active PAI-1 in these animals. PAI-1 activity as measured in control mice and at three time points post-thrombosis (2, 6 and 14 days) is demonstrated in Figure 1. Both total and active PAI-1 levels were elevated in the Vn−/− mice when compared to WT. Contrary to expectations, we found active PAI-1 elevated 36-fold compared to wild-type (**Figure 1A**) and total circulating PAI-1 to be elevated 10-fold in Vn−/− mice (**Figure 1B**). Active PAI-1 remained elevated in the Vn−/− mice following thrombosis with significant elevations at days 6 and 14, with 82-fold and 39-fold increase over WT respectively, (**Figure 1C**), with similar elevations seen of total PAI-1 (**Figure 1D**). To determine whether the results we found with regards to thrombus resolution and vein wall fibrosis were truly due to alterations in PAI-1 activity in these animals or rather a secondary function of Vn, we included a comparison cohort of PAI-1 overexpressing transgenic mice (PAI-1 Tg) with normal endogenous Vn. Levels of active circulating PAI-1 in these animals were markedly elevated at baseline (137-fold) and post-thrombosis (15 to 412 fold) compared to WT (**Figure 1A and C**). Similar elevations were seen in total PAI-1 at baseline (172 fold) and post-thrombosis compared to WT (10 to 54 fold) **(Figure 1B and D)**.

Venous thrombogenesis and thrombus resolution are unaffected by Vn−/−

Thrombus weights during acute thrombogenesis, as measured at day 2, were similar between WT and Vn−/− mice (**Figure 2A**). Thrombus resolution, as measured by thrombus weight at 6 and 14 days post-thrombosis (**Figure 2B**), was also unaffected by Vn −/−. Transgenic PAI-1 overexpressing mice exhibited impairment of thrombus resolution, with increased residual thrombus weight at 6 and 14 days post thrombosis (**Figure 2B**). However, thrombogenesis (2d) was not affected in these animals (**Figure 2A**).

Vein wall fibrosis is attenuated by excess PAI-1

Vein wall fibrosis, intimal thickness and collagen content were assessed at day 14 and collagen gene expression assessed at days 6 and 14. Vein wall fibrosis was significantly diminished in mice with elevated PAI-1 (Vn−/− mice and PAI-1 Tg) at day 14 compared to WT (**Figure 3B**). Total vein wall collagen content, as measured by Picrosirius red staining, [7] demonstrated a stepwise reduction with increasing levels of circulating PAI-1 (**Figure 3A, E-F**). Decreases in vein wall fibrosis at day 14 were accompanied by a paradoxical

increase in pro-collagen I and IIIα gene expression in Vn−/− (3.7-fold and 2.1-fold) and PAI-1 Tg (5.3-fold and 4.7-fold) mice compared to controls **(Figures 3C,D)**.

PAI-1 inhibits monocyte recruitment and influx

Monocyte chemotactic protein 1 (MCP-1) peaks during the early and middle stages of thrombus resolution, drives monocytes chemotaxis, and is implicated in clearance of residual thrombus.[39-41] At these time-points (days 2 and 6), MCP-1 levels were suppressed by ≥50% in mice with elevated PAI-1 (Vn−/− and PAI-Tg), both in the vein wall (**Figure 4A**) and in the thrombus (**Figure 4B**). This correlated with diminished vein wall (**Figures 4C, E-G)** and thrombus (**Figures 4D-G**) monocyte influx at day 14 as measured by positively staining F4/80 cells and impaired thrombus resolution (**Figure 2B**) in mice with markedly elevated circulating PAI-1 (PAI-1 Tg).

Vein wall MMP activity is inversely related to PAI-1 activity

The target of PAI-1 inhibition, plasmin, can serve as an activator of MMP-2 and MMP-9.[42-44] Furthermore, monocytes represent a source of MMPs in the post thrombotic vein wall[29] and recruitment is altered in the presence of excess PAI-1 **(Figure 4C).** In mice with PAI-1 (Vn−/− and PAI-1 Tg) elevation, total MMP-9 and -2 levels were diminished in the post-thrombotic vein wall (**Figures 5A and B**). Vein wall TIMP-1 antigen did not account for inhibition of MMPs, and was actually decreased at 6 and 14 days in Vn−/− and PAI-1 Tg mice compared to WT (**Figure 5C**).

