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Low-molecular-weight heparin modulates vein wall fibrotic response in a plasminogen activator inhibitor 1-dependent manner

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Abstract

Background—Treatment with low-molecular-weight heparin (LMWH) favorably alters the vein wall response to deep venous thrombosis (DVT), although the mechanisms remain unclear. Previous studies have suggested that LMWH alters the levels of circulating plasminogen activator inhibitor 1 (PAI-1), a known mediator of fibrosis, and may improve endogenous fibrinolysis. We hypothesized that LMWH favorably alters the vein wall response by binding of PAI-1 and acceleration of fibrinolysis.

Methods—Wild-type and PAI-1 $^{-/-}$ mice underwent treatment with LMWH after induction of occlusive DVT. Vein wall and plasma were harvested and analyzed by enzyme-linked immunosorbent assay, zymography, real-time polymerase chain reaction, and immunohistochemistry.

Results—Wild-type mice treated with LMWH exhibited diminished vein wall fibrosis (0.6 ± 0.6 vs 1.4 ± 0.2; $P < .01$; n = 5) and elevation of circulating PAI-1 (1776 ± 342 vs 567 ± 104 ρg/mL;

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AUTHOR CONTRIBUTIONS

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$P < .01$; n = 5) compared with untreated controls after occlusive DVT. PAI-$1^{-/-}$ mice treated with LMWH were not similarly protected from fibrosis, despite improved thrombus resolution. Treatment with LMWH was associated with decreased intrathrombus interleukin-lβ (68.6 ± 31.0 vs 223.4 ± 28.9 ρg/mg total protein; $P < .01$; n = 5) but did not alter inflammatory cell recruitment to the vein wall. PAI-$1^{-/-}$ mice exhibited significantly elevated intrathrombus (257.2 ± 51.5 vs 4.3 ± 3.8 ρg/mg total protein; n = 5) and vein wall interleukin-13 (187.2 ± 57.6 vs 9.9 ± 1.1 ρg/mg total protein; $P < .05$; n = 5) as well as vein wall F4/80 positively staining monocytes (53 ± 11 vs 16 ± 2 cells/5 high-power fields; $P < .05$; n = 4).

Conclusions—LMWH did not accelerate venous thrombosis resolution but did protect against vein wall fibrosis in a PAI-1-dependent manner in an occlusive DVT model. Lack of PAI-1 correlated with accelerated venous thrombosis resolution but no protection from fibrosis. PAI-1 inhibition as a treatment strategy for DVT is likely to accelerate clearance of the thrombus but may come at the expense of increased vein wall fibrosis.

Clinical Relevance—The pathophysiologic mechanism of post-thrombotic syndrome is not well understood clinically or experimentally. In this study, we evaluated the effect of the prominent fibrinolytic mechanism, plasminogen activator inhibitor 1 (PAI-1), and low-molecular-weight heparin (LMWH) on vein wall injury after thrombosis. We show here that LMWH is protective from vein wall fibrosis, but this is abrogated in PAI-1-deleted mice. This is also correlated with monocyte vein wall influx. These data support the clinical observation that LMWH may be protective from post-thrombotic vein wall injury in a PAI-1-dependent manner.

Post-thrombotic syndrome (PTS) is a major health care problem, with a confirmed diagnosis in two to six of every 10 patients affected with deep venous thrombosis (DVT).[1] Acute thrombosis elicits an inflammatory response resulting in thickened, noncompliant vein wall and damaged valves[2]. Ultimately, reflux due to the damaged valves or residual obstruction from chronic thrombus or both results in venous hypertension, fluid transudate, edema, tissue hypoxia, discoloration, and, in severe cases, ulceration.[3] The paucity of knowledge about the molecular mechanisms responsible for development of PTS is highlighted by the fact that in the modern era, therapy is focused on symptomatic relief rather than treatment of the underlying pathophysiologic process and cure of the disease.

The current standard of care for treatment of DVT is therapeutic anticoagulation with vitamin K antagonists or low-molecular-weight-heparin (LMWH).[4] However, little direct evidence exists regarding anticoagulation and subsequent development of PTS. Indirect evidence, such as prevention of thrombus extension, prevention of recurrence, and correlation between subtherapeutic anticoagulation and development of PTS, supports a protective role of anticoagulants against the development of PTS.[5,6] Some data suggest that the use of LMWH may offer superior protection against development of PTS.[7,8] The biologic mechanisms responsible for this phenomenon are not well understood but have the potential both to improve our understanding of the pathophysiologic process of PTS and to lead to identification of biochemical pathways and molecular targets that allow therapeutic intervention for the prevention or treatment of PTS.

We hypothesized that LMWH may alter vein wall fibrotic response by two possible mechanisms: directly by inhibiting coagulation and accelerating fibrinolysis,[9,10] the

mechanism by which thrombolytic therapy decreases rates of PTS in humans,[11] or indirectly by binding and altering plasminogen activator inhibitor 1 (PAI-1),[12] a mediator of vein wall fibrotic response.[13] Results of previous animal studies suggest that treatment with LMWH may augment fibrinolysis.[8,14] Alternatively, LMWH may affect vein wall remodeling in a nonthrombus-dependent mechanism through its interactions with PAI-1. PAI-1 mediates tissue fibrosis by inhibiting tissue and urokinase plasminogen activators, plasmin formation, and plasmin-dependent matrix metalloproteinase (MMP) activation as well as by affecting cellular recruitment.[15] PAI-1 gene-deleted mice with induced DVT have worsened midterm vein wall fibrosis, suggesting that PAI-1 is important for venous wall remodeling.[13] This study was undertaken to determine whether improved thrombus resolution alters vein wall fibrotic response and if the protective effect of LMWH on the vein wall occurs in a PAI-1-dependent manner.

