Immunohistochemistry of thrombi following iliac venous stenting: A novel model of venous thrombosis

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Summary

Stenting has become a common intervention for venous occlusive disease. Little is known regarding the composition of venous thrombi complicating stent placement. The optimal design of antithrombotic agents in this setting requires this knowledge. Quantitative immunohistochemistry was undertaken to define the platelet, fibrin(ogen) and leukocyte composition and spatial orientation of venous thrombi following percutaneous iliac stent placement in pigs. Venous stent thrombus size was measured by weight and scintillation detection of autologous ¹¹¹In-platelets. Thrombi were divided in segments (cephalad to caudad), sectioned and stained with monoclonal anti-platelet glycoprotein Ib or polyclonal anti-fibrin(ogen) fluorescent antibodies. Thrombus platelet content was 100-fold greater than paired whole blood samples. The caudal-most segments contained platelet-rich aggregates (p<0.05) with abundant leukocytes (p<0.0001) relative to more cephalad segments. Platelet and fibrin(ogen) content varied over an eight-fold range between segments but were directly correlated with each other (r=0.77; p<0.0001). The platelet co-localization with fibrin(ogen) is consistent with the phospholipid dependence of prothrombin activation. The abundance and caudal distribution of platelet-leukocyte aggregates indicate their preferential accretion from flowing blood early in the genesis of venous stent thrombi. These may represent novel cellular targets for the prevention and treatment of venous thrombosis.

Keywords
Venous thrombosis, vascular stents, platelets

Introduction

The clinical use of venous stents for the treatment of acute or chronic venous thrombosis and other occlusive disease processes is rapidly increasing (1–9). Little is known regarding the composition or pathogenesis of the venous thrombosis complicating stent placement. It is unclear whether thrombi occurring in this setting represent a platelet-rich process such as seen following arterial stenting or a stasis thrombus typical of the venous circulation. We have developed a porcine model of venous thrombosis which employs the percutaneous placement of a Wallstent into the iliac vein. Within two hours of stent placement, a gross thrombus is present in all stented segments and 80% are totally occluded. We sought to define the platelet, fibrin(ogen) and leukocyte composition and spatial orientation within venous thrombi generated by iliac stent placement in pigs using quantitative immunohistochemistry. The search for the optimum thromboembolic prophylaxis regimen for venous thromboembolism (VTE) remains a priority. We now provide fundamental information of thrombus composition essential for this search.

Methods

Animals

Four-month-old, pre-estrus, female pigs (n=16) of the Babcock 4-way cross stock (a mixture of Landrace, Yorkshire, Hampshire, and Duroc breeds) were purchased through the Mayo Clinic’s section of Veterinary Medicine and housed at the Mayo Institute Hills Facility. The study was approved by the Mayo Clinic Animal Care and Use Committee and conformed to the National Institutes of Health and United States Department of Agriculture guidelines.

Induction of thrombosis

Anesthesia of pigs and ¹¹¹In-platelet labeling were performed as described previously (10). To stimulate procoagulant pathways to provide a maximal thrombotic signal, both carotid arteries were first serially injured by six serial hemostat clamps of 5-second duration, interspersed with a 3-second rest period, with each subsequent injury visually abutting the prior injury site. After 30 minutes, the carotid arterial thrombi were harvested. Left femo-
Venous catheterization was then accomplished percutaneously, and an 8x30-mm self-expanding Wallstent (Boston Scientific) was deployed into the left common iliac vein. Thrombi were allowed to propagate for 2 hours before harvesting the stented venous segment (without ligation) into 2-methylbutane (liquid nitrogen). Stented venous segments were assessed for $^{111}$In-platelet content (Minaxi Autogamma Counter 5000 series, Packard). Venous stents were carefully incised longitudinally and the lumen carefully assessed for presence of macroscopic thrombi. The incised self-expanding stent struts then unfolded presenting the thrombus for easy and complete extraction. Thrombi were then weighed and archived at –70°C.

