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Recanalization and flow regulate venous thrombus resolution and Matrix metalloproteinases expression *in vivo*

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Abstract

Objective—We examined the role of thrombus recanalization and ongoing blood flow in the process of thrombus resolution by comparing two murine *in vivo* models of deep venous thrombosis.

Design of study—In CD1 mice, we performed surgical inferior vena cava (IVC) ligation (stasis thrombosis), stenosis (thrombosis with recanalization) or sham procedure. We analyzed thrombus weight over time as a measure of thrombus resolution, and quantified the mRNA and protein levels of Membrane-Type Matrix Metalloproteinases (MT-MMPs) as well as effectors of the plasmin complex at day 4, 8 and 12 post-surgery.

Results—Despite similar initial thrombus size, the presence of ongoing blood flow (stenosis model) was associated with a 45.91% subsequent improvement in thrombus resolution at day 8, and 12.57% at day 12, as compared with stasis thrombosis (ligation model). Immunoblot and real-time PCR demonstrated a difference in MMP-2 and MMP-9 activity at day 8 between the two models ($P=.03$ and $P=.006$ respectively), as well as a difference in MT2-MMP gene expression at day 8 ($P=.044$) and day 12 ($P=0.03$) and MT1-MMP protein expression at day 4 ($P=.021$). Histological analyses revealed distinct areas of recanalization in the thrombi of the stenosis model compared to the ligation model, as well as the recruitment of inflammatory cells, especially macrophages, and a focal pattern of localized expression of MT1-MMP and MT3-MMP proteins surrounding the areas of recanalization in the stenosis model.

Conclusions—Recanalization and ongoing blood flow accelerate deep venous thrombus resolution *in vivo*, and are associated with distinct patterns of MT1- and MT3-MMP expression and macrophages localization in areas of intra-thrombus recanalization.

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Conflicts of Interest

The authors declare no competing financial interests.

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Introduction

Deep vein thrombosis (DVT) is a one of the most common vascular disorders, with more than 2 million new cases in the U.S. each year. Among patients suffering from DVT, 23 to 60% of them will develop post-thrombotic syndrome (PTS) within 2 years. PTS leads to long term pain, swelling and leg ulceration secondary to valve destruction and persistent obstruction of the vein¹. Improved understanding of the molecular mechanisms of thrombus formation and resolution would aid in developing new treatments to accelerate thrombus resolution or prevent vein wall inflammation and valve damage.

An interesting feature of venous thrombosis is the process of recanalization, which leads to restoration of a flow channel even in cases of initial complete occlusion of the vein lumen by the acute thrombus². The process of recanalization of venous thrombus remains poorly understood, and is associated with the ultimate development of large channels lined with endothelial cells within the thrombus^{2,3}. These channels are significantly larger than conventional capillaries, and are thought to develop through a combination of mechanical retraction of the resolving thrombus and the proteolytic activity of inflammatory cells recruited during the process of thrombus resolution⁴.

In experimental studies, two established *in vivo* models have been described to study the processes of thrombus resolution: a stasis model utilizing vena caval ligation⁵ and a stenosis model of the vena cava with continuous minimal venous flow^{6,7}. Thrombi generated by the stenosis model have a layered composition with partial recanalization similar to human DVT. The stasis model, however, generates a more reproducible and consistent thrombus⁴ and has been used more widely to precisely elucidate the role of individual genes in the process of thrombus resolution⁷⁻¹¹. The significant difference between these two models is the presence of ongoing blood flow in the stenosis model. The purpose of this study was to define the effect and potential molecular mediators of blood flow and recanalization in thrombus resolution by comparing these two models.

The molecular basis of thrombus formation and resolution involves key roles for various matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9^{8,10,12-14}. The membrane type-MMPs (MT-MMPs) regulate the enzymatic activity of the secreted MMPs and also directly cleave several substrates of the extracellular matrix¹⁵. PAI-1 is the secreted serpin involved in the inhibition of uPA and tPA, and has been shown to be involved in thrombus resolution¹⁶. The uPA/plasmin system is also involved in the processes of thrombus formation and resolution^{7,11,17}. Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that mediates thrombus resolution *in vivo*⁹ whereas the cytokine gamma-interferon inhibits thrombus resolution¹⁰. We therefore compared the expression of these mediators in both models to determine their regulation by ongoing blood flow.