METHODS

Mouse model of DVT

C57BL/6 wild-type (WT) 8-to 10-week old mice (Jackson Laboratory, Me) or PAI-l$^{-/-}$ mice on a C57BL/6 background were anesthetized with inhaled isoflurane (2%) (Supplementary Table I, online only). A midline laparotomy was performed, the intestines were exteriorized, and the retroperitoneum was entered to expose the inferior vena cava (IVC). Infrarenal venous side branches were ligated with 7–0 Prolene suture (Ethicon, Inc, Somerville, NJ). In the total occlusion, "Venous stasis" model,[9,10,16–18] the caudal branches feeding the IVC were interrupted with electrocautery, and a 7–0 (Prolene) ligature was placed around the IVC to generate stasis. The incision was closed in a bilayer fashion with 5–0 Vicryl suture followed by VetBond skin adhesive (3M Animal Care Products, St. Paul, Minn).

All operations were performed by a single dedicated microsurgeon. At the time of harvest, the IVC and associated thrombus were removed, weighed, and measured (total thrombus weight). The thrombus was then separated from the IVC and weighed (isolated thrombus weight). The tissue was processed by formalin fixation (immunohistochemistry/histology) or snap frozen (−80°C) for processing. All procedures were approved by the University Committee on Use and Care of Animals. All work was performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

LMWH treatment protocol

To evaluate the effect of LMWH on thrombosis and thrombus resolution, mice were dosed with enoxaparin (Aventis-Pharma, Bridgewater, NJ), 6 mg/kg/d subcutaneously. The first dose was administered after the procedure, and it was given daily until the time of harvest at 2, 6, or 14 days after thrombosis. Adequacy of anticoagulation was determined by antifactor Xa levels.

Vein wall morphometries and immunohistochemistry

Infrarenal IVCs were resected en bloc with adjacent aorta and psoas muscle (n = 5), preserved in 10% formalin followed by 100% ethanol, and sectioned in paraffin.

Hematoxylin and eosin-stained sections were examined under oil immersion light microscopy (× 100), and vein wall polymorphonuclear (PMN) cell counts were performed in a blinded fashion by a board-certified veterinary pathologist according to standard pathologic criteria. Vein wall monocyte cell counts were performed after staining with F4/80 antibody (Abcam, Cambridge, Mass). Briefly, nonspecific binding sites were blocked with normal serum and primary antibody was added (1:100), followed by biotin-labeled secondary antibody (rat immunoglobulin G). An avidin, biotin, peroxidase complex was performed according to the manufacturer's instructions (Vector Laboratories Inc, Burlingame, Calif), and the slides were counterstained with hematoxylin.

Vein wall fibrosis was scored by the same dedicated board-certified veterinary pathologist in a blinded manner. A hematoxylin and eosin-stained midsection of the thrombosed IVC segment was used for comparison of all groups. Five representative high-power fields were examined circumferentially around the diameter of the vein and scored according to a previously validated scoring system (Supplementary Table II, online only).[9,10,13]

Circulating PAI-1 assay

Murine plasma was preserved in sodium citrate, and levels of active PAI-1 were determined as previously described (n = 5).[10,19] Briefly, the sample was incubated overnight in phosphate-buffered saline 1% bovine serum albumin solution (10 μL) and 5000 Luminex beads (in 30 μL phosphate-buffered saline 1% bovine serum albumin). Human urokinase plasminogen activator (rheotromb) coupled to carboxylated beads captured active murine PAI-1. Rabbit antimouse PAI-1 antibody labeled with biotin (Molecular Innovations, Novi, Mich) was added to the beads, followed by streptavidine-R-phycoerthrin (Molecular Probes, Eugene, Ore). The beads were read with Luminex 100 and compared with a standard curve that was generated by addition of known concentrations of murine PAI-1 into PAI-1 – depleted mouse plasma (Molecular Innovations).

MMP gel zymography

Gelatin substrate zymography (MMP-2 active and latent forms; n = 5 controls, n = 10 experimental) was performed on precast sodium dodecyl sulfate–polyacrylamide gels (Novex, San Diego, Calif) as previously described.[10,16,17] MMP activity was visualized as clear bands against a darkly stained background. Optical density was measured with a FOTO/ Analyst CCD camera (Fotodyne, Hartland , Wisc) and EL-Pro Analyzer software version 3.1 (Media Cybernetics, Silver Springs, Md). Results for each sample were normalized to total protein present as previously detailed.

Quantitative real-time polymerase chain reaction

Gene expression of procollagen 1 (col1a2, PPM04448E Ref Pos 4816) and procollagen III (Col3a, PPM04784B Ref Pos 4380) was determined by isolated vein wall RNA (n = 10) in TRIzol reagent as previously described.[10,17] Samples underwent reverse transcription, and the resultant complementary DNA was amplified by *Taq* polymerase in the Rotor-Gene quantitative polymerase chain reaction system (Qiagen Inc, Valencia, Calif). DNA levels of each gene of interest were measured with SYBR green intercalating dye (Roche,

Indianapolis, Ind). Gene expression was normalized to β actin (PPM02945A Ref Pos 164–183) levels for each specimen.

Antigen determination

Vein wall and thrombus for each specimen were homogenized, treated with ultrasonic sonication, and centrifuged (10,000 *g* × 5 minutes), and supernatant was collected for analysis (n = 5). Antigen determination of monocyte chemoattractant protein 1 (MCP-1), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), and interleukin (IL)-β and IL-13 by enzyme-linked immunosorbent assay was performed according to the manufacturer's protocol (R&D Systems, Minneapolis, Minn). Results were normalized to total protein by a bicinchoninic acid protein assay kit.[10,17]

Statistical analysis

Descriptive statistics of central tendency include mean ± standard error of mean and median. Statistical analysis was performed with either unpaired *t*-test with Welch correction or one-way analysis of variance between groups with Tukey multiple comparison test as appropriate. Statistical software and assistance were provided by the University of Michigan (GraphPad Software, Inc, La Jolla, Calif), and significance was reported for $P < .05$. In accordance with independent statistical review, animal numbers all were >5 on the basis of protocols and numbers needed for significance.[18,20–22] For comparison of immunologic test results, we expect at least 20% difference among treated animals, and thus for an α of .5 and power of 80%, n = 5 is sufficient, whereas among zymography, an n = 10 was needed to achieve a power of 80%.