**Thrombus immunohistochemistry**

To define the thrombus architecture as it relates to platelet, leukocyte, and fibrinogen composition, frozen thrombus samples from six representative thrombi were divided into four equal segments (arbitrarily labeled 1–4, cephalad to caudal) and sectioned at 5 microns on positively charged slides. Each section was stained with either a monoclonal anti-porcine platelet glycoprotein Ib or a rabbit polyclonal anti-porcine fibrinogen conjugated with either FITC or rhodamine. Whereas the rabbit polyclonal antibody lacks specificity for distinguishing fibrinogen from fibrin, this variable is labeled fibrin(ogen) throughout the remainder of the manuscript. Negative controls were performed with non-immune murine immunoglobulin to delineate background staining. Stained thrombus cross-sections were photographed digitally using an Axioplan 2 fluorescence microscope equipped with an Axiocam HRC camera (Carl Zeiss Inc.). To explore the biochemical and cellular architecture of venous thrombi, two distinct immunohistochemical measures were used: the area of positive staining and staining intensity. Area of positive staining was quantified on electronically stored images with NIH-image 1.62 (11). The entire section of each thrombus segment was systematically analyzed by moving from one visual field to the adjacent field using histologic landmarks. The mean percentage of positive staining area for each antibody was calculated for each segment. Fluorescence staining intensity for platelet and fibrin(ogen) content was then quantified on the same digitally captured images with Image J 1.33 software after uniform background subtraction. To assess co-localization of cellular and biochemical constituents, visual fields were realigned using histologic landmarks and positive staining and non-staining areas were compared.

**Figure 1:** Venous thrombus weights and platelet content. Two hours following deployment, stented iliac venous segments were harvested for thrombus weight and $^{111}$In platelet content (A). Platelet content within crush injured carotid arteries was nearly five-fold greater than stented iliac veins (p<0.05; B). Paired Student t-test.

**Figure 2:** Iliac vein thrombus histology. Harvested iliac venous thrombi were divided into segments (columns labeled 1–4, cephalad to caudal), sectioned and stained with hematoxylin and eosin (magnification: upper row 10X, lower row 40X). Platelets (Plt) and leukocytes (arrows) were more abundant in caudal segments whereas red cells (R) were more evident in cephalad segments.
Non-fluorescent images were photographed digitally (DC120 Eastman Kodak) at 5X magnification (Carl Zeiss Inc.). Leukocyte quantity was assessed manually for each section.

**Statistics**
All values are presented as mean ± SD. Paired students t-test was used to compare platelet, fibrin(ogen) and leukocyte content between various segments within a thrombus. Correlation between continuous variables was assessed by ANOVA using JMP software. Statistical significance was set at p<0.05.

**Results**
Two hours after iliac venous stent deployment, visible thrombus was notable in all harvested venous segments, and 80% of all thrombi were occlusive. The average thrombus weight was 318 mg (SD ± 206 mg) with an average platelet count of 626 x 10⁶ cm² (SD ± 474 x 10⁶ cm²) (Fig. 1A). The venous thrombus platelet content was 100-fold greater than anticipated compared to the platelet content of equal volumes of paired whole blood samples taken from each animal (p<0.05). The mean platelet content of venous stent thrombi was on average one fifth of the platelet content of injured carotid arteries (2,922 x 10⁶ cm² ± 2,116 x 10⁶ cm²) (Fig. 1B; p<0.05).

Gross morphology of these thrombi revealed small channels evident throughout the body of all occlusive thrombi. To define the architecture, thrombi were divided into four equal segments (arbitrarily labeled 1 – 4, cephalad to caudad). Microscopic evaluation of segments stained with hematoxylin and eosin revealed platelet-rich aggregates with abundant leukocytes in the caudal-most segments (Fig. 2). Typical “lines of Zahn” were evident in the more cephalad segments which were red-cell rich with a paucity of either platelets or leukocytes.