Material and Methods

Thrombus resolution models

CD1 mice between 6–10 weeks old and of similar size were subjected to inferior vena caval (IVC) ligation or stenosis via midline laparotomy under isoflurane anesthesia (2%). All lumbar branches of the IVC from the renal veins to the iliac bifurcation were cauterized. In the ligation model, a 7-0 silk suture was tied around the IVC just below the renal veins^{12, 18}. In the stenosis model, this suture was tied around both the IVC and a 4-0 Prolene suture. A neurosurgical vascular clip was applied to the dissected vena cava for 15s on 2 occasions, then the Prolene suture was removed after the silk suture had been secured to create a reproducible stenosis of less than 1% of the native vena cava diameter^{6,7}. For the sham controls, the suture was simply wrapped around the IVC just below the renal veins and left untied¹². Animals were sacrificed at days 4, 8, and 12 and the IVC and thrombus were harvested from the renal veins to the iliac bifurcation. At harvest, the animal and IVC thrombus were weighed. The IVC and the thrombus were then frozen and stored at -80°C for RNA and protein analyses or placed in formalin followed by ethanol for histological analysis. All procedures were performed in accordance with Institutional Animal Care and Use Committee approved protocols.

Immuno-histochemistry

Immunohistochemical staining was performed on the paraffin embedded tissue sections (5 µm) with standard techniques for H&E, MT1-MMP (Novus Biologicals), MT3-MMP (Chemicon International), macrophages using Mac-2 (Cedarlane) and neutrophils using Ly6B.2 (ABD Serotec). Sections were taken from the midsection of the thrombus at least 5 mm away from the suture region to avoid inflammation in this region. Vein wall area was evaluated by measuring the area between the tunica adventitia and the endothelial cells layer with ImageJ software¹⁹. Vein wall thickness was determined as a pixel lengths using ImageJ¹⁹. In a blinded fashion, positive cells in 5 high power fields (hpf, 400X) in the vein wall area were counted and totaled. A minimum of 3 animals were analyzed at each time point in each model.

Immunoblotting and MMP activity

For each cohort, harvested IVC and thrombus tissue samples were lysed in Tissue Protein Extraction Reagent (Thermo Scientific). Western blotting for either actin (Santa Cruz), MT1-MMP (Novus Biologicals), MT3-MMP (Chemicon International and Sigma), MT2-MMP (Chemicon International), uPA (Pierce), PAI-1 (American Diagnostica) or HO-1 (AbCam) was performed and analyzed as previously described¹². Densitometry analysis was assessed with ImageJ¹⁹ and normalized to actin.

MMP-2 and MMP-9 activities were analyzed by gelatin zymography as previously described¹². Optical densities were determined and normalized to total amount of protein.

Real time PCR

Total RNA was extracted from homogenized samples with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA according to the First Strand Synthesis Kit (Roche)

manufacturer's instructions. Real-time PCR amplifications were performed with LightCycler 480 SYBR Green I Master Kit (Roche). To normalize expression data, 36B4 was used as an internal control for each sample. Primers sequences are listed in Table 1.

Flow Cytometry of Thrombus

To overcome the regional variability intrinsic to histological analysis of thrombus sections, we used flow cytometry of the entire thrombus to quantify macrophages and neutrophils content. On day 4 and 8 post-surgery, thrombi were cut into 1mm pieces, then processed into single cell suspensions using collagenase II at 1 mg/mL (Worthington) and tPA at 20 micrograms/mL (EMD Millipore). Cells were counted, then stained for macrophages and activated neutrophils with Cd11b (ABD Serotec) and for neutrophils with Ly-6B.2 (ABD Serotec) according to the manufacturer's instructions. Draq5 (cell Signaling Technology) was used to identify nucleated cells and subtract debris from digestion. Single stained controls were used for compensation and fluorescence-minus-one controls were used to determine gates. Flow cytometry analyses were performed with a Becton-Dickinson FACS Calibur flow cytometer and post-collection data processing were performed with FlowJo analysis software (Tree Star, Inc).