RESULTS

Thrombus resolution is accelerated with PAI-1 gene deletion but not by addition of LMWH

To explore whether LMWH augments endogenous fibrinolysis,[14,23] we measured thrombus weights at 2, 6, and 14 days. Thrombogenesis, as measured by isolated thrombus weight, peaks at 2 days in our model, and these values served as the reference point for maximum thrombus weight (100%; Fig 1, *A*). The percentage of remaining thrombus was calculated at days 6 and 14 and demonstrated that PAI-1 gene deletion resulted in significantly augmented thrombus resolution ($P < .0001$ [6 days] and $P < .01$ [14 days]; n = 50–55 [WT], 25–26 [WT + LMWH], 34–37 [PAI-$1^{-/-}$], 19–24 [PAI-$1^{-/-}$ + LMWH]; Fig 1, *B*), consistent with findings from a previous study.[13] However, WT and PAI-$1^{-/-}$ mice treated with LMWH, despite having lower initial thrombus burden than genotype controls (Fig 1, *A*), did not demonstrate accelerated venous thrombus resolution (P = NS; Fig 1, *B*).

Treatment with LMWH is associated with PAI-1 -dependent decrease in vein wall fibrosis

Mice treated with LMWH (WT + LMWH) had significant protection from vein wall fibrosis at 14 days after thrombosis compared with genotype controls as measured by a histologic scoring system ($P < .02$; n = 5; Fig 2, *A*)[9]. This was associated with a threefold increase in total circulating PAI-1 ($P < .001$; n = 4–6; Fig 2, *B*) and an approximately 10-fold increase in procollagen I and IIIα gene expression ($P < .05$; n = 5–10; Fig 2, *C* and *D*).

To determine whether the protective effect of LMWH on end-organ fibrosis was related to circulating PAI-1 levels[13] or initial thrombus burden,[24] a second set of animals with PAI-1 gene deletion (PAI-$1^{-/-}$) were treated with LMWH (PAI-$1^{-/-}$ + LMWH) to generate a circumstance in which initial thrombus burden would approximate that of the WT animals treated with LMWH (*P* = NS compared with WT + LMWH; n = 23; Fig 1, *A*), but PAI-1 levels would not be affected by administration of LMWH (*P* = NS compared with WT; n = 5; Fig 1, *B*). These animals demonstrated a vein wall response identical to that of the WT mice (without LMWH) with regard to vein wall procollagen gene expression (Fig 2, *C* and *D*) and fibrotic changes(Fig 2, *E* and *F*), suggesting that presence of PAI-1 is essential to vein wall phenotype.

Vein wall MMP activity elevated with PAI-1 deficiency

MMP-2 is associated with post-thrombotic vein wall remodeling in both humans[25] and rodent models of venous thrombosis[16] and has been implicated as an important determinant of the vein wall fibrotic response.[17] To determine whether elevation of PAI-1 levels affected MMP activity through inhibition of MMP activator plasmin, we measured vein wall MMP-2 during peak activity, at 6 days after thrombosis. Compared with untreated and treated (LMWH) controls, the PAI-$1^{-/-}$ mice exhibited increased MMP-2 activity by zymography (WT vs PAI-$1^{-/-}$: *P* = .02, n = 10; WT + LMWH vs PAI-$1^{-/-}$ + LMWH: *P*< .05, n = 4–10; Fig 3, *A*). Pro-MMP-2 activity was similarly elevated in PAI-1-deficient mice compared with WT genotype controls (WT vs PAI-$1^{-/-}$: *P*= .01, n = 9–11; WT + LMWH vs PAI-$1^{-/-}$ + LMWH: *P* =.0001, n = 3–10; Fig 3, *B*). Importantly, nonthrombosed mice MMP-2 activity (WT = 0.00 ± 0.00; WT + LMWH = 0.00 ± 0.00; PAI-$1^{-/-}$ = 2.86 ± 2.86; PAI-$1^{-/-}$ + LMWH = 0.00 ± 0.00; *P*= NS; n = 3–5) and pro-MMP-2 activity (WT = 0.21 ± 0.21; WT + LMWH = 0.00 ± 0.00; PAI-$1^{-/-}$ = 0.00 ± 0.00; PAI-1 + LMWH = 0.00 ± 0.00; P= NS; n = 4–5) did not vary, suggesting that the alteration in MMP activity was specific to thrombotic injury and not inherent to the PAI-$1^{-/-}$ genotype.

TIMP-1 is the primary inhibitor of MMP-2. Analysis of protein levels of TIMP-1 failed to show depressed levels in the PAI-1-deficient mice that could account for corresponding increased MMP activity and in fact demonstrated increased levels in the PAI-$1^{-/-}$ + LMWH mice compared with WT + LMWH controls (*P* = .01; n = 4– 5; Fig 3, C).

Monocyte recruitment is altered in PAI-$1^{-/-}$ but not WT mice treated with LMWH

Early (PMN) and late (monocyte) inflammatory cell infiltrate was measured in the vein wall. PMN recruitment at day 2 was not significantly altered by PAI-$1^{-/-}$ or addition of LMWH (*P*= NS; n = 4–5; Fig 4, *A*). Monocyte recruitment to areas of tissue damage can be either proinflammatory or anti-inflammatory and may play a key role in organ fibrosis.[26] Monocytes ($F4/80^{+}$ cells) were significantly elevated in PAI-$1^{-/-}$ vein walls, whether or not they received LMWH (Fig 4, *B*). To determine if monocyte recruitment was affected, levels of MCP-1 were measured in the vein wall at days 2 and 6 after thrombosis, and monocytes were counted in the vein wall at 14 days after thrombosis. Whereas MCP-1 levels varied little among groups (n = 4–5; Fig 4, *C*), there was a significant increase in monocytes recruited to the vein wall of the PAI-$1^{-/-}$ mice (WT vs PAI-1: 2.6-fold increase, *P*= .14, n = 4; WT + LMWH vs PAI-1 + LMWH: 3.3-fold increase, *P* = .04; Fig 4, *D-F*).

