Vein wall re-endothelialization after deep vein thrombosis is improved with low-molecular-weight heparin

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Objective: **Vein wall endothelial turnover after stasis deep vein thrombosis (DVT) has not been well characterized. The purpose of this study was to quantify re-endothelialization after DVT and determine if low-molecular-weight heparin (LMWH) therapy affects this process.**

Methods: **Stasis DVT was generated in the rat by inferior vena cava ligation, with harvest at 1, 4, and 14 days. Immunohistologic quantification of vascular smooth muscle cells and luminal endothelialization was estimated by positive staining for α-smooth muscle actin and von Willebrand factor, respectively. In separate experiments, rats were treated either before or after DVT with subcutaneous LMWH (3 mg/kg daily) until harvesting at 4 and 14 days. The inferior vena cava was processed for histologic analysis or was processed for organ culture after the thrombus was gently removed. The vein wall was stimulated in vitro with interleukin-1β (1 ng/mL), and the supernatant was processed at 48 hours for nitric oxide. Cells were processed by real-time polymerase chain reaction for endothelial nitric oxide synthase, inducible nitric oxide synthase, cyclooxygenase-1 and -2, and thrombomodulin at 4 and 14 days, and collagen I and III at 14 days. Comparisons were done with analysis of variance or *t* test. A $P < .05$ was significant.**

Results: **Thrombus size peaked at 4 days, whereas luminal re-endothelialization increased over time (1 day, 11% ± 2%; 4 days, 23% ± 4%; 14 days, 64% ± 7% (+) von Willebrand factor staining; $P < .01$, n = 3 to 4, compared with non-DVT control). Similarly, vascular smooth muscle cell staining was lowest at day 1 and gradually returned to baseline by 14 days. Both before and after DVT, LMWH significantly increased luminal re-endothelialization, without a difference in thrombus size at 4 days, but no significant difference was noted at 14 days despite smaller thrombi with LMWH treatment. Pretreatment with LMWH was associated with increased vascular smooth muscle cell area and recovery of certain inducible endothelial specific genes. No significant difference in nitric oxide levels in the supernatant was found at 4 days. At 14 days, type III collagen was significantly elevated with LMWH treatment.**

Conclusions: **Venous re-endothelialization occurs progressively as the DVT resolves and can be accelerated with LMWH treatment, although this effect appears limited to the early time frame. These findings may have clinical relevance for LMWH timing and treatment compared with mechanical forms of therapy. (J Vasc Surg 2008;47:616-24.)**

Clinical Relevance: **How the vein wall endothelium responds after deep vein thrombosis (DVT) has not been well documented owing to limited human specimens. This report shows that low-molecular-weight heparin accelerates or protects the endothelium and preserves medial smooth muscle cell integrity after DVT, but that this effect is limited to a relatively early time period. Although most DVT prophylaxis is pharmacologic (a heparin agent), use of nonpharmacologic measures is also common. The use of heparin prophylaxis, compared with after DVT treatment, and the acceleration of post-DVT re-endothelialization require clinical correlation.**

The pathobiology of deep vein thrombosis (DVT) resolution is complex, inflammatory in nature, and results in vein wall injury. The mechanical and biochemical effect of the thrombus, in juxtaposition to the vein wall directs this process. Clinically, post-DVT sequelae include post-thrombotic syndrome, recurrent DVT, and pulmonary embolism (PE). At the organ level, DVT resolution is accompanied by valvular dysfunction and stiff vein walls that manifest as venous hypertension with pain, swelling, and sometimes recalcitrant ulceration.[1,2] Heparin anticoagulation is the standard of care for DVT treatment and is proven to significantly reduce the risk of PE, as well as recurrent DVT.[3,4] A recent report suggests that rapid anticoagulation with low-molecular-weight heparin (LMWH) may decrease the incidence of post-thrombotic syndrome, but the mechanisms are not clear, and patient heterogeneity in outcomes exists.[5]

The mechanical and inflammatory insult caused by an acute DVT is now fairly well characterized in animal models.[6] Common amongst all models is the early influx of polymorphonuclear (PMN) neutrophils, followed by monocytes, thrombus neovascularization, and fibrosis. Both the lysing thrombus as well as the mechanical effect of the thrombotic occlusion likely causes vein wall endothelial damage.

Arterial injury models suggest that more rapid and complete endothelialization (return of endothelium after

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denudation) after injury is associated with lesser neointimal hyperplasia, a form of fibrotic injury. For example, exogenous administration of granulocyte-monocyte colony stimulating factor and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase agents have been shown to accelerate re-endothelialization and reduce vessel injury.[7,8] Modarai et al[9] have shown that circulating endothelial progenitor cells increase after experimental DVT and localize to the resolving thrombus.[9] The vein wall is also characterized by different endothelial markers than arterial endothelium, with less tissue plasminogen activator, similar thrombomodulin (TM), but more von Willebrand factor (vWF) expression.[10,11] To our knowledge, no experimental models of DVT have yet characterized the vein luminal endothelial response over time.

The contribution of the basement membrane and vascular smooth muscle cells (VSMCs) in directing and supporting endothelialization after injury has been described in the arterial system,[10,12,13] but less so in the venous system. Vascular injury promotes VSMC proliferation, migration, and promotes a cellular synthetic state.[11,14,15] Exposure of the basement membrane proteins to circulating blood after injury also stimulates thrombus formation. For example, factor XI is an integral component of the subendothelial matrix and is a proximal enzyme for the intrinsic pathway of thrombosis. Thus, another pathologic aspect of endothelial loss in the venous system may be potentiation of thrombosis. Lastly, the vein wall, unlike the arterial system, is subject to larger distending forces but lower pressures, and these biomechanical factors may also affect the injury response.

The goal of the current study is to delineate the endothelial response of the vein wall to stasis thrombosis, both in vivo and in vitro, and to evaluate the effect of LMWH treatment before and after thrombosis. We hypothesized that vein wall re-endothelialization would be independent of DVT size and hastened by treatment with LMWH.