Distinctive regional differences were apparent between thrombus segments by immunohistochemical analysis of platelet and fibrin(ogen) content (Fig. 3). The caudal-most segment (seg-
ment 4) contained more platelet-rich aggregates (Fig. 4A; p<0.05) and abundant leukocytes (Fig. 4B; p<0.0001) relative to the more cephalad segments. Cephalad thrombus segments were dominated by red blood cells encased by fibrinogen. Leukocytes were 7- to 10-fold more numerous in the caudal-most platelet-rich segments relative to the cephalad segments. Although the fibrinogen content was greater in segment 4 (caudal) compared to segment 2 (p<0.05), the fibrinogen content was comparable to segments 1 and 3 (Fig. 4C).

Measures of fluorescence staining intensity revealed distinct co-localization of thrombus platelet and fibrinogen deposition. A significant correlation (r=0.77; p<0.0001) between platelet and fibrinogen staining intensity was observed (Fig. 5).

To determine whether performing an acute arterial crush injury prior to iliac venous stent placement alters the venous thrombotic response, a stent was placed in one iliac vein two hours prior to carotid injury. Thirty minutes following bilateral carotid injury, a second stent was then placed in the contralateral iliac vein which was harvested two hours later. The platelet content and thrombus weights of harvested venous segments were compared. This experimental sequence was completed in two pigs. For one pig, the thrombus weight was 442 mg and platelet content was 830 x 10^6 platelets/cm^2 for the initially stented iliac segment while the subsequent iliac segment was 264 mg and 2,652 x 10^6 platelets/cm^2, respectively. For the second pig, the thrombus weight and platelet content for the initially stented iliac segment was 341 mg and 2,370 x 10^6 platelets/cm^2, respectively while the subsequent iliac segment was 444 mg and 1,940 x 10^6 platelets/cm^2. In summary, no systematic change in either the venous thrombus weight or platelet content comparing the second to the first venous segment could be appreciated.

**Discussion**

This study provides the first description of the regional cellular and biochemical elements within thrombi generated after deployment of a venous stent. The principal finding is a longitudinal variability of platelet and leukocyte content within these thrombi. Platelets and leukocytes, and to a lesser extent fibrin, are not evenly distributed throughout the length of a venous stent thrombus. The caudal end of the thrombus is comprised primarily of leukocytes and platelets. Fibrinogen and red cells with “lines of Zahn” morphology typify the more prominent cephalad “tail” of the thrombus which is relatively devoid of platelets and extends throughout the length of the stent. Both microscopic and macroscopic channels extend throughout the length of these thrombi suggesting the possibility of seepage even through apparently occlusive thrombi.

While “stasis” may be an important variable in the formation of the red-cell-rich thrombotic tail, platelet and leukocyte accretion can only occur in the setting of flowing blood. The abundance and caudal distribution of platelets and leukocytes indicate their preferential accretion from flowing blood early in the genesis of venous thrombi. Moreover, the platelet content of these thrombi is 100-fold greater than paired whole blood samples underscoring an enrichment process not possible in a static environment. Co-localization of leukocytes and platelets in the caudal segments of these thrombi could occur through P-selectin mechanisms. Selected inhibition of P-selectin has been shown to reduce venous thrombus volume (12). Whether the platelet recruits the leukocyte or vice versa remains to be determined. The two hour time-span of these experiments, however, is too brief for leukocyte enrichment to occur through migration. Venospasm resulting from platelet secretion of vasoactive amines could enhance the venous obstruction process in venous thrombi occurring in humans. While this remains a plausible hypothesis for the contribution of venous thrombogenesis, the use of a stent in this animal model precludes this effect at least within instrumented segments. Following luminal occlusion by the platelet-leukocyte aggregate, seepage of clotting factors activated on phospholipid membranes within the platelet aggregate could lead to thrombosis of adjacent static blood. This mechanism could account for the more cephalad thrombus histology. The cephalad thrombus segments appear primarily composed of red blood cells encased within fibrinogen sacs. Furthermore, it is plausible that caudal thrombus propagation following stasis may also occur, though not appreciated in our specimens.

The search for the optimum thromboembolic prophylaxis regimen for VTE primarily focuses on anticoagulants. We have found that platelets and leukocytes are present and may be principal in the initiation of venous thrombosis. To date however, platelet inhibition with arachidonate antagonism has been largely ineffective in clinical trials suggesting that alternate pathways of platelet activation and accretion may be involved. We now provide fundamental information of thrombus composition essential for this search. These remain important potential targets for both thrombosis prophylaxis and treatment. Furthermore, the role of leukocyte inhibition in this process remains attractive (12).