Statistical Analysis

Experiments were performed at least three times with samples from different animals. Data were compared with ANOVA or unpaired *t* tests as appropriate. Differences were considered significant at $P < .05$ and differences are reported as mean $\pm$ standard error.

Results

Thrombus weight and morphology in ligation and stenosis models

Both the stenosis and ligation models consistently produced thrombus, and no thrombus were noted in mice undergoing sham operation. Thrombus was limited to the cava and did not extend to the bifurcation in either model. At day 4, there was no statistical difference in thrombus weight between the ligation and stenosis models. However, at day 8, the thrombi weight observed for mice undergoing stenosis surgery was statistically less ($8.68\text{mg} \pm 0.3$, $n=5$) than for the mice undergoing ligation surgery ($12.61\text{mg} \pm 0.72$, $n=6$) ($P=.001$) (Figure 1A). This corresponds to a 45.91% improvement in thrombus resolution for the stenosis model in comparison to the stasis model. The same pattern was observed at day 12, with a mean thrombus weight of $3.91\text{mg} \pm 0.29$ for mice undergoing stenosis surgery ($n=9$) compared to $5.67\text{mg} \pm 0.51$ for the mice undergoing ligation surgery ($n=12$), $P=.014$. This is a 12.57% improvement in thrombus resolution for the stenosis model when compared to stasis thrombosis. By normalizing the percentage of thrombus weight at day 8 and 12 to the initial thrombus weight at day 4, the stenosis model demonstrated quicker resolution ($53.56\% \pm 2.05$ at day 8 and $79.72\% \pm 1.07$ at day 12) than the ligation model ($36.71\% \pm 4.04$ at day 8 and $70.82\% \pm 2.58$ at day 12) ($P=.007$ at day 8, $P=.01$ at day 12) (Figure 1A).

In order to determine if thrombus resolution occurred through different pathways in the two DVT models, we first examined the morphology of the thrombi. As shown in Figure 1B, thrombi at day 12 from the stenosis model presented some areas of distinct recanalization

(arrows) which were not seen for the ligation model. Also, while comparing the pattern of cellular infiltration, it appeared that in the ligation model, cells were predominantly at the periphery of the thrombi, while in the stenosis model they seemed more centrally located (Figure 1B). In assessment of vein wall injury, both vein wall area and thickness were similar between the two models, however, more mononuclear cells were observed in the vein wall of the ligation model ($P=.005$) (Figure 1B).

MMP activity and MT-MMP mRNA expression in thrombus resolution

At day 8, which is the midpoint of the thrombus resolution process, there was an increased activity level in the ligation model compared to the stenosis model for both MMP-9 ($n=3$, $P=.006$) and MMP-2 ($n=3$, $P=.03$). At day 4 and 12, the difference in MMP-2 and MMP-9 activity between the two models was not statistically significant (Figure 1C).

The changes in various MT-MMP mRNA levels in the ligation and stenosis models relative to sham operation are shown in Figure 2. MT1-MMP mRNA expression was increased in both models at day 8 and day 12. MT2-MMP mRNA decreased in both ligation and stenosis with more sustained decreases noted in ligation. MT3-MMP mRNA decreased at day 4 in both models. MT4-MMP mRNA expression decreased in both models at day 4 and at day 12, the MT4-MMP expression in the ligation model was higher than in the stenosis model. No difference was found for MT5-MMP gene expression between all groups. It was however difficult to establish some comparison as the numbers of copies were at the limit of detection for MT5-MMP and MT6-MMP. A trend of increased MT6-MMP gene expression in the stenosis model at day 4 and 8 in comparison to ligation, however this did not reach statistical significance, whereas decreased MT6-MMP was noted in the ligation model (relative to sham) on day 4.