Genetic alterations in Vn and PAI-1 result in altered inflammatory cytokine milieu

Pro-inflammatory cytokine IL-1β is upregulated in the post-thrombotic vein wall[41] and augments PAI-1 production by endothelial cells.[45-47] We found that in animals with very high levels of PAI-1, peak vein wall IL-1β upregulation was attenuated (**Figure 6A**). Consistent with the anti-fibrotic phenotype of the PAI-1 Tg mice, we found a reduction of IL-13 in the vein wall at early and mid-time points post-thrombosis (**Figure 6B**). The Vn−/− mice exhibited an intermediate vein wall cytokine profile: IL-1β levels approximated those of the PAI-1 Tg mice and IL-13 levels approximated the WT.

PAI-1 Tg vein walls are not anti-fibrotic *ex vivo*

Given that PAI-1 over expressing vein wall fibrosis was significantly reduced, we asked if the altered genotype affected the fibrotic response *ex vivo*. We used our organ culture model[37] to assess the response to fibrotic mediator TGF-β (**Figure 7**). Consistent with the increases seen *in vivo* with increased pro-collagen I and IIIα gene expression in response to thrombosis compared to WT **(Figure 3C,D)**, the *ex vivo* cultured vein wall demonstrated an increase in pro-collagen gene expression in response to fibrotic stimulation with TGF-β **(Figure 7A,B).** Additional proteins associated with fibrotic injury, MMP-2 **(Figure 7C)** and α-SMA[37] **(Figure 7D),** demonstrated increased relative gene expression in PAI-1 Tg mice vein wall, suggesting that the anti-fibrotic effect of PAI-1 over expression was not due to fundamental changes in vein wall gene expression, but rather the anti-fibrotic effect of circulating PAI-1.

DISCUSSION

Vein wall fibrosis after DVT, clinically manifested as PTS, is a problem with no known direct therapy. Thus, defining the mechanisms of vein wall fibrosis is important translationally. In this study, we have shown the following: (1) contrary to findings in arterial injury models[17-19], the presence of Vn does not influence the amplitude of thrombogenesis or thrombus resolution; (2) Vn−/− mice have increased circulating active and total PAI-1; (3) elevation of PAI-1 is protective against vein wall fibrosis and inversely correlates with vein wall MMP activity; (4) elevation of PAI-1 is associated with decreased monocyte infiltrate and altered cytokine profile; (5) these effects do not appear to be due to genotype specific alterations in the vein wall but rather to the anti-fibrotic effect of circulating PAI-1.

A significant role of PAI-1 in vascular thrombosis and thrombus resolution has been confirmed in animal and human studies alike.[48] Consistent with previous data suggesting that PAI-1 gene deletion does not affect venous thrombogenesis, we found here that PAI-1 over-expression failed to impact initial thrombus weight.[49] However, the role of PAI-1's “cofactor” Vn remains understudied, with few animal studies evaluating thrombotic potential in Vn−/− mice, and none specifically evaluating the deep venous system.[17-19] In a small vessel model of photochemically-induced VT, thrombogenesis was impaired in Vn−/− mice.[17] This contrasts to our data which demonstrates that in the deep vein system, at the time of peak thrombogenesis, there was no difference between thrombus weight and size in Vn−/− and WT mice. The discrepancy between these results suggest that Vn may function very early in thrombogenesis, as the photochemical model was used to study early thrombus formation at a very early time point (3-24 hours), or that Vn is less essential to stasis-induced thrombus formation. Furthermore, Vn deletion does not hasten thrombus resolution, in contrast with PAI-1−/− mice.[7] These findings are supported by elevation of active PAI-1 levels in Vn −/− mice, perhaps due to inability of PAI-1 to remain localized to site of fibrin formation or extracellular matrix via its interaction with Vn. Transgenic overexpression of PAI-1, which produced levels 3 to 17 times higher than the Vn−/−mice, resulted in incremental inhibition of thrombus resolution with larger VT, suggesting that the effect of PAI-1 on thrombus resolution is likely dose dependent.