Divergent IL-1β and IL-13 response in vein wall with LMWH treatment and PAI-1 gene deletion

Studies have demonstrated that heparins and LMWHs have the ability to alter the local cytokine milieu by alterations in proinflammatory cytokines and[27] profibrotic cytokines.[28] We measured proinflammatory IL-1β and profibrotic cytokine IL-13 to test this hypothesis in our model. We found no differences in vein wall IL-1β (Fig 5, *A*), but intrathrombus IL-1β was significantly reduced in the WT mice treated with LMWH at days 2 and 6 and in PAI-l$^{-/-}$ mice treated with LMWH at day 6 ($P < .05$; n = 4–5; Fig 5, *B*). Remarkably, IL-13 was markedly upregulated in both the thrombus (day 6; Fig 5, *C*) and vein wall (days 6 and 14; Fig 5, *D*) in the PAI-1$^{-/-}$ group compared with all others ($P < .05$; n = 4–5), suggesting that PAI-1 gene deletion significantly alters profibrotic mediators in response to thrombosis. This effect was diminished by the addition of LMWH, with significantly decreased IL-13 levels in the PAI-1 $^{-/-}$ vein walls (all $P < .05$; n = 4–5; Fig 5, *C* and *D*).

DISCUSSION

The current standard of care for treatment of DVT is anticoagulation, which prevents thrombus extension, embolization, and recurrence. However, there are no specific therapies to target vein wall remodeling and fibrosis, which ultimately results in PTS in a significant proportion of patients.[1,2,29] Some data suggest that treatment with LMWH is associated with a decreased incidence of PTS.[8] Understanding of the molecular mechanisms by which LMWH affects the vein wall response may identify key processes to prevent PTS. In this study, using experimental DVT, we demonstrate that (1) LMWH does not accelerate fibrinolysis, (2) augmentation of fibrinolysis and smaller thrombus size do not decrease tissue fibrosis, (3) LMWH is protective against vein wall fibrosis in a PAI-1-dependent manner, and (4) alterations in local MMP activity are associated with the tissue fibrotic response.

LMWH decreased the initial thrombus size in our model of DVT, which could account for less initial vein wall injury and subsequent decreased fibrotic response. Interestingly, fibrinolysis in animals treated with LMWH was not augmented, as has been reported in nonocclusive models of thrombosis, such as the rabbit jugular vein model.[14] Accelerated fibrinolysis and rapid clearance of the thrombus achieved by infusion of plasminogen activators correlate with improved quality of life in patients with iliofemoral DVT,[30,31] suggesting that the degree of mechanical obstruction may determine the degree of vein wall injury and ultimately the development of PTS. PAI-1 gene deletion allowed us to mimic a prothrombolytic environment by disinhibiting the tissue plasminogen activator/urokinase plasminogen activator/plasmin axis. PAI-1 gene deletion in combination with LMWH resulted in a smaller initial thrombus size and increased rate of the thrombus resolution, but it did not improve vein wall fibrotic response. These data are congruent with other studies reporting that a smaller thrombus does not correlate with a favorable vein wall response.[13,17,32,33] Our results suggest that the beneficial effect of LMWH on the vein wall fibrotic response is independent of thrombus size but dependent on the presence of PAI-1.

PAI-1 levels were elevated in LMWH-treated mice, probably because of the ability of LMWH to complex with PAI-1.[34,35] The antifibrotic effect of LMWH was abrogated in

PAIT$^{-/-}$ mice, despite improved thrombus clearance, suggesting a critical role for PAI-1 in mediating vein wall response. We demonstrate an inverse relationship between PAI-1 levels and MMP activity. The proteolytic activity of MMP-2[17] plays a key role in modulating post-thrombotic vein wall remodeling and fibrosis. Taken together, these data suggest that occlusive thrombi, as many patients have at the time of diagnosis, result in vein wall damage sufficient to increase local MMP activity, which is amplified in the absence of PAI-1.

Inflammatory cells represent an important source of MMPs in the post-thrombotic vein wall. During thrombogenesis, PMNs represent a major source of MMP-9,[36] and during thrombus resolution, influxing monocytes as well as smooth muscle cells supply MMP-2.[22] Experimentally, early post-thrombosis inflammatory cellular influx is decreased with LMWH treatment.[37] In our model, we found that early PMN influx was nonsignificantly decreased in both WT and PAI-I$^{-/-}$ LMWH-treated mice, correlating with smaller thrombi found in these animals. In contrast, late monocyte influx did not correlate with thrombus size but was significantly elevated in PAI-1$^{-/-}$ + LMWH mice, perhaps because of competition of PAI-1 with monocyte surface receptor urokinase plasminogen activator receptor for binding to extracellular matrix protein vitronectin. Binding of PAI-1 displaces urokinase plasminogen activator receptor and inhibits monocyte adhesion.[38] MCP-1 levels were not elevated in these animals, suggesting that the monocyte influx was not related to thrombus size or altered chemokine milieu. An important area of future research will be determining the relative contribution of PAI-1 activity and inflammatory cell influx to MMP-mediated vein wall remodeling.

Improved vein wall fibrosis in WT + LMWH mice coincided with increased gene expression of type I and IIIα procollagen gene expression. Previous experiments have confirmed that LMWH treatment results in less collagenolysis,[20] decreased fibrotic response,[39] and improved endothelial recovery,[33] and thus increased collagen gene expression may represent normal or advantageous vein wall healing response. Intrathrombus IL-1β was decreased in animals treated with LMWH in our study, perhaps secondary to a diminished inflammatory response in the setting of a smaller thrombus size. Interestingly, vein wall IL-1β was not similarly decreased, and thus vein wall inflammatory cytokine response occurred independently of magnitude of thrombosis. Ex vivo, IL-1β– stimulated LMWH-treated vein wall demonstrates an increased collagen gene expression compared with controls,[33] suggesting that LMWH may modulate endothelial response to local cytokines. LMWH did not decrease profibrotic IL-13 in our study as demonstrated by others,[39] but we did demonstrate markedly elevated IL-13 in PAI-1$^{-/-}$ mice. To our knowledge, this is the first time a relationship between PAI-1 and IL-13 has been described in vascular fibrosis.

