

INFLAMMATION-DEPENDENT THROMBOSIS

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1. ABSTRACT

Vessel wall endothelial damage initiates a local inflammatory response, which promotes a prothrombotic state driven by tissue factor, adhesion molecules, and pro-inflammatory cytokines. Understanding how natural inflammatory mechanisms promote a procoagulant state, may lead to the development of new pharmacological interventions targeted at thrombosis.

2. INTRODUCTION

Thrombosis as described by Virchow (1856) is influenced by a triad of factors consisting of 1) endothelial injury, 2) blood stasis or turbulent flow, and 3) blood hypercoagulability (1). Vessel wall endothelial damage initiates a local inflammatory response, which promotes a prothrombotic state driven by tissue factor, adhesion molecules, and pro-inflammatory cytokines. Stewart *et al.* in 1974 hypothesized that vascular inflammation and thrombosis are interrelated (2). Stewart's original hypothesis suggested that initiating factors that promote

thrombosis cause the activation of both leukocytes and platelets. This activation promotes the adhering and layering of leukocytes and platelets on the thrombus, leading to its amplification (2). Current research in vascular biology supports this hypothesis.

The vascular inflammatory response initially is protective by nature; its role is to promote the recruitment of inflammatory cells for the removal of bacteria and endotoxins. However, local and systemic inflammation can produce a prothrombotic environment driven by tissue factor, adhesion molecules, pro-inflammatory cytokines and prothrombotic microparticles.

These molecular events occurring in and around injured vascular endothelium have proven to be a factor in the pathogenesis of several cardiovascular diseases (hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure) (3). Endothelial dysfunction is a term used to identify several pathological conditions that

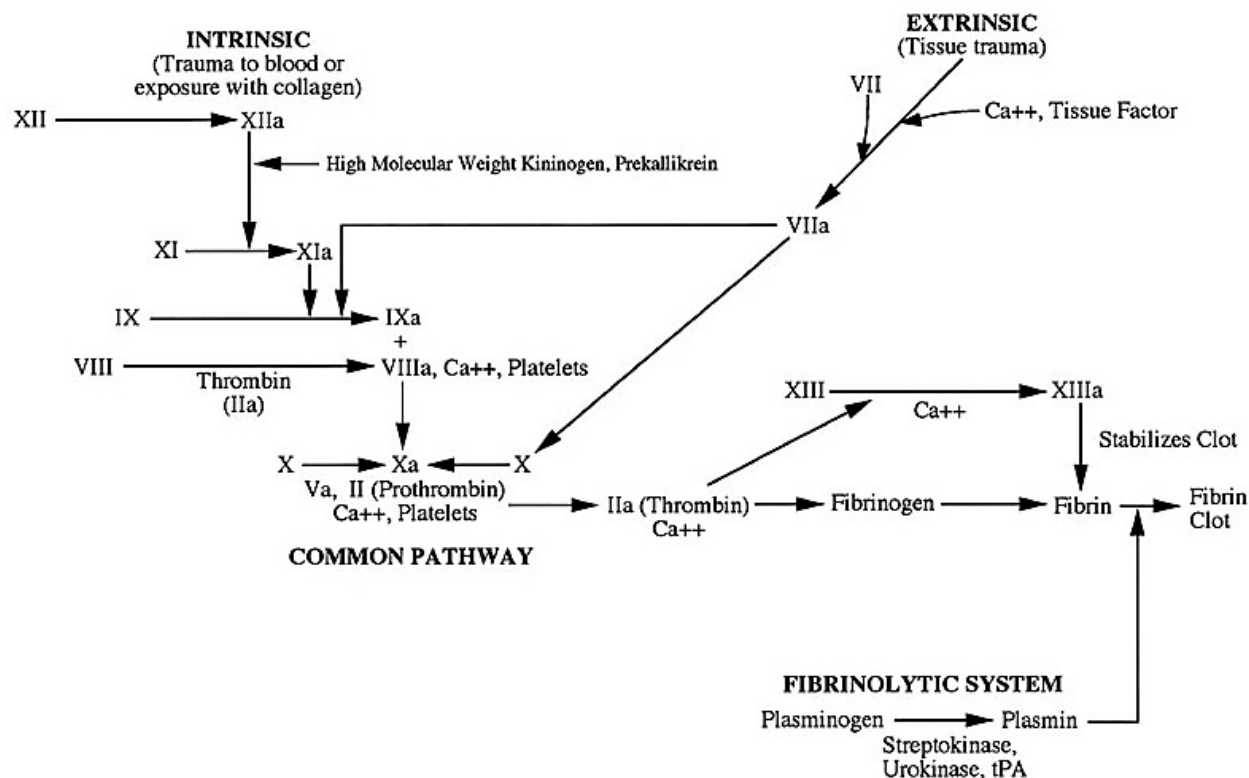


Figure 1. Secondary Hemostasis: Coagulation factors that activate both the Intrinsic and Extrinsic coagulation pathways. (Modified with permission, 11).