Although genetic deletion of PAI-1 is associated with increased fibrinolysis by secondary increasing plasmin activity and smaller VT, an increase in vein wall fibrosis was observed.[7] PAI-1's role in organ fibrosis is complex, and dependent on the animal model. For example, divergent results with PAI-1 deletion exist in arterial injury models, with increased medial hyperplasia after wire injury[50] or decreased medial hyperplasia after ferric chloride injury.[51] From the current study, the mechanisms of PAI-1 anti-fibrotic effect is likely two-fold; first, significantly decreased MMP-2 and -9 activity in the post-thrombotic vein wall was found. Elevation of both MMPs is associated with post-thrombotic vein wall fibrosis, [37, 52, 53] and a reduction is consistent with attenuated fibrotic injury. Second, fewer macrophages (and consistently, decreased MCP-1) were found in the vein wall and thrombus of PAI-1 Tg and Vn−/− mice. While macrophages may have pro-fibrotic or anti-fibrotic activities[54], it is likely in the current experiments that less pro-fibrotic signaling was present in the PAI-1 Tg mice. In lung injury models, IL-1β[55] and IL-13[56] are pro-

fibrotic. Consistently, in the current model, IL-1β and IL-13 were significantly elevated in WT mice post-thrombosis, and reduction of these pro-fibrotic mediators may also account for the observed reduction in fibrotic injury.

Binding of PAI-1 to its "cofactor" Vn stabilizes and confers PAI-1 to a longer half-life, contributing to local anti-fibrinolytic activity but also indirectly enhancing the anti-adhesive functions of PAI-1. PAI-1 competes with monocyte surface receptor urokinase plasminogen activator receptor (u-PAR) for the NH_2 terminus of Vn.[8] Binding of PAI-1 displaces u-PAR and inhibits monocyte adhesion.[57] Vn also exhibits a variety of PAI-1-independent activities, including increasing cellular migration[13] and vascular permeability.[58] Interestingly, the vein walls from PAI-1 Tg mice were not intrinsically resistant pro-fibrotic stimulus. Indeed, using a low TGF-β dose, we found PAI-1 Tg vein walls were more likely to have increased collagen gene expression and fibrotic gene markers of MMP-2 and α-SMA. This suggests that the anti-fibrotic phenotype associated with elevated PAI-1 in our model was not the result of genotype specific decreased vein wall susceptibility to fibrosis , but more likely the anti-fibrotic effect of circulating PAI-1.

Post thrombotic vein wall injury is complex, and new data suggests that compression therapy may not be truly effective.[59] New and specific therapies are needed, particularly since aggressive thrombolysis may be limited to specific good risk patients and carries a risk of life threatening hemorrhage.[60] In this study we demonstrated that increased circulating PAI-1, either from gene deletion of PAI-1 ligand Vn or in setting of transgenic over-expression, is associated with attenuated fibrosis of the post-thrombotic vein wall. While it is unlikely that exogenous PAI-1 would be practical as it would impair thrombus resolution, interruption of PAI-1-fibrin interaction via specific targeting of vitronectin[6161] has the potential to decrease vein wall fibrotic response without affecting thrombus resolution, and represents a future area of study.

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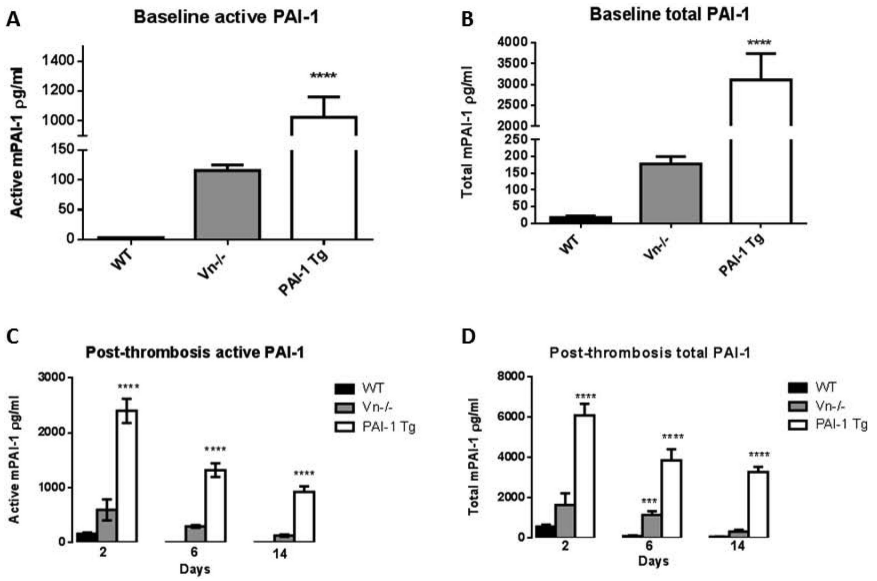

Figure 1.