METHODS

Animal model. Male Sprague-Dawley rats (250-350 g) were used for all studies, and all protocols were approved by the University of Michigan Animal Care Committee. For all surgical procedures, the rats underwent general anesthesia with isoflurane and oxygen, with full physiologic monitoring. Thrombosis was induced by inferior vena cava (IVC) ligation to produce a stasis thrombus as described.[16-18] Briefly, a laparotomy with ligation of the IVC below the renal veins and all visible side branches was performed. At 1, 4, and 14 days, the thrombosed IVC was carefully dissected and removed for histologic analysis, and separate tissue studies were done after thrombus vein wall separation at 4 and 14 days. The control for this group was a sham operation with no IVC ligation, harvested at 4 days.

For treatment groups, the thrombosed control was IVC ligation, with daily saline injection subcutaneously (SubQ), and harvest at 4 and 14 days. The prethrombosis group received the LMWH Lovenox (AstraZeneca, Chicago, Ill), 3 mg/kg SubQ 1 hour before IVC ligation and every day postoperatively, with harvest on day 4 or 14 days. The post-thrombosis LMWH treatment group received LMWH, 3 mg/kg SubQ on days 2 and thereafter postoperatively, with harvest on day 4 or 14. The dosages were based on prior experimental studies in rats.[19,20] This strategy allowed comparison of the timing of the LMWH, and we acknowledge that the prethrombosis dosing was not at prophylactic levels.

Deep vein thrombosis resolution. A reliable measure of thrombus resolution is its weight-to-length measure, harvested fresh.[17,19,21]

Immunohistochemical staining. Immunohistochemical staining was performed on the paraffin-embedded tissue sections (10 μm) as described.[16,22] The midsection of each thrombosed vein was used for consistency of analysis. No significant differences were found comparing the proximal or distal segments of vein (not shown), but an intact full vein wall for analysis was most consistently present in the midsection. The antibodies used were anti-vWF (Rabbit Anti-Human, Chemicon, Temecula, Calif) anti–α-smooth muscle actin (Mouse Monoclonal αSMA, Abcam, Cambridge, Mass), anti-ED-1 (1:100; Mouse Anti-Rat CD68, Serotec, Raleigh, NC) and anti-PMN (1:500, Accurate Chemical, Westbury, CT). A species-specific ABC peroxidase kit for rabbit, goat, or mouse (Vector Laboratories Inc, Burlingame, Calif) was used according to the manufacturer's instructions for the secondary antibody and subsequent steps.

Color development was performed with diaminobenzidine (DAB, Vector Laboratories). The slides were counterstained hematoxylin only with the ED-1 and PMN stains. To quantify monocyte (ED-1) and PMN cellular-positive staining, direct blinded counting of cells in five high-power fields (×1000 original magnification) radially around the IVC wall was performed.

Von Willebrand factor and α-smooth muscle actin analysis. Permanent histologic sections that underwent the described immunohistochemical staining with antibodies vWF and α-SMA application underwent standard color development timing to allow consistency in color development as described.[22,23] This limits variability in stain quality, and no hematoxylin counterstain was applied. These stained sections were captured using the Zeiss Axio Imager M1 microscope, Axio Cam, and Axiovision 4.6 software (Berlin, Germany) under ×200 objective. Images were acquired using a mosaic technique to facilitate analysis of a complete image of IVC wall and clot (when present). Positive vWF-stained cell layer measurements along the lumen of the vein wall were made using the curve spline tool. This allowed direct measure of the total circumference and correction for shape irregularities. Area measurements using the outline tool were done for α-SMA–stained IVC wall and corrected to IVC size using the wall circumference measurement, yielding cross-sectional area.

In vitro vein wall culture and analysis. The IVCs were harvested at 4 and 14 days in the sham (4 days only), ligated control, and LMWH treatment groups, and placed in cold Dulbecco phosphate buffered saline with 2× penicillin/streptomycin/glutamine (Gibco, Invitrogen, Carls-

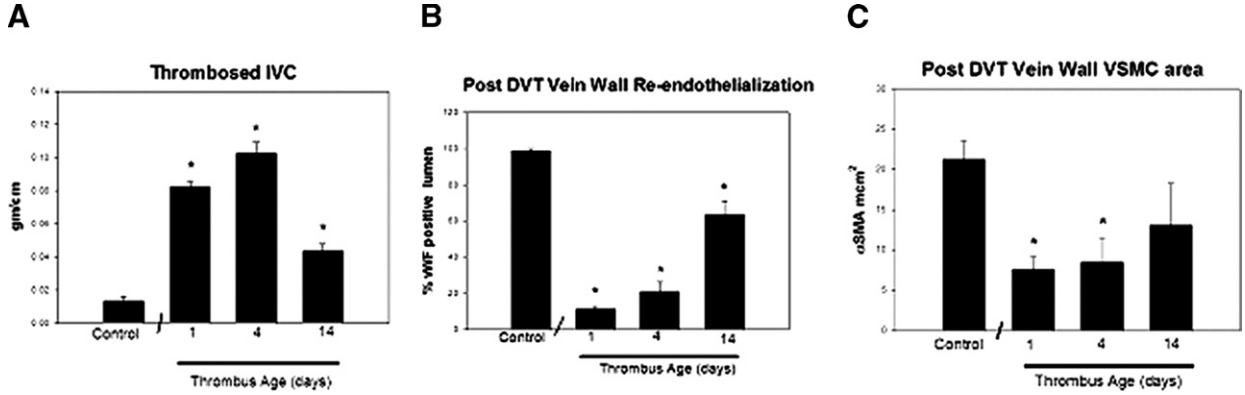

Fig 1. A, Thrombus size peaks at day 4 after ligation. Sham operation group is the inferior vena cava *(IVC)* weight-to-length measure only (n = 5-8). **P* < .05. **B,** Percentage of luminal staining positive for von Willebrand factor *(vWF)* over time after stasis thrombosis or sham control (n = 3-4).**P* < .05. **C,** Cross-sectional area of α-smooth muscle actin (α-*SMA*) staining in medial vein wall, corrected for circumference, after stasis thrombosis or sham control (n = 4).**P* < .05. *DVT,* Deep vein thrombosis; *VSMC,* vascular smooth muscle cells. Error bars signify the standard error.