LMWH is associated with anti-inflammatory properties independently of anticoagulant properties,[33,37,40] which may play a role in decreasing the incidence of PTS after DVT.[8] These data, congruent with others,[32,33] suggest that the size of the thrombus is not the main determinant of the vein wall fibrotic response. This has important implications translationally, as current therapies are aimed at decreasing residual thrombus and preventing thrombus extension rather than mediating the molecular response. We do acknowledge the limitations of animal models and in particular that the factor of venous hypertension is not the same as in humans. We demonstrate that the antifibrotic effect of

LMWH is dependent on the presence of PAI-1, and thus the beneficial effect of accelerated fibrinolysis by PAI-1 inhibition may be offset by undesirable vein wall response. Similarly, no data exist on whether unfractionated heparin or warfarin would confer a similar benefit. However, data do exist in humans that LMWH may be more efficacious in humans in preventing PTS.[7] Lastly, the use of novel oral anticoagulants for the treatment of DVT is an important emerging area of study as the effect on post-thrombotic vein wall remodeling and fibrosis remains unknown despite widespread adoption into clinical practice.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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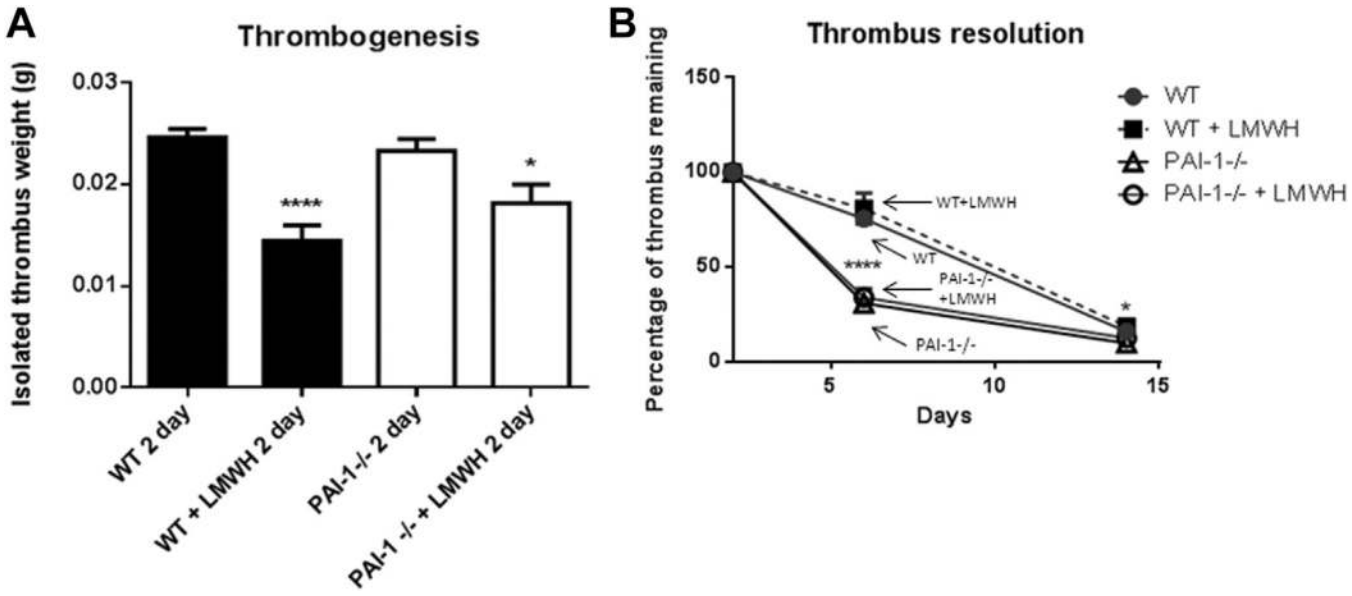

Fig 1.

Peak thrombus weight, measured during thrombogenesis (day 2 after surgery), was significantly diminished in the low-molecular-weight heparin (*LMWH*)-treated groups wild type (*WT*) + LMWH ($P < .0001$) and plasminogen activator inhibitor 1 $(PAI\text{-}1)^{-/-}$+ LMWH ($P < .05$) compared with genotype controls. There was no difference between WT and $PALI^{-/-}$ mice with respect to thrombus weight (n ≥25, all groups) (**A**). Peak thrombus weight at 2 days was assigned 100%, and remaining thrombus at 6 and 14 days was displayed as a percentage to calculate thrombus resolution over time. During thrombus resolution, a significantly smaller percentage of thrombus remained in mice with $PALI^{-/-}$ genotype (untreated and treated with LMWH) at days 6 ($P < .0001$) and 14 ($P < .05$) compared with WT and WT + LMWH mice (**B**).