Both active and total PAI-1 were elevated in transgenic PAI-1 overexpressing mice (PAI-1 Tg) compared to WT (n=5 for all groups) at baseline (**A, B**) and following induction of deep venous thrombosis (**C, D**), with intermediate levels of PAI-1 in the Vn−/− depleted mice (*** $p<0.001$, **** $p<0.0001$).

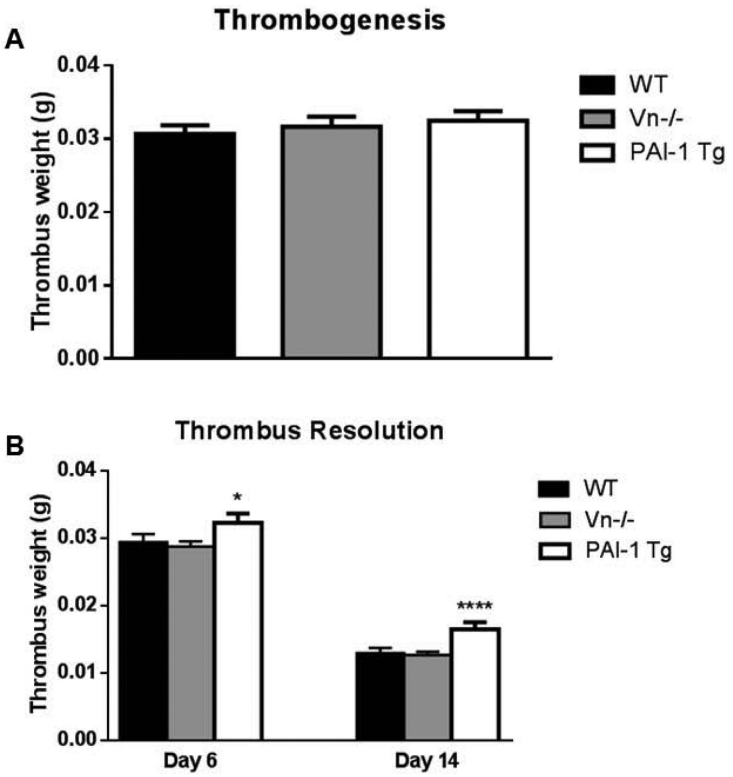

Figure 2.

Neither Vn−/− gene deletion nor PAI-1 over-expression affected thrombogenesis, as measured by thrombus weight 2 days post thrombosis, in the deep venous system (**A**, all n>25). Thrombus resolution was impaired in the PAI-1 Tg mice but not in the Vn−/− mice (**B**, all n>25, *p<0.05, ****p<0.0001).

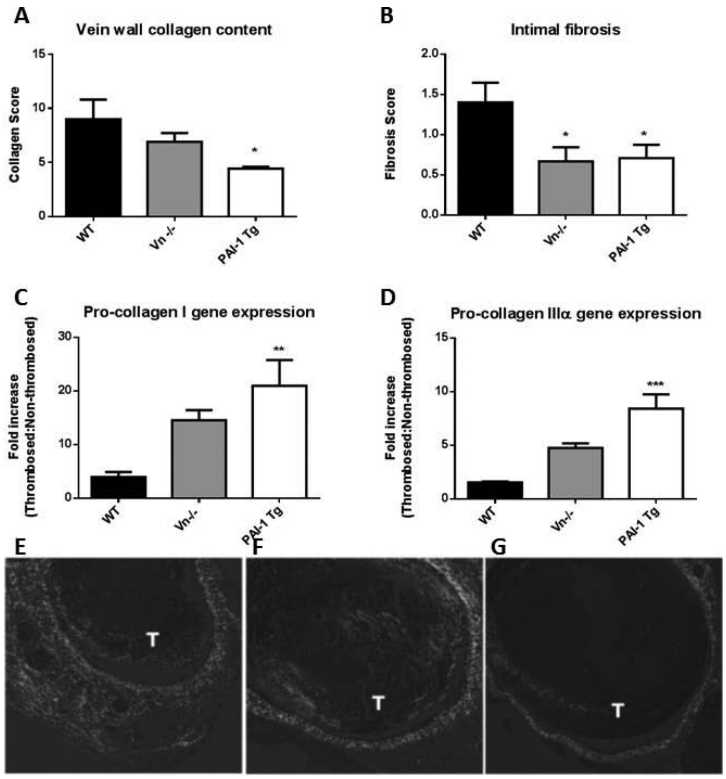

Figure 3.