bad, Calif) and then cleaned using a dissecting microscope. The IVCs were cut lengthwise, weighed, and each half was cut into 3 to 4 pieces and then placed in Dulbecco Modified Eagle Medium (Gibco) with interleukin 1-β (1 ng/mL, Mouse Recombinant, Sigma Aldrich, St. Louis, Mo) in a 12-well plate.[24-26] The tissue culture was incubated at 37°C in 5% carbon dioxide for 48 hours. The wet weight of each lengthwise IVC tissue sample was measured and then frozen at −70°C for polymerase chain reaction (PCR) processing. Culture media was spun at 5000*g* and the supernatant removed and frozen at −70° C.

Quantitative real time polymerase chain reaction. Expression of α-actin, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), thrombomodulin (TM), cyclooxygenase (COX) -1 and -2, and procollagen I, III, was determined from the harvested in vitro cell tissue, as follows:

Messenger RNA (mRNA) was isolated by treatment of vein wall segments with TRIzol reagent and reverse transcribed by incubating with Oligo-(dT) primer and M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, Calif) at 94°C for 3 minutes, followed by 40°C for 70 minutes.

The resultant complimentary DNA was amplified by Taq Polymerase (Promega, Madison, Wisc) in the SmartCycler quantitative PCR system (Cepheid, Sunnyvale, Calif).

Primers sequences were derived using Primer Premier Software (Premier Biosoft International, Palo Alto, Calif) based on primary mRNA sequences from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Relative mRNA expression was calculated by the formula 2 − (Ct target gene − Ct reference gene) and cycle lengths used were within the exponential phase of the PCR.[27] The following sequences were used:

iNOS Sense–CCC GAA ACG CTA CAC T, Anti-Sense–CGG CTG GAC TTC TCA C.

eNOS Sense–GTT TGT CTG CGG CGA TGT, Anti-Sense–GTG CGT ATG CGG CTT GTC.

TM Sense–ACC AGT CGC CTC CAC TTT, Anti-Sense–TTC TCG CAC GGC TTC TC.

COX-1 Sense–GAA CCA AAG GGA AGA AGC, Anti-Sense–GCA AAG AAA GCA AAC AAG A.

COX-2 Sense–CAG GTC ATC GGT GGA G, Anti-Sense–AGG TGC TCA GGG ACG.

Col I Sense–AGG GAC ACA GAG GTT TCA GTG, Anti-Sense–ACC ATT GGC ACC TTT AGC ACG.

Col III Sense–CCA CCC TGA ACT CAA GAG CGG, Anti-Sense–CCA TCC TCT AGA ACT GTG TAA GTG.

Nitric oxide assay. The vein wall tissue culture supernatant was harvested and stored as described. Nitric oxide was measured in the culture media using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman, Ann Arbor, Mich) according to manufacturer instructions.

Statistical analysis. All data are represented as mean ± SE. Unpaired Student *t* test and one-way analysis of variance were used as appropriate for comparison between the groups at their individual time points and appropriate controls. Calculations were done with SigmaStat software (SPSS, Chicago, Ill) A $P < .05$ was assigned significance.

RESULTS

Deep vein thrombosis resolution is accompanied by loss of endothelium and vascular smooth muscle cell staining. The thrombosed IVC size was greatest at 4 days, and trended down by 14 days (n = 5 to 8; Fig 1, *A*). Note the shams were purely the IVC weight. Post-DVT re-endothelialization, as measured by the percentage of luminal vWF-positive staining, showed a steady increase from 1 to 14 days, with only 17% luminal staining at day 1, but

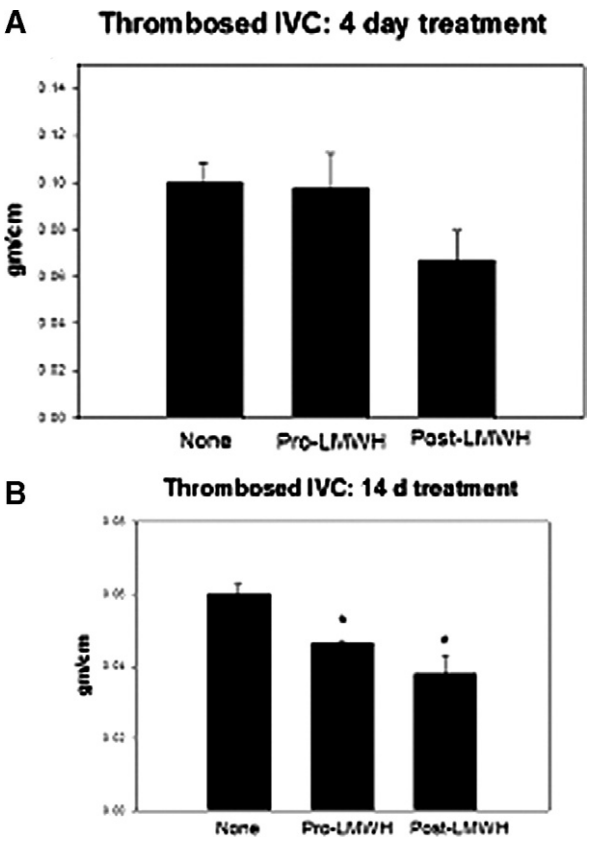

Fig 2. A, Thrombus size with vehicle treatment prethrombosis and post-thrombosis low molecular weight heparin *(LMWH)* treatment. No significant difference was observed. **B,** Thrombus size with vehicle, and prethrombosis and post-thrombosis LMWH treatment. Treatment with LMWH before and after thrombosis was associated with smaller thrombi (n = 6-8). **P* < .05. *IVC,* Inferior vena cava. Error bars signify the standard error.

returning to 63% of the lumen by day 14; all were significantly less than the sham (n = 3 to 4; *P* < .05; Fig 1, *B*). Similarly, α-SMA–positive medial vessel staining, reflective of VSMC area, showed a significant decrease on days 1 and 4 by 64%, and 60% (n = 4, *P* < .05), respectively, with return toward sham at 14 days (Fig 1, *C*).