MT-MMP protein expression in the stenosis and ligation models of thrombus resolution

MT1-MMP protein expression was decreased for the ligation model while compared to the sham operation ($P=.023$) as well as compared to the stenosis model at day 4 only ($P=.021$) (Figure 3). MT2-MMP protein expression was increased in the stenosis model at day 12 by comparison with sham model ($P=.038$) (Figure 3). An increase of MT3-MMP protein level was found at day 8 in the ligation model compared to the sham model ($P=.026$) (Figure 3). Effective antibodies for mouse MT4-MMP and MT5-MMP were not available and immunoblots MT6-MMP did not show any expression (data not shown), consistent with the low mRNA levels.

As some differences in the MT-MMPs protein expression between the stenosis and ligation model were found, we decided to study the localization of some of these MT-MMPs in the different thrombi. We first identified with CD31 staining the presence of endothelium lined channels within the thrombi. As show in Figure 4A (arrow), areas of recanalization were only observed within the thrombi of the stenosis model. We then looked at the localization of MT1-MMP and MT3-MMP within the thrombi in these two models (Figure 4B). In the stenosis model, both proteins seem to be expressed mostly around the recanalization area, while in the stenosis model, no distinct pattern could be determined (Figure 4B). This would

suggest a possible role for MT1-MMP and MT3-MMP in the development of recanalization and thrombus resolution in the stenosis model.

uPA, PAI-1 and HO-1 protein expression in the different models

No significant difference in uPA protein expression or in total and active UPA levels (determined by ELISA, data not shown) was found at day 4, 8 and 12 in both the sham operation samples and the DVT samples (Figure 5). PAI-1 protein expression was significantly induced at each time point in both DVT models compared to sham operation, but no difference was found between the stenosis and ligation models (Figure 5). At day 4, thrombi produced in the stenosis model exhibited greater staining for PAI-1 than thrombi produced in the sham operation ($P=.05$).

At day 8, the expression of heme oxygenase-1 was increased in both stenosis and ligation models compared to sham operation, but no difference was noted between both models (sham vs stenosis: $P=.014$, sham vs ligation: $P=.019$) (Figure 5). Gamma-interferon has been shown to regulate thrombus resolution *in vivo*¹⁰. We did not detect a difference in thrombus gamma-interferon levels between the stenosis and the ligation models (determined by ELISA, data not shown).

Inflammatory response in the two models

Thrombus resolution requires inflammatory cellular recruitment^{20,21}, including a migration of neutrophils, followed by macrophages and other leukocytes^{21,22}. We used flow cytometry to quantify the thrombus macrophage and neutrophil populations. No difference was found between the two models for neutrophils and macrophages at day 4 and day 8 (Figure 6A). We then examined the spatial localization of macrophages in both models (Figure 6B). There was a striking concentration of macrophages and to a lesser degree, neutrophils, around the area of recanalization. These results suggest that the difference in thrombus resolution found between the two models could not be explained by a quantitative difference in cellular composition within the thrombi, but rather by a specific pattern of distribution and recruitment of the inflammatory cells and protein expressed in the areas of recanalization associated with ongoing blood flow in the stenosis model.

Discussion

The present study is the first to examine the role of thrombus recanalization and ongoing blood flow in the resolution of deep venous thrombosis, and to study potential protease mechanisms responsible for the differences noted. We found a significant increase in the rate of thrombus resolution when thrombus was exposed to ongoing blood flow (stenosis model). Interestingly, while measuring the expression of MMP-2 and -9, we found an increase in the ligation model in comparison with the stenosis model, which differs from what would be suspected and therefore cannot explain the difference in thrombus resolution. The increased MMP-2 and MMP-9 activity in the ligation model was not accompanied by increased MT1-MMP, the canonical cell surface activator of MMP-2. Other proteases, including thrombin, can activate proMMP-2, and these may account for the increased MMP-2 and MMP-9 activity noted in the ligation model.

All the MT-MMPs, with the exception of MT4-MMP, are capable of activating pro-MMP-2 in various *in vitro* and *in vivo* models^{15,23,24}. However, the primary activator of pro-MMP-2 is MT1-MMP, as shown by the marked deficits in MMP-2 activation in *in vivo* studies in which MT1-MMP is decreased^{25,26}. We observed an up-regulation of MT1-MMP gene expression and a down-regulation of other MT-MMP genes, especially MT2-MMP and MT4-MMP, between sham surgery and the two models. We have previously reported that thrombus resolution induces MMP-2 and MT1-MMP expression and activity in a ligation model¹². Therefore, a further increase in MT1-MMP protein expression noted in the stenosis model in the present study may be responsible for the accelerated thrombus resolution observed with ongoing blood flow.