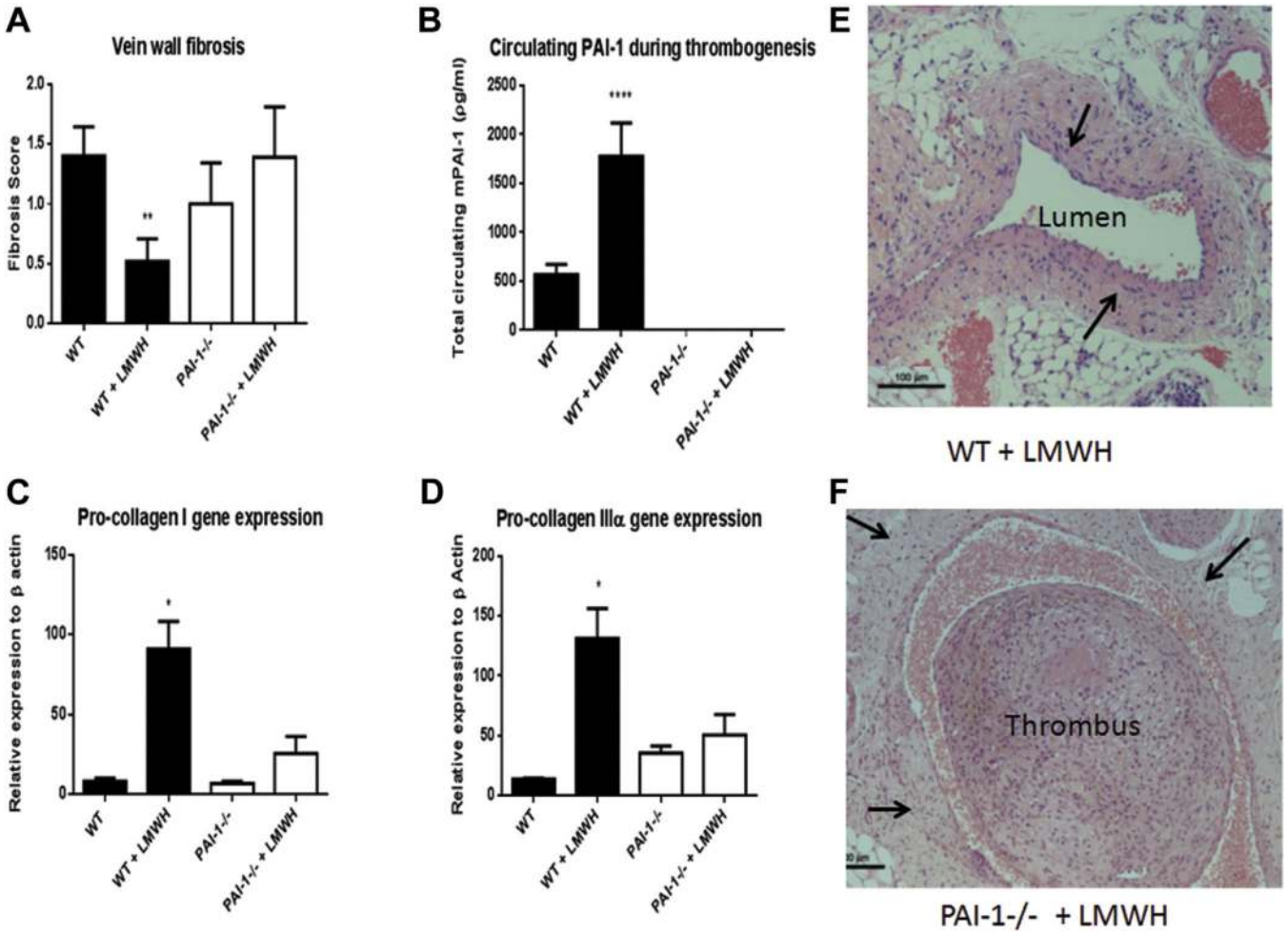

Fig 2.

A, Wild type (*WT*) mice dosed treated with enoxaparin (WT + low-molecular-weight heparin *[LMWH]*) were protected from post-thrombotic vein wall fibrosis at 14 days compared with WT mice ($P = .009$; n = 5). B, The protective effect of LMWH correlated with significantly elevated ($P < .0001$; n = 5) levels of circulating plasminogen activator inhibitor 1 (*PAI-1*) during thrombogenesis as measured at 2 days after surgery. Mice gene-deleted for PALI had no appreciable circulating levels of PALI (**B**, WT vs PALI$^{-/-}$ and PALI$^{-/-}$ + LMWH; P = NS; n = 5) and were not similarly protected from fibrosis (**A**, WT vs PAI-1$^{-/-}$ and PAI-1 + LMWH; P = NS; n = 5). **C** and **D**, Procollagen I and IIIα gene expression was elevated in WT + LMWH mice compared with both untreated controls and PAI-1$^{-/-}$ mice treated with LMWH ($P < .05$; n = 5–10), whereas no difference existed between WT and PAI-1$^{-/-}$ or PAI-1 + LMWH (P = NS; n = 5–10). **E** and **F**, Photomicrographs (×100) of WT + LMWH (E) displaying the relatively normal architecture of the vein wall intima and media, lacking significant fibrosis; in contrast, no discrete layers are present in the PAI-1$^{-/-}$ + LMWH (F); the *arrows* denote vessel wall fibrosis; *scale bar* =100 μm.