The second noteworthy finding of this study is a colocalization of platelets and fibrinogen) within venous thrombus seg-
ments (Fig. 5). Coagulation-factor activation occurs as a “cascade” of proenzymes which are cleaved to their active form. This cascade of enzyme activation and amplification requires the assembly of activation complexes on the phospholipid membrane of activated platelets. These activation complexes include activated platelet phospholipid membrane, activating enzyme and proenzyme, an activated cofactor, and calcium. Exposed tissue factor within the vessel wall initiates the cascade by binding circulating factor VIIa which in turn cleaves factors IX and X to their active forms (IXa and Xa, respectively). Factor Xa then combines with factor Va on the platelet phospholipid membrane to form the prothrombinase complex, which cleaves prothrombin to thrombin. Thrombin then orchestrates thrombosis by inducing further platelet granule secretion, cleaving fibrinogen to fibrin, and activating factor XIII which crosslinks fibrin strands to form a stable thrombus. Therefore, the significant correlation between platelet and fibrin content is to be expected.

We believe that this is the first description of an animal model of venous stent thrombosis. Within two hours of percutaneous deployment of a self-expanding stent into the iliac veins of pigs, a gross thrombus was present in all of stented segments and 80% of stented segments were totally occluded. This venous thrombosis model employs neither stasis nor stenosis. Indeed, these findings argue that venous stasis is not necessary for the genesis of venous thrombosis as previously reported (13–16). Furthermore, venous stenosis related to wall injury or vasoreactivity is uniformly prevented by the presence of the stent. The stented segment imposes uniform vascular geometry and flow characteristics. The only luminal impediment in this model is propagating thrombus. Morphology of these thrombi is consistent with published reports of venous thrombosis in humans (13). For these reasons, this model may be appropriate for studying venous thrombosis mechanisms.

There are several limitations of this study worth noting. Whereas this model involves venous stenting in pigs, the results may not be generalized to humans. The thrombotic response to stent placement in pigs may be more robust than would be anticipated in humans. The outcomes of balloon angioplasty and stenting in 455 limbs was reported in patients with chronic venous outflow stenoses (17). After a median follow-up of three months, 13 of 324 limbs (4%) were occluded. In six patients, stent occlusion occurred in the immediate postoperative period. In this study, however, patients with acute deep venous thrombosis or malignancy were excluded from participation, and no information was provided regarding procedural and subsequent anticoagulant therapy. In-stent restenosis had developed in 80% of these patients at 42 months. It was acknowledged that the nature of in-stent stenosis is not known and may include thrombotic mechanisms, neointimal hyperplasia or both (18). Thrombotic outcomes appear to be higher when stenting is combined with thrombolytic therapy for acute venous thrombosis. For example, 77 patients with lower-extremity thrombosis were treated with thrombolytic therapy (19). Of these, stents were placed in 45% of limbs. Although procedural thrombolytic therapy and heparin was described, no information was provided regarding subsequent anticoagulation at hospital discharge. Furthermore, the primary patency rate was significantly lower for stented compared to non-stented limbs (54% vs. 75%; p=0.01) at one year. We therefore believe that the search for the optimum thromboembolic prophylaxis regimen for VTE in this setting remains a priority. Second, we employed carotid crush injury prior to venous stent deployment to increase the thrombotic milieu. The rationale for this experimental design includes our desire to exploit prior venous thrombosis models which employ either mechanical or biochemical means of augmenting the thrombotic milieu. Furthermore, the development of clinical venous thrombosis includes exposure to either major surgery or major trauma. Our model of arterial injury incorporates both. Upon further study, however, we have found that venous thrombosis can be adequately initiated prior to carotid injury. Lastly, all thrombi were harvested within two hours of stent deployment. We therefore cannot comment on the rapidity of thrombus formation or the thrombus evolution with maturation beyond this time frame.

References