In vivo vein wall response to LMWH treatment before and after thrombosis. Given the baseline kinetics of the poststasis DVT vein wall re-endothelialization, the greatest change seemed to occur between 4 and 14 days. We chose these two time points to further analyze. These two time points also represent an acute-to-chronic phase of vein wall remodeling after DVT.[6,16,17]

At 4 days, treatment with LMWH either before or after thrombosis did not significantly affect overall thrombus size compared with the nontreated thrombosed controls (Fig 2, *A*). By 14 days, the thrombus size was significantly reduced with LMWH treatment before or after thrombosis compared with thrombosed control by 23% to 37% (n = 4 treatments; n = 6 controls, *P* < .05; Fig 2, *B*).

Monocyte influx, estimated by ED-1 staining, was not significantly altered by LMWH treatments compared with thrombosed control at either 4 or 14 days (4 day thrombosed control. 20 ± 7; pre-LMWH, 15 ± 4; post-LMWH, 27 ± 9; cells/5 hpf, n = 4 treatment; n = 7 controls; *P* > .05). There was a fourfold reduction in overall vein wall monocytes at 14 days compared with the 4-day time point (thrombosed control, 5 ± 2; pre, 5 ± 1; post, 6 ± 2, cells/5 hpf). Sham controls showed few monocytes present in the vein wall with no thrombus present (1.4 ± 1 cells/5 hpf; n = 4). No significant difference was observed in PMN vein wall influx at 4 days and was not analyzed at 14 days given the low vein wall numbers at this time point[28] (data not shown).

At 4 days, the prethrombosis and post-thrombosis LMWH treatment groups had significantly greater luminal vWF-positive staining compared with thrombosed controls (n = 4, treatments; n = 7, controls; *P* < .05; Fig 3, *A-D*). At 14 days, compared with ligated controls, there was no significant difference in luminal vWF-positive staining, and all groups ranged between 65% and 70% positive staining of the vein lumen (n = 6).

Similarly, at 4 days, α-SMA medial vein wall–positive staining was reduced in all groups (compared with sham) in the thrombosed control, and post-DVT LMWH treatment, but not with the pre-LMWH treatment (n = 5 treatment; n = 8 controls; Fig 4, *A-D*). No significant differences in the α-SMA-positive staining area were found at 14 days between groups (n = 5 to 6).

In vitro vein wall response of prophylactic and low-molecular-weight heparin treatment after deep vein thrombosis. To determine the induction of endothelial gene expression from the thrombus-conditioned vein wall tissue culture, we evaluated one nonendothelial related gene and several endothelial related genes.[10] The gene induction was driven in vitro by a standard proinflammatory agent, IL-1β.[24,25]

Evaluating a nonendothelial derived gene, *iNOS,* we found no significant differences in expression at 4 days and overall low gene expression consistent with the sham nonthrombosed control. In contrast, at 14 days, only the thrombosed control showed significantly increased *iNOS* expression (n = 5 to 6, *P* < .05; Fig 5, *A*), whereas LMWH treatment after thrombosis was associated with significantly lower gene expression compared with sham control.

Evaluating endothelial specific genes at 4 days, eNOS and TM showed a similar expression pattern. The thrombosed group and post-thrombosis LMWH groups had significantly reduced eNOS gene expression compared with the sham control (n = 4, treatment; n = 6 control; *P* < .05). However, prethrombosis LMWH treatment was associated with a nonsignificant reduction in eNOS expression compared with the sham control (*P* = .12; Fig 5, *B*). At 14 days, overall eNOS expression was reduced approximately sixfold compared with the 4-day time point, and no differences between the thrombosed control, prethrombosis, and post-thrombosis treatment LMWH groups were found.

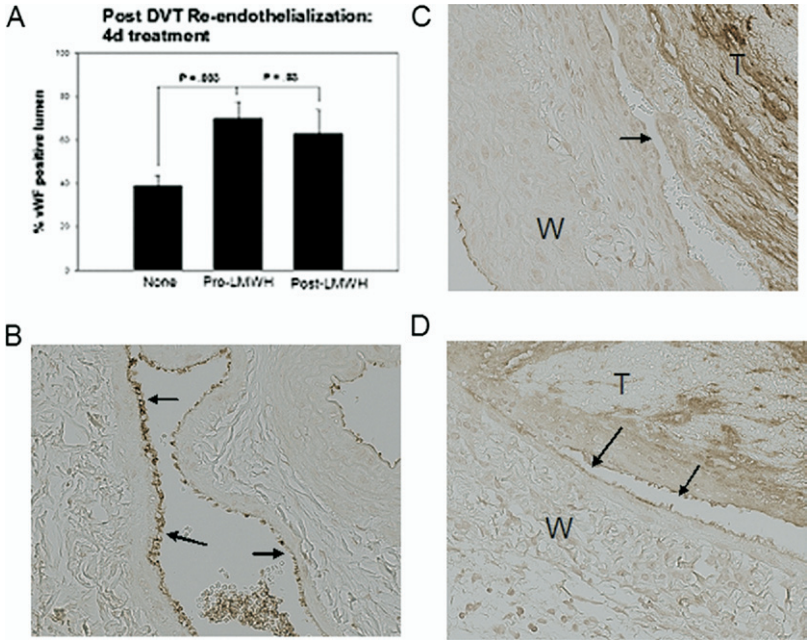

Fig 3. A, Graphic representation of vehicle (none), and prethrombosis and post-thrombosis low-molecular-weight heparin *(LMWH)* effect on percentage of positive von Willebrand factor *(vWF)* luminal staining at 4 days. A significantly greater luminal area was positive for vWF with LMWH treatment (n = 4-7). Error bars signify the standard error. *DVT,* Deep vein thrombosis. **B,** Example of sham inferior vena cava stained for vWF to denote the endothelial layer. Brown stain indicates the endothelium *(arrows)* at ×400 original magnification. **C,** Vehicle (none) thrombosed inferior vena cava. A large thrombus is seen with luminal staining and a small amount of luminal vWF staining *(arrow),* at ×400 original magnification. *W,* Vein wall. **D,** Prethrombosis LMWH treatment. Note large thrombus *(T)* but more luminal positive vWF staining *(arrows)* at ×400 original magnification. *W,* Vein wall.