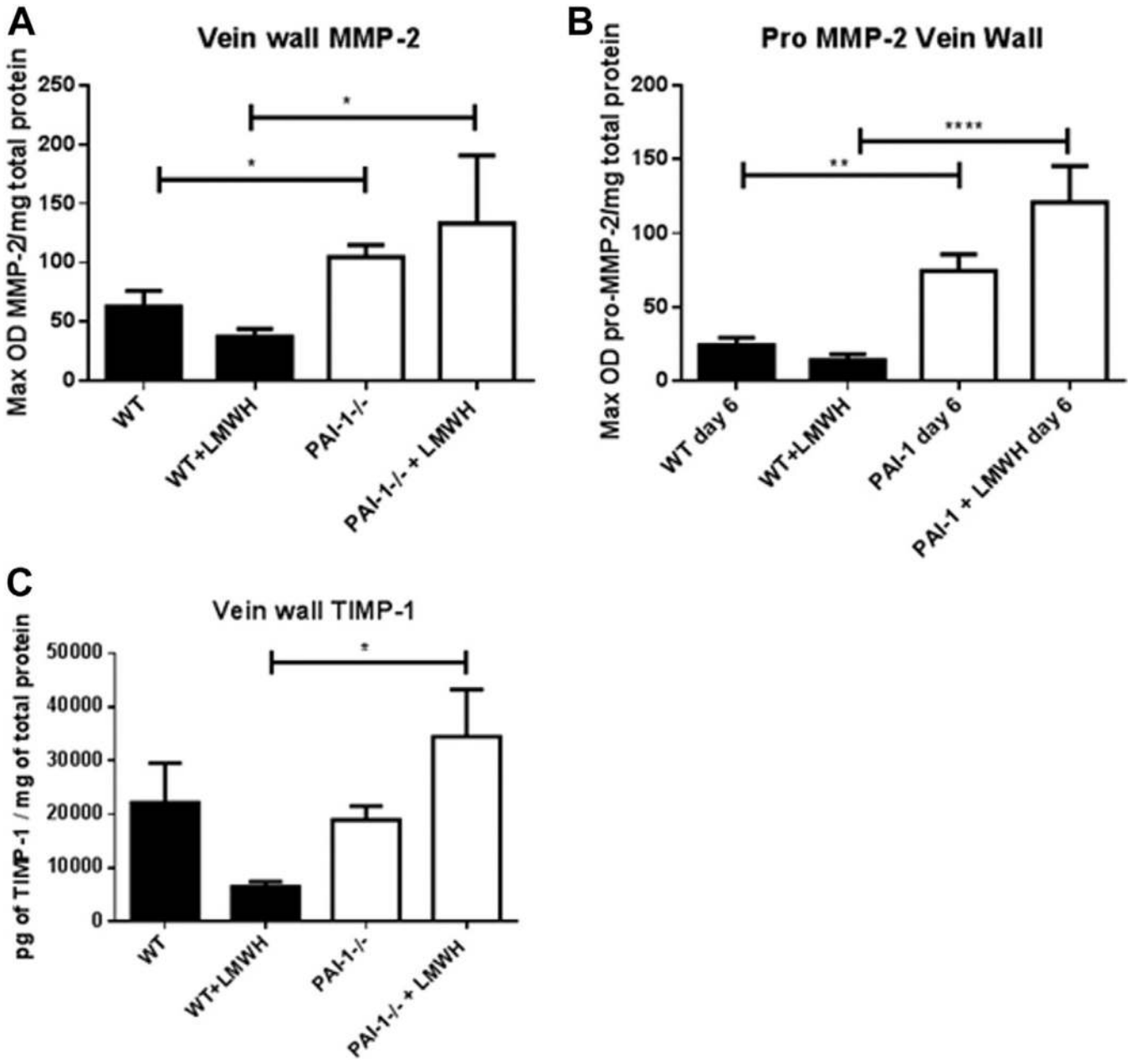

Fig 3.

Matrix metalloproteinase 2 (*MMP-2*) activity during thrombus resolution (6 days) was elevated in the plasminogen activator inhibitor 1 (*PAI-1*) animals compared with wild type (*WT*) (untreated and treated) controls ($P < .05$; n = 5–10). Low-molecular-weight heparin (*LMWH*) treatment did not significantly alter the levels of MMP-2 activity (**A**). A similar trend was demonstrated in pro-MMP-2 levels (**$P < .01$; ****$p < .0001$; n = 5–10) (**B**). Vein wall tissue inhibitor of matrix metalloproteinase 1 (*TIMP-1*) did not vary inversely with MMP-2 levels (P = NS; n = 5) (**C**). *OD*, Optical density.

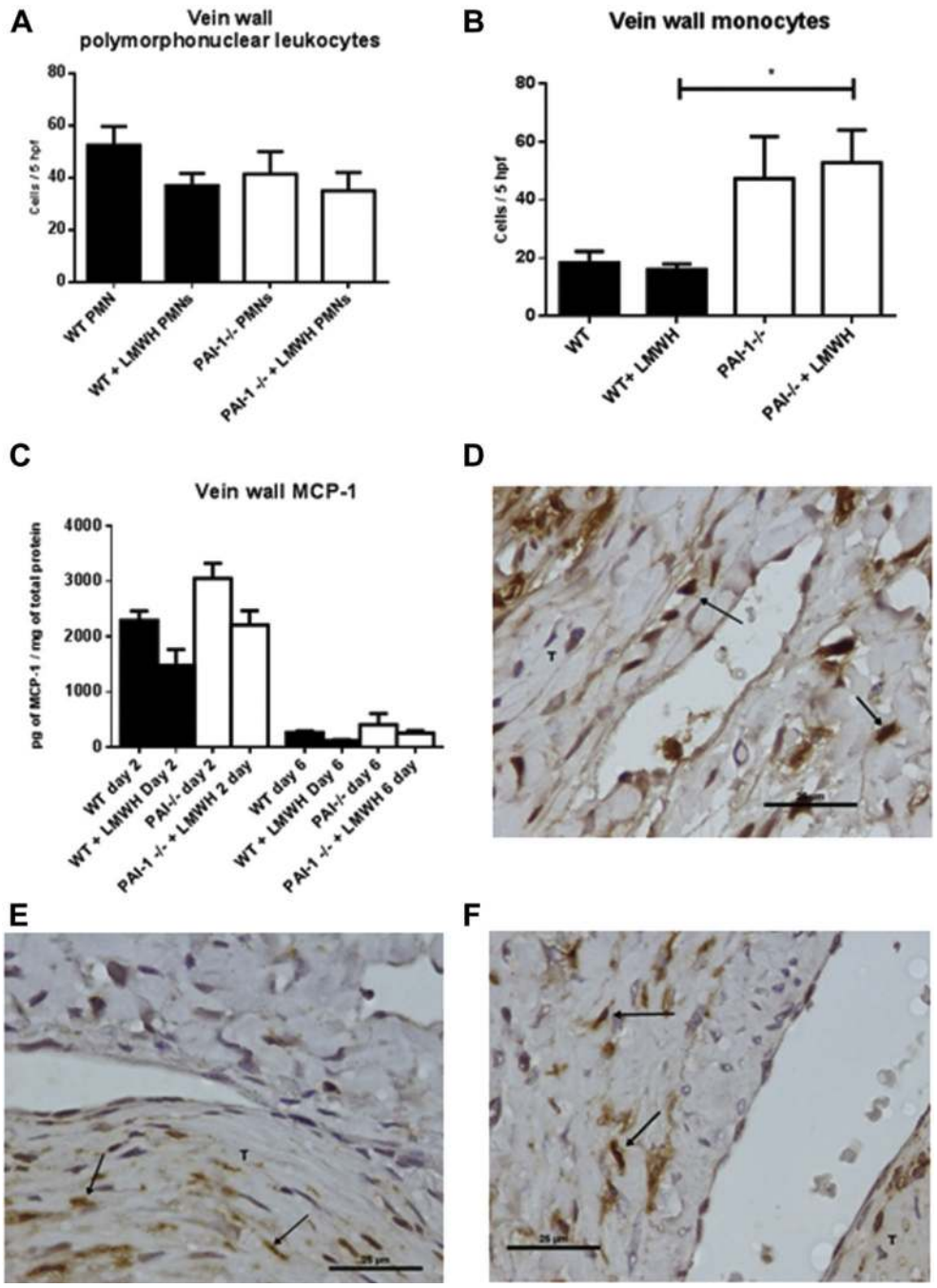

Fig 4.