At two weeks post-thrombosis collagen content was inversely related to PAI-1 activity with PAI-1 Tg mice exhibiting nearly a 50% reduction compared to WT at day 14 **(A, E-G,** n=5, *p<0.05). Cellular fibrotic changes (**B,** n=5) were inversely associated with levels of PAI-1. Compared to WT, collagen I and IIIa gene expression was increased in mice with lowest vein wall collagen deposition **(C,D,** n=5-10, **p<0.01, ***p<0.001). Decreased collagen content is evident in vein wall stained with Sirius Red in Vn **(F)** and PAI-1 Tg **(G)** compared to WT **(E,** T=thrombus).

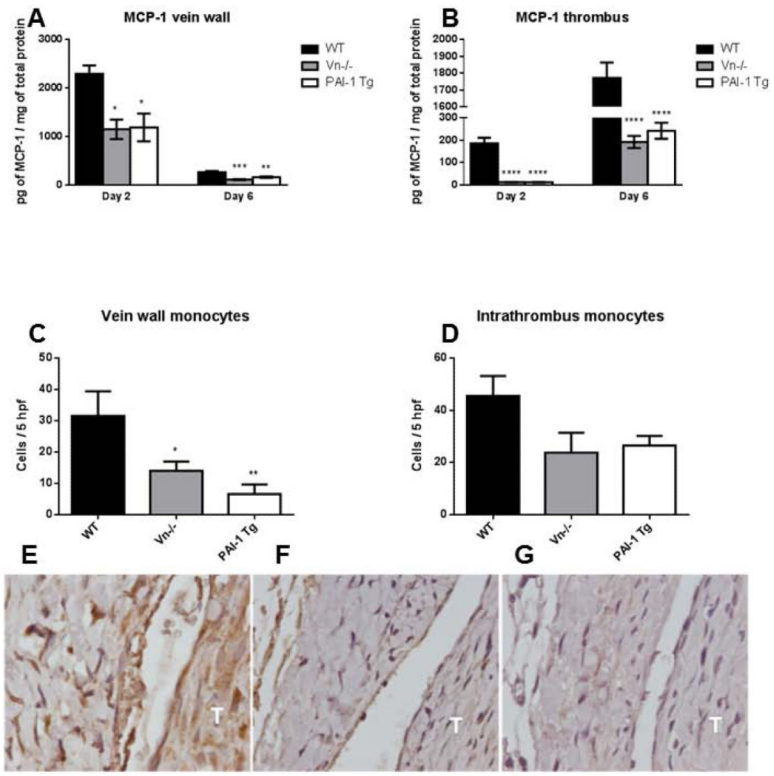

Figure 4.

MCP-1 was significantly decreased in Vn−/− and PAI-1 Tg mice in both the vein wall **(A)** and thrombus at days 2 and 6 **(B**, n=5-10). Vein wall monocyte influx **(C)** but not intrathrombus monocyte influx **(D)** at day 14 was significantly suppressed in Vn−/− and PAI-1 Tg mice (n=4-5). **E-G,** representative photomicrogaphs at 100x of WT **(E)**, Vn−/− **(F)** and PAI-1 Tg **(G)** mice stained with monocyte specific antibody F4/80. *T*, thrombus, *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by analysis of variance with Dunnett's multiple comparison test.

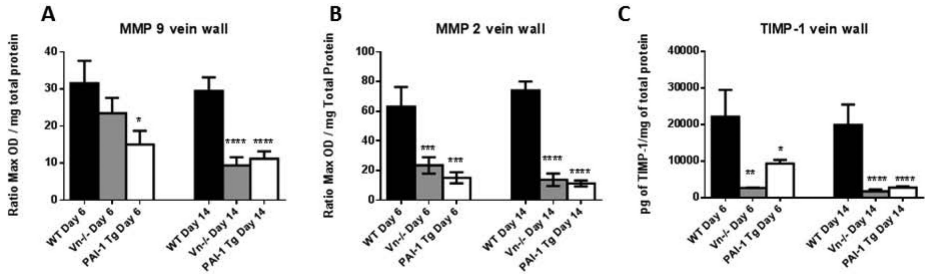

Figure 5.