can lead to altered coagulation, inflammation, impaired vascular growth, and vascular remodeling (4). This process is associated with a decrease in nitric oxide and an increase in oxidative stress, which is a promoter of the inflammatory process (5, 6). Risk factors consisting of acute and chronic infection, local immune reaction or permanent factors like hypertension, diabetes, obesity, hyperhomocysteinemia, and more can induce endothelial cell dysfunction, promoting tissue factor to activate the clotting cascade (7-9). Additionally, the interaction of tissue factor expression on the surface of monocytes facilitates monocyte-platelet and monocyte-endothelial interactions through P-selectin binding mechanisms (10). This cascade driven by inflammatory mediators and tissue factor leads to the acceleration of fibrin formation and deposition into a developing thrombus (10) (Figure 1, 11).

3. THE EPIDEMIOLOGY OF VASCULAR THROMBOSIS

Arterial thrombosis and its associated clinical diseases make it the leading cause of death in the world today (12). In the peripheral circulation, its incidence is reported to be approximately 2 cases/10,000 people per year with a morbidity and mortality rate greater than 20-25% (13, 14). In the coronary circulation, the majority of myocardial infarctions are caused by arterial thrombosis, with fresh thrombus superimposed on a ruptured or eroded atherosclerotic plaque. Acute myocardial infarction is responsible for a staggering loss of life in our country and

is the number one killer of individuals at a premature age (15).

Deep venous thrombosis (DVT) remains a serious health care problem in this country with over 250,000 patients affected yearly and at least 200,000 diagnosed yearly with pulmonary embolism (PE), although these figures are conservative (16-18). Treatment costs to the United States health care system are in the billions of dollars per year just for the acute treatment of venous thrombosis, not including the monetary cost spent on the treatment of the sequelae of DVT (chronic venous insufficiency) and PE (chronic pulmonary hypertension) (19-21). Chronic venous insufficiency, a major complication of DVT, affects between 400,000 and 500,000 patients with skin ulcerations and six to seven million patients with manifestations of chronic venous disease including stasis pigmentation and stasis dermatitis. It has been reported that up to 28% of the patients evaluated after having an iliofemoral DVT developed marked edema and skin changes that lead to venous ulceration over time. Approximately 1% of adults and 3% to 4% of those over age 65 years once had or now have an ulcer of venous etiology (22).

4. ADHESION MOLECULES, INFLAMMATION, AND THROMBOSIS

Selectins are glycoproteins that are expressed by leukocytes, activated endothelial cells and platelets. The role of selectins is to mediate the initial adhesion