Histological analysis demonstrated localized expression of MT1-MMP and MT3-MMP (Figure 4B). These proteins were predominantly localized to areas of recanalization within the thrombus in the stenosis model, while their expression was uniformly distributed throughout the thrombus in the ligation model (Figure 4B). This distinct and focal expression of these proteases around areas of endothelial recanalization suggests a potential molecular mechanism for the differences in thrombus resolution noted with the ongoing flow model (stenosis). *In vivo* studies have shown that neovascularization through the appearance of channels within the body of the thrombus is a key component of thrombus resolution^{2,27}. Interestingly, in a human arterial mural thrombus, MT1-MMP expression was also localized to neovessels of a recanalized thrombus²⁸. The key role of recanalization in thrombus resolution is also supported by the findings that angiogenic therapy accelerates thrombus resolution in ligation models^{29,30} but not in stasis models³, suggesting that the presence of channels is a prerequisite for effective pro-angiogenic enhancement. Therefore, these data suggest that the focal activity of MT-MMPs around areas of recanalization, rather than a quantitative difference in overall gene or protein expression, may be responsible for the enhanced thrombus resolution observed in the stenosis model with ongoing blood flow. The proteolytic targets of MT1-MMP and MT3-MMP in these areas of recanalization can include other proenzymes (e.g. proMMP-2) as well as extracellular matrix proteins. Fibrin is the principal component of fresh thrombus, and MT1-MMP can cleave fibrin as can MT3-MMP. These findings are summarized in the diagram of the two models of thrombus resolution (Figure 7).

In this study we also observed increased expression of MT6-MMP in the stenosis model when compared with the ligation model. MT6-MMP is a newly identified and relatively poorly characterized member of the MT-MMP family and is expressed almost exclusively in neutrophils³¹. Recent studies show that MT6-MMP cleaves and activates CXC and CC chemokines and can thus enhance neutrophil and macrophage recruitment³². Early stages of thrombus resolution involve the sequential recruitment of neutrophils, macrophages, endothelial cells and myofibroblasts^{2,33}. Furthermore, studies utilizing experimental neutropenia have shown that neutrophils are important for thrombus resolution^{21,34}. Although the total absence of blood flow around the thrombus in the ligation model would suggest impaired influx of neutrophils into the resolving thrombus, our analysis using flow cytometry or histology did not demonstrate any difference in neutrophil or macrophage composition in the thrombi between the two models. The increased MT6-MMP expression

levels found in the stenosis model may result from alterations in neutrophil activation and expression of MT6-MMP.

We evaluated the effect of ongoing blood flow and recanalization on vein wall injury and only found differences in vein wall mononuclear cell infiltration without differences in vein wall area or thickness. In addition, we did not see any difference in macrophage counts in the vein wall between the two models (data not shown). This suggests that recanalization (in these mouse models) decreases wall inflammation, as would be expected with more rapid recanalization.

Previous studies have shown that the administration of pro-angiogenic cytokines (IL-8 and VEGF) enhanced thrombus resolution in the stenosis model, suggesting an important role for recanalization and neovascularization during this process^{2,30}. Our finding of areas of endogenous recanalization with focal macrophage accumulation and MT-MMP expression within the stenosis thrombi help explain and support these findings as well as provide a potential mechanism for the increased rate of thrombus resolution in this model. These two models have distinct features which mimic different aspects of human thrombus resolution. The consistency of thrombus formation and inflammatory reaction of the ligation model allow precise and consistent delineation of the contribution of individual genes to inflammation and thrombus resolution^{9,35}. The recanalization noted with the stenosis model may more closely mimic human venous thrombi, the majority of which resolve via recanalization. Therapeutic strategies to augment this process are perhaps better tested in this model. Another recent model for thrombus resolution with ongoing blood flow has been recently reported utilizing electrolytic injury³⁶. This model appears to have approximately 63% thrombus resolution between day 2 and day 11, which is similar to our stenosis model. It is not clear whether this model demonstrates accelerated thrombus resolution as it has not been compared directly with a ligation model, and the expression of MT-MMP and MMP's in this model is an exciting area for future investigation.