The TM gene-induced expression pattern was similar to the eNOS expression pattern (Fig 5, *C*). The prethrombosis LMWH treatment group had a nonsignificant reduction in TM expression compared with the sham control, whereas the thrombosed group and the post-thrombosis LMWH group showed significantly reduced TM gene expression (n = 6 treatments; n = 8 controls; $P < .05$). In contrast to the eNOS pattern, the sham, prethrombosis, and post-thrombosis treatment LMWH groups were not significantly different at day 14, and overall low gene expression was found (not shown). In contrast, the thrombosed control had a 10-fold increased TM gene expression (n = 4 treatment; n = 7 controls; $P < .05$).

Evaluation of COX-1 and COX-2 gene expression showed no significant differences among sham, thrombosed, and the prethrombosis and post-thrombosis LMWH groups at 4 or 14 days (data not shown).

As the vessel wall remodels in the chronic phase, collagen turnover occurs.[29] Collagen production is dependent on the synthetic activities of VSMC. To reflect this process, we evaluated collagen gene expression at the 14-day time point. No significant difference in collagen I gene expression was found when comparing all groups. However, collagen III expression was significantly elevated in the prethrombosis and post-thrombosis treatment LMWH group compared with the sham (n = 5 treatment; n = 8 control; $P < .05$). The thrombosed group was not significantly different then the sham control group (Fig 5, *D*).

Because eNOS gene expression was different between treatments at 4 days, NO was evaluated. We found that compared with the shams, all three groups had similar levels of nitrite/nitrate, with no significant differences among the groups. All were lower than the nonthrombosed sham control group by approximately 40% (n = 5 to 6, $P < .05$).

DISCUSSION

These experiments suggest that stasis DVT causes an initial loss of endothelium that recovers towards sham controls within 14 days and affects the underlying VSMC integrity. Further, prethrombosis and, to a lesser extent, post-thrombosis treatment with LMWH may accelerate re-endothelialization or protect the venous endothelium and preserve the medial VSMC integrity. This effect was most striking at 4 days after thrombosis and less so at 14 days, despite a smaller thrombus in the LMWH treatment groups. This suggests some disconnect between the beneficial effect of heparin and thrombolysis; that is, smaller thrombi do not necessarily portend greater endothelial recovery.

Heparin agents have pluripotent effects beyond anticoagulation. For example, LMWH, by way of sialyl Lewis antigen, may block selectin interactions.[30] Low-molecular-

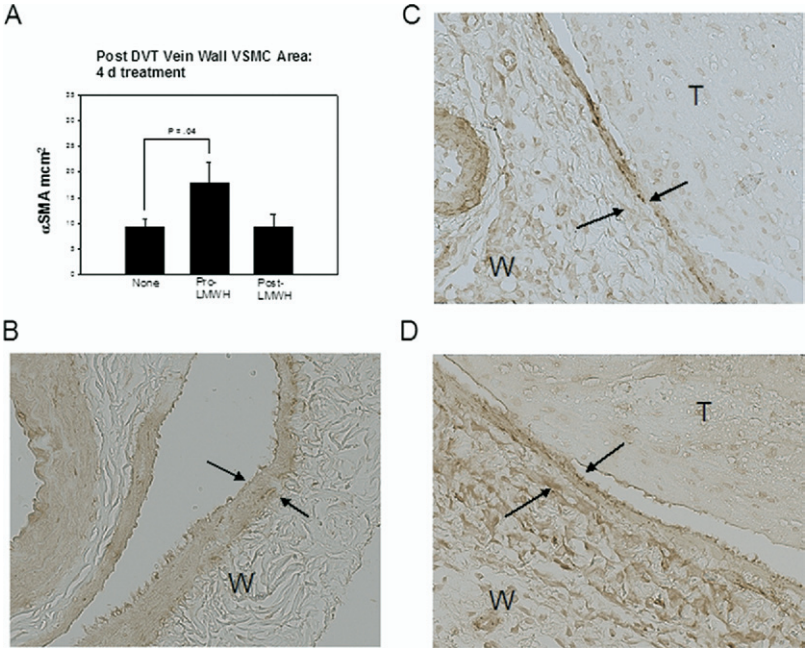

Fig 4. A, Graphic representation of vehicle (none), and prethrombosis and post-thrombosis low-molecular-weight heparin *(LMWH)* treatment on vein wall α-smooth muscle actin *(α-SMA)* area staining at 4 days (n = 5-8). *DVT,* Deep vein thrombosis; *VSMC,* vascular smooth muscle cell. **B,** Example of sham control vein, showing brown medial area of α-SMA staining *(arrows)* at ×400 original magnification. *W,* Vein wall. **C,** Vehicle (none) treatment example showing thin brown α-SMA area, at 4 days *(arrows)* at ×400 original magnification. *T*, Thrombus; *W,* vein wall. **D,** Prethrombosis LMWH treatment. Not larger and more diffuse α-SMA area *(arrows)* at ×400 original magnification. *T*, Thrombus; *W,* vein wall.

weight heparin has other anti-inflammatory actions as well, involving inhibition of lymphocyte and chemokine responses that are likely also at work in this system.[30-32] A recently published study using a temporary clip model of experimental thrombosis suggested that LMWH had a greater anti-inflammatory effect than urokinase plasminogen activator, as measured by vein wall tumor necrosis factor-α expression.[33]

Arterial injury models suggest that the presence of endothelium plays a major role in vessel wall healing and recovery; for example, in mice with impaired endothelialization after mechanical injury, increased neointimal hyperplasia is observed.[7,8] We cannot answer whether the prethrombosis LMWH protected the vein wall from endothelial loss, increased local endothelial regeneration, or increased endothelial progenitor cells to migrate to the injured area. We did not find significant positive staining for CD 133 or vascular endothelial growth factor receptor-2, two markers of endothelial progenitor cells, at 1, 4, or 14 day time points in preliminary studies, suggesting local regeneration or endothelial protection may play more of a role than circulating progenitor cells.