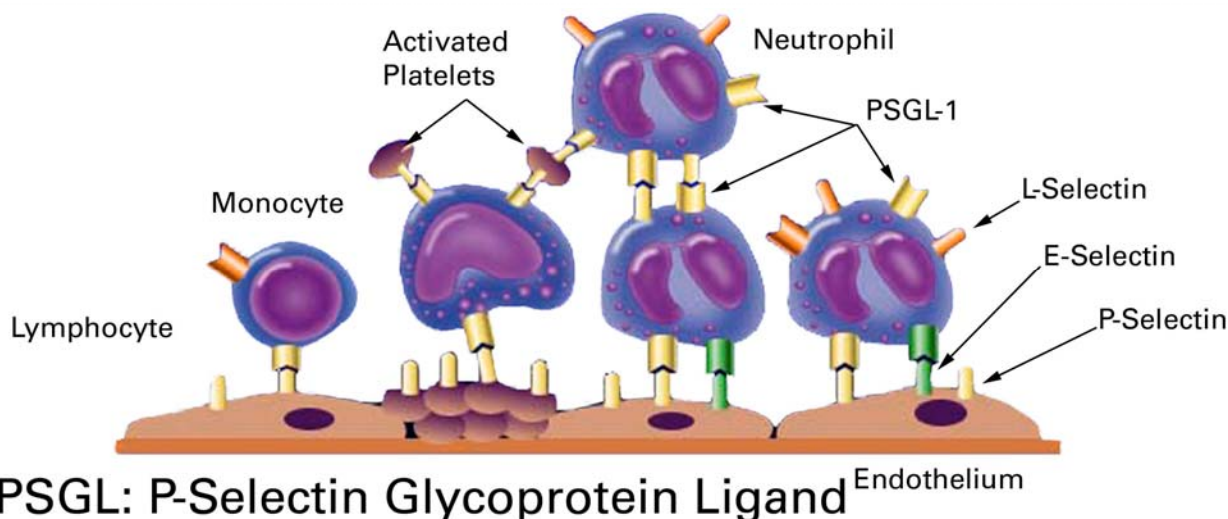


Figure 2. Leukocytes and activated endothelial cells express selectins. They recognize specific carbohydrate sequences and are responsible for the rolling and tethering of leukocytes onto stimulated vascular endothelium.

interactions of leukocytes stimulated by physiological changes in blood flow at sites of vascular endothelium injury. Presently three selectins have been identified: P-selectin, E-selectin, and L-selectin. All selectins have been shown to recognize the sialyl Lewis^x (sLe^x) carbohydrate ligands. The adhesion molecule P-selectin, which is present in platelet α -granules and endothelial cell Weible-Palade bodies (23), is up-regulated early during thrombosis promoting vein wall inflammation in multiple animal models (24).

Thrombogenic and inflammatory mediators such as thrombin, leukotrienes, and histamine induce the rapid translocation of P-selectin to the surface of endothelial cells and platelets. The receptor for P-selectin is a glycoprotein expressed on the surfaces of most hematopoietic cells termed P-selectin glycoprotein ligand-1 (PSGL-1, 25) (Figure 2). Additionally, P-selectin:PSGL-1 interactions are responsible for thrombus amplification. This receptor is associated with adhesion interactions responsible for the initial rolling of neutrophils along stimulated vascular endothelium. This receptor has a high affinity for P-selectin, and a lesser affinity for E- and L-selectin.

We have defined the importance of P-selectin to thrombosis using a mouse model of stasis induced venous thrombosis. In mice, vein wall neutrophils are significantly elevated above sham control animals at day 2 after thrombosis, monocytes are significantly elevated above sham controls at day 6 after thrombosis, while total inflammatory cell counts are significantly elevated at both time points. As measured by vein wall ELISA, P-selectin is up-regulated as early as 6 hours after thrombus induction, while E-selectin is up-regulated at day 2 after thrombosis. Selectins remain elevated through day 6 after thrombosis, and mRNA activity precedes protein elevations (24). Mice that are gene deleted in both P-selectin and E-selectin, show statistically decreased thrombus fibrin staining associated with decreased thrombus

weights compared to controls evaluated 2 and 6 days post thrombosis in the same mouse model (25).

5. MEDIATORS OF INFLAMMATION

Pro-inflammatory cytokines like interleukin-1 β , interleukin-6, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor- β have been shown to be up-regulated during thrombogenesis (10). After an inflammatory stimulus, the progression of the inflammatory response reflects a balance between anticoagulant and prothrombotic activity. This balance is shifted toward the procoagulant state by the ability of these molecules to down-regulate anti-thrombotic proteins (thrombomodulin and protein C pathway) while up-regulating prothrombotic proteins (such as primarily tissue factor)(26).

The expression of TF circulating on monocytes has been shown to be important in acute thrombotic events (10, 27). TF expression on monocyte surfaces promotes monocyte interactions with activated platelets and endothelial cells leading to fibrin formation and deposition into the developing thrombus. Cell culture investigations using both monocytes and endothelial cells preps, can be stimulated by tumor necrosis factor (TNF), interleukin-1, MCP-1, or interleukin-6 to express TF on their cell surfaces (28, 29). Complement activation generates the C5b9 complex, which if deposited on cells causes exposure of phosphatidylserine on the surface of cells, thus initiating the activation of coagulation cascade (30). Recent investigations have also shown that activation of platelets can cause the release of CD40 ligand, which in turn has pro-inflammatory activities and augments the thrombogenic response (31).