The late detrimental effects of post-thrombotic syndrome after DVT on venous function have generally been attributed to venous valve destruction, fibrosis or dysfunction³⁷. However more recent large studies of patients with persistent post-thrombotic syndrome utilizing intravascular ultrasound have demonstrated an obstructive component amenable to intervention in a surprising plurality of patients^{38,39}. This suggests that impaired, incomplete or maladaptive resolution of the initial DVT with persistent luminal obstruction plays a significant pathological role in post-thrombotic syndrome. The increased inflammatory cells in the vein wall noted in the ligation model, along with the decreased thrombus resolution, may represent this incomplete resolution. Thus, strategies to accelerate thrombus resolution may have clinical benefit in patients, and increasing blood flow across the region of DVT may be one means of accelerating thrombus resolution and potentially decreasing post-thrombotic syndrome. While it seems intuitive that ongoing blood flow would result in improved thrombus resolution, there are no prior studies examining this important question *in vivo*. We identify both a distinct acceleration of thrombus resolution as well as a cellular and molecular mechanism that is potentially responsible. The residual thrombus in patients subjected to pharmacomechanical recanalization may also respond biologically in a manner more similar to our stenosis model than stasis thrombosis. Studies

from our group have recently demonstrated that upper extremity exercise increases lower extremity blood flow in both healthy volunteers as well as patients with DVT⁴⁰. We are currently prospectively enrolling DVT patients in a randomized study of exercise to increase blood flow and potentially prevent later development of post-thrombotic syndrome. The data in this report provide proof-of-concept and a scientific basis to support such clinical studies. These findings also delineate cellular and molecular targets (e.g. macrophages and MT1-MMP in areas of recanalization) which may serve as potential therapeutic targets for thrombus resolution.

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Clinical Relevance

Incomplete thrombus resolution after deep venous thrombosis (DVT) is important in the development of post-thrombotic syndrome. This study directly examines the role of ongoing blood flow and recanalization in the resolution of deep venous thrombosis in vivo. We find that the presence of ongoing blood flow enhances thrombus resolution and is associated with expression of key matrix metalloproteinases in areas of recanalization within the thrombus. These findings help define the physiology of thrombus resolution and provide potential therapeutic targets to accelerate this clinically important process for patients with DVT.

Figure 1.

(A) Left panel: Thrombus weight over time in CD1 mice after sham, stenosis or ligation surgery (Day4: n=5–6; Day 8: $P=0.001$, n=5–12; Day 12: $P=0.014$, n=9–13). Right panel: Clot resolution at day 8 and 12, represented as the percentage of thrombus weight normalized to the initial thrombus weight at day 4 (Day 8: $P=0.007$; Day 12: $P=0.01$). (B) Left panel: Representative H&E for both stenosis and ligation models at day 12 (Magnification= 100X). Right panel: Vein wall area and thickness for both stenosis and ligation models. Quantitative analyses of the number of mononuclear cells in 5 high power field (Magnification x400, n=3, $P=0.05$). (C) Gelatin zymography of thrombus protein lysates at day 4, 8 and 12.

Densitometry are shown as relative activity compared to sham surgery (Day 4: MMP-9 sham vs stenosis $P=.006$, $n=2-3$), (Day 8: MMP-2 sham vs ligation $P=.032$; MMP-2 stenosis vs ligation $P=.03$; MMP-9 sham vs stenosis $P=.044$; MMP-9 sham vs ligation $P=.001$; MMP-9 stenosis vs ligation $P=.005$; $n=2-3$) , (Day 12: MMP-2 sham vs stenosis $P=.044$; MMP-2 sham vs ligation $P=.015$; MMP-9 sham vs ligation $P=.026$, $n=2-3$).

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Figure 2.