A report in a rabbit jugular thrombosis model suggested prophylactic treatment with unfractionated heparin was protective for endothelial cells.[34] A report by Modarai et al[9] suggests progenitor cells occupy the resolving thrombus, but it was not apparent whether these cells were present at the vein wall interface or whether they contribute to endothelial recovery. Lastly, the endothelial protective effect of LMWH was probably not mediated by monocytes, because no differences were found in absolute numbers at either 4 or 14 days by LMWH treatment within the vein wall.

We analyzed the vein wall segments in vitro to determine the IL-1β–inducible endothelial response genes that are less easily defined with whole in vivo vein segment analysis because less thrombus and peri-IVC tissue is present with an in vitro tissue culture system. Specifically, we hoped to be able to define the functional state of the endothelium (tissue culture) to complement the immunohistologic findings. These strategies produce somewhat different but overall consistent results.

First, prethrombosis LMWH treatment for experimental stasis thrombosis was associated with the greatest endothelial recovery. This notion is supported by the significantly increased luminal vWF-positive staining, the greater α-SMA area, as well as the nonsignificant decrease in specific endothelial homeostatic genes, eNOS and TM.

Second, post-thrombosis treatment with LMWH was associated with greater luminal vWF staining but was not associated with preserved eNOS or TM gene expression compared with nonthrombosed IVC tissue culture.

Third, in vitro tissue culture analysis suggested diminished endothelial gene expression related to the stasis

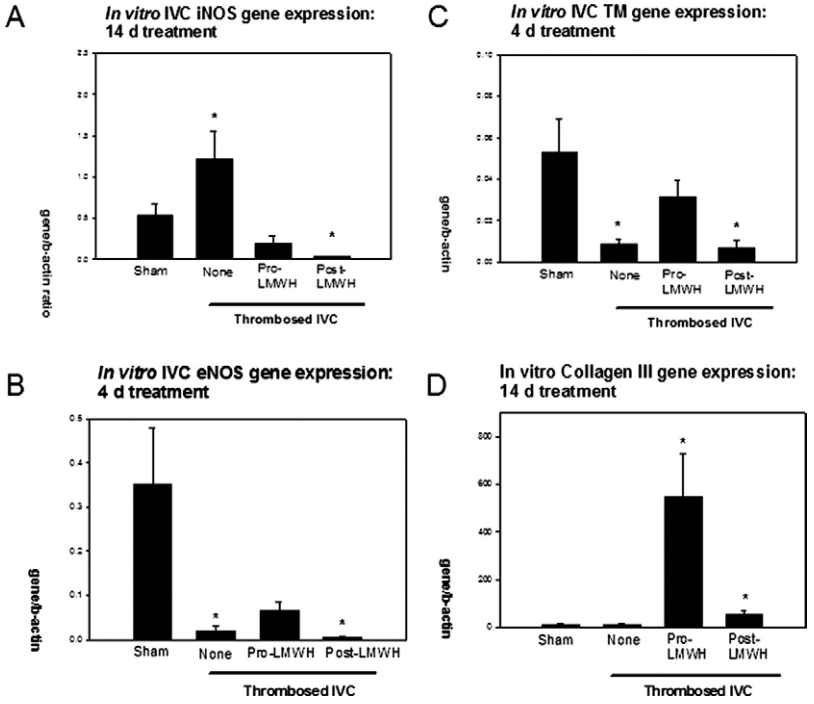

Fig 5. Interleukin 1β (IL-1β; 1 ng/mL) 48-hour stimulated post-thrombosis vein wall tissue culture. Harvested tissue was at 4 days or 14 days before harvest. **A,** Inducible nitric oxide synthase *(iNOS)* 14-day gene expression shows greater expression in the vehicle (none) thrombosed inferior vena cava *(IVC)* and significantly lesser expression with post-thrombosis low-molecular-weight heparin *(LMWH)* treatment (n = 5). $*P < .05$. **B,** Endothelial nitric oxide synthase *(eNOS)* 4-day gene expression shows significantly reduced expression with vehicle and thrombosis LMWH treatment compared with sham (n = 4-6). $*P < .05$. **C,** Thrombomodulin *(TM)* 4-day gene expression shows significantly reduced expression with vehicle and post-thrombosis LMWH treatment compared with sham (n = 6) $*P < .05$. **D,** Collagen III expression at 14 days shows significantly increased expression with prethrombosis and post-thrombosis LMWH treatment compared with sham (n = 4-5). $*P < .05$.

thrombus injury compared with shams. It is likely the damage is both mechanical with the stretch, but probably more so from the persistent thrombus–vein wall interface. Thrombus leukocytes release proteinases, as well as proinflammatory factors that may alter the basement membrane. Recent data suggest the mechanisms of thrombogenesis (stasis vs nonstasis) and the thrombus itself primarily drives the injury.[35] The local environment of the exposed basement membrane components are also important; namely, a high fibronectin/fibrin concentration produces a less hospitable environment for endothelium.[13] The endothelial protective effects were not as striking at 14 days. Thus, persistence of thrombus, which occurs in this stasis model, may negate the early beneficial effect of LMWH.[36]

Preservation of the endothelium paralleled preservation of the VSMC layer (α-SMA area) and was observed with prethrombosis, and to a lesser extent, post-thrombosis LMWH treatment at 4 days. The α-SMA antigen is also a marker for VSMC contractible phenotype, which is the physiological vasculae state.[15] After injury, a synthetic phenotype predominates with loss of α-SMA expression and is responsible for the fibrotic response and hyperplasia that can result. Although no difference in VSMC area was observed at 14 days by immunohistology, IL-1β–induced type III collagen gene expression was significantly increased with prethrombosis and, to a lesser extent post-thrombosis LMWH treatment at 14 days. It is unlikely that a true increase in collagen occurs in vivo, and indeed, prior work has suggested less fibrosis with LMWH,[20] as well as less collagenolysis.[35] The fact that prethrombosis and post-thrombosis LMWH treatment was associated with increased collagen III gene expression also implies this may be part of the normal healing response after DVT. Not surprisingly, sham vein wall had a very low inducible type collagen III gene expression and suggests the thrombus primes the vein wall response.