6. TISSUE FACTOR

Tissue factor is a membrane-bound protein (46-kDa) that triggers thrombin generation by forming a

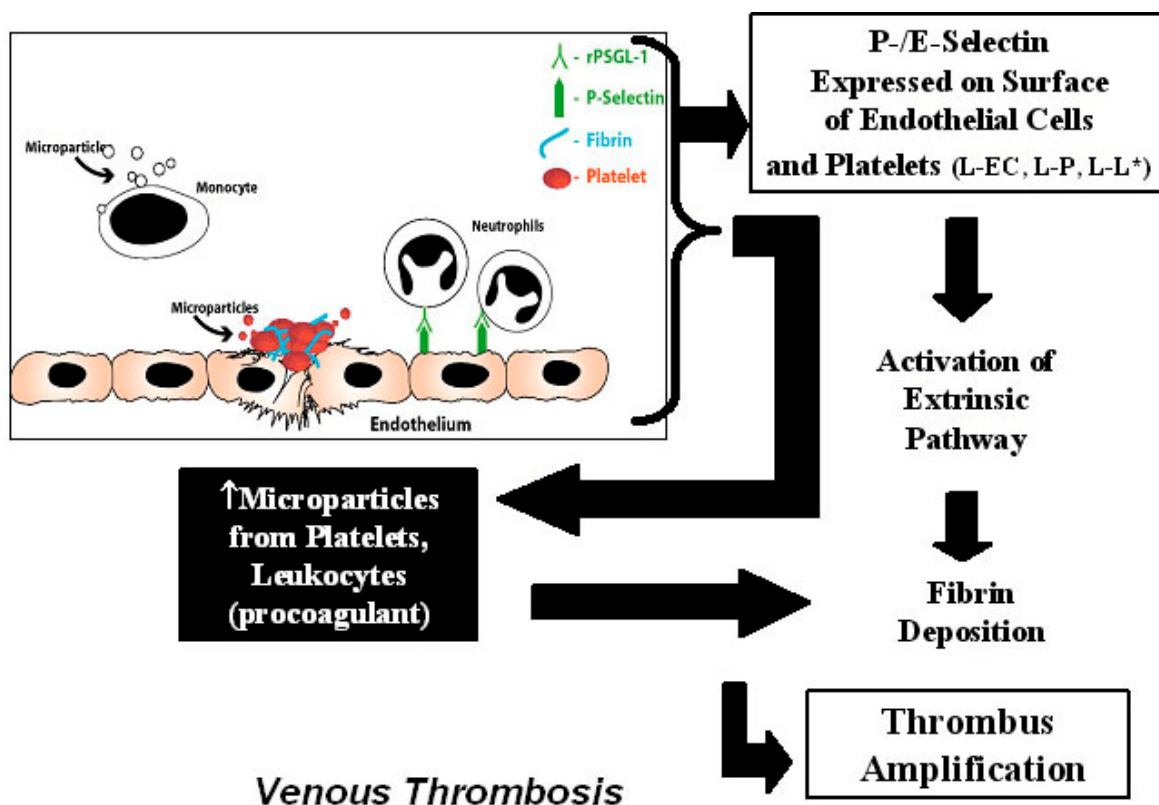


Figure 3. Proposed mechanism of venous thrombosis involving the amplification of thrombus formation by the elaboration of microparticles and the thrombogenic nature of inflammatory cells (especially monocytes). (Modified with permission, 25).

complex with factor VIIa which triggers the activation of the coagulation cascade (32, 33). Vascular injury and various disease states can promote the exposure of TF within the vessel wall to blood flow, thus leading to the initiation of thrombosis (34). It has been hypothesized that the dynamics of thrombus also propagation support a role for circulating TF as the diffusion distance from the vessel wall to the luminal surface of the thrombus increases (35).