MT-MMPs gene expression during thrombus resolution, shown as relative expression compared to sham surgery.

MT1-MMP: Day 8, Sham vs stenosis, $P=.003$, Sham vs ligation, $P=.001$; Day 12, Sham vs stenosis, $P=.004$, Sham vs ligation, $P<.001$. MT2-MMP: Day 4, Sham vs ligation, $P=.033$; Day 8, stenosis vs ligation, $P=.044$; Day 12, stenosis vs ligation, $P=.03$. MT3-MMP: Day 4, Sham vs stenosis, $P=.047$, Sham vs ligation, $P=.008$. MT4-MMP: Day 4, Sham vs stenosis, $P=.009$, Sham vs ligation, $P=.001$; Day 12, stenosis vs ligation, $P=.005$. MT6-MMP: Day 4, Sham vs ligation, $P=.033$. (Day 4, $n=4-5$; Day 8, $n=3-6$; Day 12, $n=3-7$).

Figure 3.

MT-MMPs protein expression during thrombus resolution, normalized to actin and shown as relative expression compared to sham surgery (A) Day 4. (B) Day 8. (C) Day 12. (D) MT1-MMP, Day 4: Sham vs ligation, $P=.02$; stenosis vs ligation, $P=.021$. MT2-MMP, Day 12: Sham vs stenosis, $P=.038$; MT3-MMP, Day 8: Sham vs ligation, $P=.026$ ($n=2-3$).

Figure 4.

(A) Representative immuno-histochemistry for H&E and endothelial cells (CD31 positive cells) for both stenosis and ligation models at day 12 (Magnification= 100X and 400X).

(B) Representative immuno-histochemistry for MT1-MMP and MT3-MMP for both stenosis and ligation models at day 12 (Magnification= 100X and 400X).

Figure 5.

uPA, PAI-1 and HO-1 protein expression normalized to actin during thrombus resolution and represented as relative expression compared to sham surgery. (A) Day 4: PAI-1 Sham vs stenosis, $P=.05$; $n=2-3$. (B) Day 8: PAI-1 Sham vs stenosis, $P=.019$; PAI-1 Sham vs ligation, $P=.008$; HO-1 Sham vs stenosis, $P=.014$; HO-1 Sham vs ligation, $P=.019$; $n=2-3$. (C) Day 12. Arrow shows PAI-1 protein, arrow head represents a non-specific band.

Figure 6.

(A) Flow cytometry results for macrophage and neutrophils content on day 4 and 8 thrombi (n=2–6). (B) Representative immuno-histochemistry for control IgG, macrophages (mac-2) and neutrophils (Ly-6B.2) on day 12 thrombus sections (Magnification 100X and 400X).

Figure 7.

Diagram of potential mechanisms occurring during neovascularization

Table 1

Real-time PCR primer list.

Primer	Sequence
36B4 <i>Forward</i>	GCGACCTGGAAGTCCAACACTAC
36B4 <i>Reverse</i>	ATCTGCTGCATCTGCTTGG
MT1-MMP <i>Forward</i>	TGGTGGCTGTGCATGAGTTG
MT1-MMP <i>Reverse</i>	GTGACCCTGACTTGCTTCCATA
MT2-MMP <i>Forward</i>	ACACCCATTTCGACGCAGAT
MT2-MMP <i>Reverse</i>	TGTCGGTATCCATCCACTGGT
MT3-MMP <i>Forward</i>	CCCATTTTGATTCTGATGAGC
MT3-MMP <i>Reverse</i>	CTGCTACAAGAAATAAATCGTTTCC
MT4-MMP <i>Forward</i>	TGTTATCTCCCTCTCTTGGCC
MT4-MMP <i>Reverse</i>	GCCCACCTTTATTCCAGGTGTT
MT5-MMP <i>Forward</i>	TATCATGGCTCCCTTCTACCAATAC
MT5-MMP <i>Reverse</i>	CTGCGGACCGGGAGTGT
MT6-MMP <i>Forward</i>	TGGCTGTCTGGGCTACTGAA
MT6-MMP <i>Reverse</i>	GGTAGGCCCGAGCAAAGTG