Vein wall MMP-9 **(A)** and -2 **(B,** all n=8-12, ***p<0.001, ****p<0.0001) activity is diminished during thrombus resolution (6d and 14d) in Vn−/− and PAI-1 Tg mice compared to WT controls. TIMP-1 levels were similarly decreased in PAI-1 Tg mice and Vn−/− vein wall **(C,** n=5-10 *p<0.05, **p<0.01).

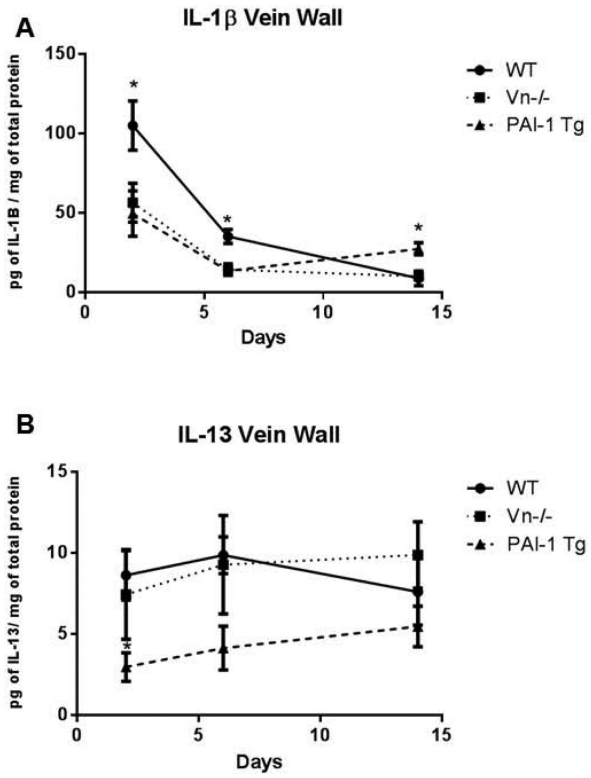

Figure 6.

IL-1β was reduced in the vein wall at early and mid-time points post thrombosis **(A,** all n=5-10, p*<0.05**)** in Vn−/− and PAI-1 Tg mice. PAI-1 Tg but not Vn−/−mice demonstrated a reduction in IL-13 at 2 days post thrombosis **(B)**.

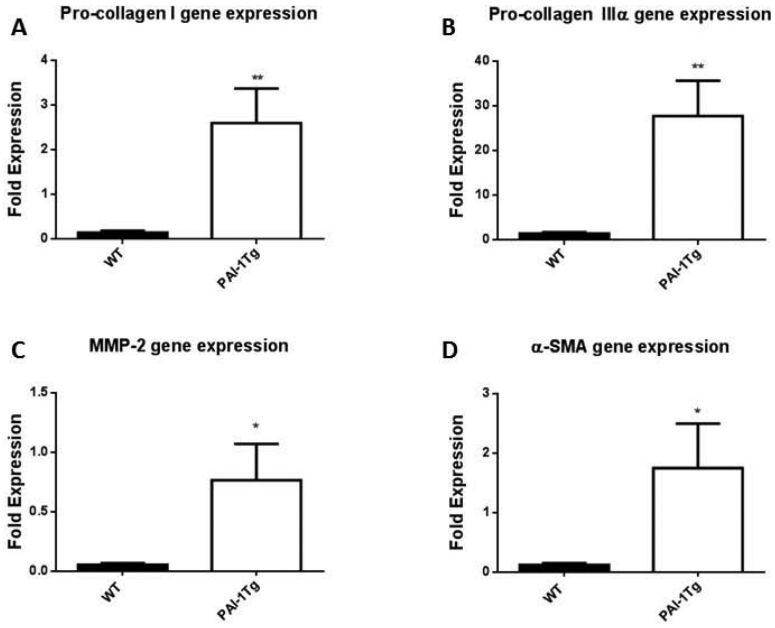

Figure 7.

Ex vivo vein wall response to pro-fibrotic stimuli with TFG-β demonstrates divergent response between WT and PAI-1 Tg mice. As compared with saline treated controls,, TGF-β administration was associated with a significant increase in pro-collagen I, IIIα,MMP-2 and α-SMA gene expression in PAI-1Tg vein wall compared to WT **(A-D,** all n=5, *p<0.05, **p<0.01).