Although the cellular origin of circulating TF is unknown, there is evidence to suggest that leukocytes and leukocyte-derived microparticles are a major source (36). By use of a whole blood assay for TF activity, procoagulant activity in normal individuals can be determined showing the presence of TF on mononuclear cells (37). The deposition of leukocyte-associated TF has been demonstrated in thrombi formed on collagen-coated slides or pig arterial media and in human thrombi *in situ* (38, 39). Furthermore, TF can be transferred from leukocytes to platelets *in vitro* via an interaction involving CD15-expressing membrane microparticles and P-selectin (40). There is a growing body of evidence in favor of a role for leukocyte-derived TF in thrombosis based on *in vitro* studies, coupled with observational data of increased circulating TF-positive leukocyte-derived microparticles in various disease states associated with thrombosis (22, 41-43). Additionally, it is also unknown whether these leukocytes merely transport TF or actually express it (31). However, a recent study evaluating both mouse models of

arterial and venous thrombosis modulated tissue factor expression using gene-targeting and bone marrow transplantation technology, found that TF in the vessel wall and not TF from leukocytes was more important for thrombus formation (44). This suggests that TF production by leukocytes may play less of a role in the process of thrombus amplification than TF from the vessel wall in the *in-vivo* situation.

7. MICROPARTICLES AND THROMBOGENESIS

Microparticles (MPs) are described as fragments of phospholipids from cell membranes that are hypercoagulable, and have been found to modulate a number of inflammatory cell vessel wall interactions. Recent investigations suggest that MPs, which are prothrombotic in part by virtue of tissue factor on their surface (39, 45), are extremely important in early venous thrombogenesis, especially those MPs derived from leukocyte origin. When there is vascular injury that initiates inflammation and thrombosis, selectins are expressed on the surface of endothelial cells and platelets. The interaction between selectins and their leukocyte receptors stimulates fibrin formation (46). Procoagulant MPs, derived more from activated leukocytes and less from activated platelets produced are recruited to the area of thrombosis (47), where they amplify coagulation via tissue factor and factor VIIa (40, 45, 48-51) (Figure 3). The colocalization of fibrin, platelets, and leukocytes in the developing thrombus (45, 52) and the contribution of P-

selectin to leukocyte-platelet interactions to generate tissue factor (53) support their central role of inflammation in thrombogenesis. Platelet-derived MPs are involved in venous thrombosis in the syndrome of heparin-induced thrombocytopenia (54).

Less is known regarding leukocyte-derived MPs than platelet derived MPs, although two studies suggest that these MPs are associated with endothelial cell activation and cytokine gene induction as indicated by elevations in interleukin-6, MCP-1, TNF, and a factor Xa procoagulant response mediated by c-Jun N-terminal kinase (JNK)1 signaling pathway (55, 56). Microparticles have been found to impair endothelial cell nitric oxide transduction from endothelial cells (57). Additionally, MPs derived from endothelial cells were found to induce monocyte tissue factor antigen and mRNA release, partially dependent on the interaction of the firm adhesion receptor ICAM-1 with its counter-receptor beta-2 integrin (58). The procoagulant nature of microparticles induced by P-selectin has been demonstrated in a recent manuscript (23). In this study, P-selectin:PSGL-1 interactions led to the development of procoagulant microparticles rich in tissue factor that were able to reverse bleeding in Factor VIII deficient mice. These microparticles, when fluorescently labeled, were recruited into the growing thrombi within 1 minute of ferric chloride induced injury to venules (23).

A recent study has reported that microparticles from pericardial blood of cardiac surgery patients were found to be highly thrombogenic in a rodent IVC thrombosis model (59). Of interest and importance, MPs have also been found to be present in normal healthy individuals. They have been hypothesized to have an anticoagulant function by promoting the generation of low amounts of thrombin which activates protein C, supporting protein C's anticoagulant function (60). Microparticles have also been suggested to play a role in the inflammatory response during severe sepsis, and their reduction was found to correlate with organ dysfunction and mortality (61). Thus, MPs are important in both normal homeostasis and pathophysiology. Further research is warranted in order to standardize both the identification and quantification of circulating cell-derived microparticles (62, 63).

8. INFLAMMATION AND ARTERIAL THROMBOSIS

Arterial thrombosis, whether acute or chronic, is initiated by damage to the vascular endothelium, which promotes an inflammatory response. An example of this is the pathophysiology of atherosclerosis. The early stage of atheroma development is marked by the deposition and accumulation of lipid within the arterial wall. This creates a local endothelial inflammatory response, which leads to inflammatory cell extravasation into the intima layer. Activated inflammatory cells initiate adhesion interactions initiated by P- and E-selectin on the stimulated vascular endothelium. Alpha and beta integrins, members of the cellular adhesion molecule family (CAMs), are responsible for firm adhesion of inflammatory cells to stimulated endothelium. This acute event eventually leads to chronic

intimal injury characterized by a lipid core rich in degenerating inflammatory cells, cholesterol crystals, and tissue factor generated from active macrophages (macrophage colony stimulating factor [M-CSF] (64). Inflammatory cells secrete cytokines, growth factors, and promote the migration and proliferation of smooth muscle cells. These smooth muscle cells produce enzymes that degrade elastin and collagen. These events weaken the fibrous cap and leads to rupture, leading to massive tissue factor expression, coagulation cascade activation, platelet aggregation, activation, and thrombus amplification (65).