The associated homeostatic gene product of eNOS is NO, an essential mediator for vasodilation and antithrombotic properties of the vein wall.[37,38] Of interest was that supernatant NO levels were all reduced in vein tissue exposed to thrombus. Although LMWH preserves eNOS expression at 4 days, it was still reduced compared with sham IVC tissue, and the diminished levels likely reflect this fact. Similarly, TM confers an anticoagulant state to the endothelium, in part, through its protein C interac-

tion.[39,40] The increased TM expression at the 14-day time point may reflect a lag in re-endothelialization that occurs in the setting of no LMWH treatment. Of interest was that the major genes associated with prostaglandin production, COX-1 and COX-2, were not induced differentially after DVT. Cyclooxygenase inducibility by IL-1β has been documented but may drive cellular differentiation in vitro as well as prostaglandin synthesis.[26] Alternatively, endothelial cells early after injury may not rely on the COX pathway until a later chronic time point for prostaglandin production. For example, chronic inhibition of COX-2 affects late but not early arterial remodeling.[41,42]

CONCLUSION

The clinical translation of this experimental study is that prethrombosis and, to a lesser extent, post-thrombosis LMWH treatment may enhance early re-endothelialization or protect the vein wall from endothelial loss. This may explain why recurrent thrombosis is less likely with this agent, in addition to its direct anticoagulation effect, and has been suggested by others as well.[35] A recent retrospective study also suggested that rapid, timely DVT treatment may positively affect development of post-thrombotic syndrome.[42] These data also beget the clinical question of whether patients who develop a DVT who are receiving prophylactic LMWH may have less long-term risk of post-thrombotic syndrome than those receiving purely mechanical prophylaxis.

AUTHOR CONTRIBUTIONS

Conception and design: DM, GU, TW, PH
Analysis and interpretation: DM, PH
Data collection: EL, CL, VS
Writing the article: DM, PH
Critical revision of the article: DM, EL, CL, VS, GU, TW, PH
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REFERENCES

1. Prandoni P, Lensing AW, Cogo A, Cuppini S, Villalta S, Carta M, et al. The long-term clinical course of acute deep venous thrombosis. Ann Intern Med 1996;125:1-7.
2. Delis KT, Bountouroglou D, Mansfield AO. Venous claudication in iliofemoral thrombosis: long-term effects on venous hemodynamics, clinical status, and quality of life. Ann Surg 2004;239:118-26.
3. Bates SM, Ginsberg JS. Clinical practice. Treatment of deep-vein thrombosis. N Engl J Med 2004;351:268-77.
4. Snow V, Qaseem A, Barry P, Hornbake ER, Rodnick JE, Tobolic T, et al. Management of venous thromboembolism: a clinical practice guideline from the American College of Physicians and the American Academy of Family Physicians. Ann Intern Med 2007;146:204-10.
5. Hull RD, Pineo GF, Brant RF, Mah AF, Burke N, Dear R, et al. Long-term low-molecular-weight heparin versus usual care in proximal-vein thrombosis patients with cancer. Am J Med 2006;119:1062-72.
6. Wakefield TW, Henke PK. The role of inflammation in early and late venous thrombosis: Are there clinical implications? Semin Vasc Surg 2005;18:118-29.
7. Gennaro G, Menard C, Michaud SE, Rivard A. Age-dependent impairment of reendothelialization after arterial injury: role of vascular endothelial growth factor. Circulation 2003;107:230-3.
8. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, et al. Statin therapy accelerates reendothelialization - a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002;105:3017-24.
9. Modarai B, Burnand KG, Sawyer B, Smith A. Endothelial progenitor cells are recruited into resolving venous thrombi. Circulation 2005;111:2645-53.
10. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res 2007;100:158-73.
11. Hedin U, Roy J, Tran PK, Lundmark K, Rahman A. Control of smooth muscle cell proliferation–the role of the basement membrane. Thromb Haemost 1999;82(suppl 1):23-6.
12. Aguilera CM, George SJ, Johnson JL, Newby AC. Relationship between type IV collagen degradation, metalloproteinase activity and smooth muscle cell migration and proliferation in cultured human saphenous vein. Cardiovasc Res 2003;58:679-88.
13. Davis GE, Senger DR. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res 2005;97:1093-107.
14. Dollery CM, Humphries SE, McClelland A, Latchman DS, McEwan JR. Expression of tissue inhibitor of matrix metalloproteinases 1 by use of an adenoviral vector inhibits smooth muscle cell migration and reduces neointimal hyperplasia in the rat model of vascular balloon injury. Circulation 1999;99:3199-205.
15. Thyberg J, Hultgardh-Nilsson A. Fibronectin and the basement membrane components laminin and collagen type IV influence the phenotypic properties of subcultured rat aortic smooth muscle cells differently. Cell Tissue Res 1994;276:263-71.
16. Henke PK, Varga A, De S, Deatrick CB, Eliason J, Arenberg DA, et al. Deep vein thrombosis resolution is modulated by monocyte CXCR2-mediated activity in a mouse model. Arterioscler Thromb Vasc Biol 2004;24:1130-7.
17. Wakefield TW, Strieter RM, Wilke CA, Kadell AM, Wrobleski SK, Burdick MD, et al. Venous thrombosis-associated inflammation and attenuation with neutralizing antibodies to cytokines and adhesion molecules. Arterioscler Thromb Vasc Biol 1995;15:258-68.
18. Henke PK, Varma MR, Deatrick KB, Drewyer NA, Lynch EM, Moore AJ, et al. Neutrophils modulate post-thrombotic vein wall remodeling but not thrombus neovascularization. Thromb Haemost 2006;95:272-81.
19. Myers DD Jr, Henke PK, Bedard PW, Wrobleski SK, Kaila N, Shaw G, et al. Treatment with an oral small molecule inhibitor of P selectin (PSI-697) decreases vein wall injury in a rat stenosis model of venous thrombosis. J Vasc Surg 2006;44:625-32.
20. Thanaporn P, Myers DD, Wrobleski SK, Hawley AE, Farris DM, Wakefield TW, et al. P-selectin inhibition decreases post-thrombotic vein wall fibrosis in a rat model. Surgery 2003;134:365-71.
21. Varma MR, Moaveni DM, Dewyer NA, Varga AJ, Deatrick KB, Kunkel SL, et al. Deep vein thrombosis resolution is not accelerated with increased neovascularization. J Vasc Surg 2004;40:536-42.
22. Henke PK, Pearce CG, Moaveni DM, Moore AJ, Lynch EM, Longo C, et al. Targeted deletion of CCR2 impairs deep vein thrombosis resolution in a mouse model. J Immunol 2006;177:3388-97.
23. Sullivan VV, Hawley AE, Farris DM, Knipp BS, Varga AJ, Wrobleski SK, et al. Decrease in fibrin content of venous thrombi in selectin-deficient mice. J Surg Res 2003;109:1-7.
24. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA, Jr. Interleukin-1 activation of vascular endothelium. Effects on procoagulant activity and leukocyte adhesion. Am J Pathol 1985;121:394-403.
25. Kaplanski G, Fabrigoule M, Boulay V, Dinarello CA, Bongrand P, Kaplanski S, et al. Thrombin induces endothelial type II activation in vitro: IL-1 and TNF-alpha-independent IL-8 secretion and E-selectin expression. J Immunol 1997;158:5435-41.
26. Maier JA, Hla T, Maciag T. Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. J Biol Chem 1990;265:10805-8.

27. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 2002;30: 503-12.
28. Varma MR, Varga AJ, Knipp BS, Sukheepod P, Upchurch GR, Kunkel SL, et al. Neutropenia impairs venous thrombosis resolution in the rat. J Vasc Surg 2003;38:1090-8.
29. Deatrick KB, Eliason JL, Lynch EM, Moore AJ, Dewyer NA, Varma MR, et al. Vein wall remodeling after deep vein thrombosis involves matrix metalloproteinases and late fibrosis in a mouse model. J Vasc Surg 2005;42:140-8.
30. Wang L, Brown JR, Varki A, Esko JD. Heparin's anti-inflammatory effects require glucosamine 6-O-sulfation and are mediated by blockade of L- and P-selectins. J Clin Invest 2002;110:127-36.
31. Ranjbaran H, Wang Y, Manes TD, Yakimov AO, Akhtar S, Kluger MS, et al. Heparin displaces interferon-gamma-inducible chemokines (IP-10, I-TAC, and Mig) sequestered in the vasculature and inhibits the transendothelial migration and arterial recruitment of T cells. Circulation 2006;114:1293-300.
32. Downing LJ, Strieter RM, Kadell AM, Wilke CA, Greenfield LJ, Wakefield TW. Low-dose low-molecular-weight heparin is anti-inflammatory during venous thrombosis. J Vasc Surg 1998;28:848-54.
33. Carr JA, Cho JS. Low molecular weight heparin suppresses tumor necrosis factor expression from deep vein thrombosis. Ann Vasc Surg 2007;21:50-5.
34. Trabucchi E, Foschi D, Marazzi M, Abelli P, Andriuoli G, Lami F, et al. Heparin prevents stasis-induced thrombosis through protection of the venous endothelial cells: an electron microscopic study in rabbits. Haemostasis 1991;21:37-44.
35. Henke PK, Varma MR, Moaveni DK, Dewyer NA, Moore AJ, Lynch EM, et al. Fibrotic injury after experimental deep vein thrombosis is determined by the mechanism of thrombogenesis. Thromb Haemost 2007;98:1045-55.
36. Stenberg B, Bylock A, Risberg B. Effect of venous stasis on vessel wall fibrinolysis. Thromb Haemost 1984;51:240-2.
37. Rabelink TJ, Luscher TF. Endothelial nitric oxide synthase: host defense enzyme of the endothelium? Arterioscler Thromb Vasc Biol 2006;26:267-71.
38. Blackman DJ, Morris-Thurgood JA, Atherton JJ, Ellis GR, Anderson RA, Cockcroft JR, et al. Endothelium-derived nitric oxide contributes to the regulation of venous tone in humans. Circulation 2000;101: 165-70.
39. Waugh JM, Yuksel E, Li J, Kuo MD, Kattash M, Saxena R, et al. Local overexpression of thrombomodulin for in vivo prevention of arterial thrombosis in a rabbit model. Circ Res 1999;84:84-92.
40. Kim AY, Walinsky PL, Kolodgie FD, Bian C, Sperry JL, Deming CB, et al. Early loss of thrombomodulin expression impairs vein graft thromboresistance: implications for vein graft failure. Circ Res 2002;90: 205-12.
41. Rudic RD, Brinster D, Cheng Y, Fries S, Song WL, Austin S, et al. COX-2-derived prostacyclin modulates vascular remodeling. Circ Res 2005;96:1240-1247.
42. Henke PK, Ferguson E, Varma M, Deatrick KB, Wakefield GT, Woodrum DT. Proximate versus nonproximate risk factor associated primary deep venous thrombosis: clinical spectrum and outcomes. J Vasc Surg 2007;45:998-1003; discussion 1003-4; quiz 1005-7.

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