A, Vein wall polymorphonuclear (*PMN*) leukocyte influx was not different among groups at day 2 after thrombosis. **B**, Vein wall monocytes were significantly elevated in plasminogen activator inhibitor 1 $(PAI\text{-}1)^{-/-}$ mice at day 14 after thrombosis by 2.6-fold compared with wild type (*WT*), although this did not reach significance. Similarly, PALI + low-molecular-weight heparin (*LMWH*) mice had increased monocytes (3.3-fold) compared with WT controls ($P < .05$; n = 5). **C**, The influx of monocytes in these mice did not correspond with elevation of vein wall chemokine monocyte chemoattractant protein 1 (*MCP-1*). **D-F**,

Representative photomicrographs of WT, WT + LMWH, and PAI-$1^{-/-}$ + LMWH at day 14 after thrombosis. Slides treated with antibody against monocyte-specific cell marker F4/80. Histologic images ×100; the *arrows* mark representative monocytes. *hpf*, High-power field; *T*, thrombus.

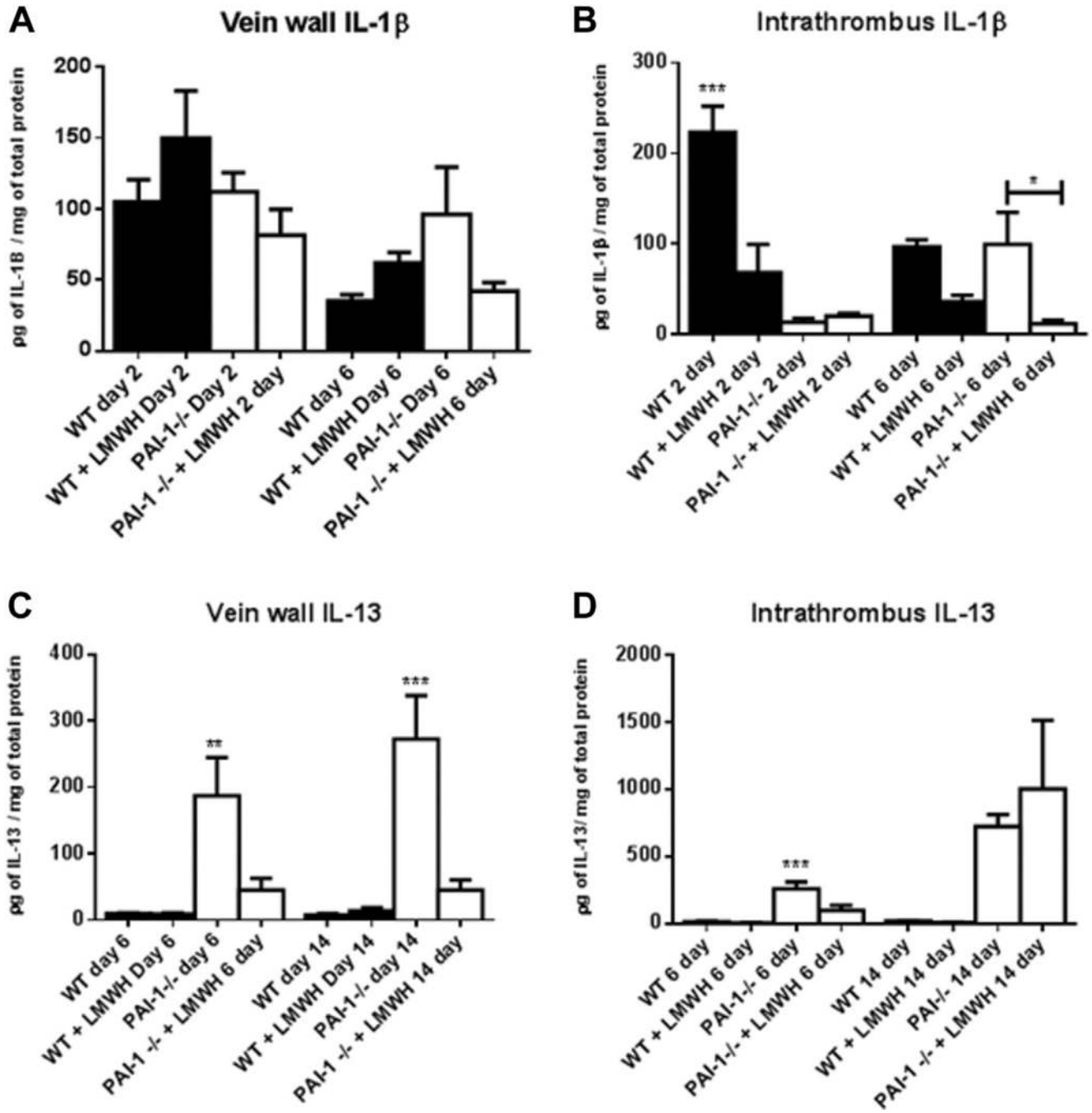

Fig 5.

Vein wall interleukin (IL)-Iβ did not differ significantly among the groups after thrombosis (**A**), although the addition of low-molecular-weight heparin (*LMWH*) decreased intrathrombus IL-1β in wild type (*WT*) animals at 2 days and in plasminogen activator inhibitor 1 $(PAI\text{-}1)^{-/-}$ animals at 6 days (**B**). IL-13 was consistently elevated in the vein wall (**$P < .01$; ***$p < .001$; n = 5) of PAI-$1^{-/-}$ mice compared with WT controls and was

diminished by the addition of LMWH (**C**), with a similar trend seen at day 6 in the thrombus (**D**).