9. INFLAMMATION AND VENOUS THROMBOSIS

Venous thrombosis is associated with a significant inflammatory response. This inflammatory response involves acute to chronic changes that lead to thrombus amplification, vein wall damage, and eventual thrombus organization.

An early elevation in neutrophils is associated with an increase in tumor necrosis factor- α levels within the blood vessel wall. There is a steady and progressive rise in vein wall cytokines such as MCP-1. The rise is presumably the stimulus for a monocytic influx into the vein wall and thrombus recanalization. Pro-inflammatory, anti-inflammatory cytokines and chemokines are involved in determining the ultimate vein wall inflammatory response to the thrombus, and adhesion molecules such as P-selectin and E-selectin also play a role in the process.

Venous thrombosis results in thromboembolization, luminal recanalization, or chronic occlusion with scarring. When rapid and complete thrombus resolution occurs, valvular function is better preserved and the sequelae of chronic venous insufficiency are lessened. If this does not occur, the thrombosed vein may resolve, partially restoring the vessel lumen. However, the subsequent intraluminal scarring entraps the valvular mechanism resulting in valvular incompetence, or leads to vein wall fibrosis, preventing the normal function of the valve mechanism. Finally, venous thrombosis more rarely results in complete long-acting fibrous luminal obliteration.

Inflammatory cells are important to the process of thrombus recanalization and organization. Although it may seem intuitive that a decrease in inflammation will decrease thrombogenesis, once clot forms the presence of neutrophils is important for thrombus recanalization. In a study using an antibody to neutrophils, animals rendered neutropenic developed significantly larger thrombi (65). Neutropenic cancer patients are not protected from DVT, and multiple neutropenic episodes are significantly associated with recurrent venous thromboembolism (VTE) in patients with malignant disease who require filter placement due to a failure of, or contraindication to, anticoagulation (66). We have also noted an inverse correlation between thrombus neutrophils and thrombus weight in mouse thrombi, especially prominent in the center of the thrombus (unpublished observation). The presence of monocytes and urokinase-type plasminogen

activator (uPA) has found to be important in later thrombus organization and recanalization. Injection of MCP-1 into thrombi in a rat DVT model resulted in enhanced thrombus resolution (67). Thrombi have been found to contain increasing amounts of both tissue plasminogen activator (tPA) and uPA activity as they resolve (59) and this activity are expressed by invading monocytes (68). In a recent study, mice gene deleted for uPA had impaired thrombus resolution with collagen deposition and cell infiltration restricted primarily to the margins of the thrombus, with few neovascular channels present. Mice gene deleted for tPA, however, were not similarly affected suggesting that it is uPA and not tPA, which is responsible for this activity. Absence of uPA was also associated with delayed monocyte recruitment into the thrombus (69, 70). Taken together, the inflammatory cell data suggests that inflammation is important for thrombus organization and recanalization, with neutrophils setting the early stage for later monocyte activity.

10. SUMMARY

There are important differences between venous and arterial thrombosis. For example, thrombi in the venous circulation are associated with a significant inflammatory response, while such a response is much less prominent in the arterial circulation. Previous research done by our laboratory suggests that selectins are important in stasis-induced venous thrombosis. Modulation of selectins effectively decreases the formation of venous thrombus in stasis-induced animal models. Of interest, our recent work evaluating the procoagulant effects of microparticles shows that specific drug therapies can influence the number and type of microparticle in the circulation. However, additional research is needed to define the factors regulating inflammation and thrombosis. Understanding the mechanisms of inflammation-induced thrombosis may lead to the development of new pharmacological interventions targeted at modulating thrombosis. Additionally, understanding more fully the differences between venous and arterial thrombosis will allow for targeted therapies; in other words, anticoagulants likely need to be developed specifically for arterial and venous thrombosis.

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