

Inflammation and thrombosis: new insights

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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

Venous thromboembolism (VTE) is a disease that encompasses both deep vein thrombosis (DVT) and pulmonary embolism (PE). Traditionally, this definition has not included superficial venous thrombosis (SVT) but there is now suggestion within the literature that SVT should be included in the definition of VTE and therefore treated accordingly (1). The factors that could lead to or interact with one another to lead to VTE were first described by Virchow in 1856 and include 1) endothelial injury, 2) blood stasis or turbulent flow, 3) and blood

hypercoagulability (2). 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 first hypothesized that vascular inflammation and thrombosis are interrelated (3). This original hypothesis suggested that prothrombotic factors lead to the activation of leukocytes and platelets. This process promotes thrombus amplification via adherence and layering of the activated platelets and leukocytes (3). 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 micro-organisms 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) (4). Endothelial dysfunction is a term used to identify several pathological conditions that can lead to altered coagulation, inflammation, impaired vascular growth, and vascular remodeling (5). This process is associated with a decrease in nitric oxide and an increase in oxidative stress, which is a promoter of the inflammatory process (6, 7). 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 (8-10). Additionally, the interaction of tissue factor expression on the surface of monocytes facilitates monocyte-platelet and monocyte-endothelial interactions through P-selectin binding mechanisms (11). This cascade driven by inflammatory mediators and tissue factor leads to the acceleration of fibrin formation and deposition into a developing thrombus (11) (Figure 1).

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

Venous thromboembolism (VTE) is a major health problem in the United States with approximately 900,000 individuals affected annually (16). The incidence of this disease has not changed significantly over the past 25 years despite improvements in prophylaxis (17). Deep venous thrombosis (DVT) affects around 380,000 patients annually while at least 520,000 are diagnosed yearly with pulmonary embolism (PE) (16) (18-20). 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 (postthrombotic syndrome) and PE (chronic pulmonary hypertension) (21-23). Postthrombotic syndrome (PTS) affects approximately 30% of patients within 5 years of a DVT episode of the lower extremities (24). PTS is the result of venous hypertension due to valvular damage accompanied by reflux, a non-compliant vein wall, and venous obstruction (25). Therefore, common clinical signs include pain, heaviness, swelling, cramping of the leg, which are usually exacerbated by standing or exercising, and in severe cases, skin ulceration (25). Approximately 1% of adults and 3% to 4% of those over age 65 years once had or now have an ulcer of venous etiology (26). Various risk factors for the

development of VTE have been identified both in animal models and human studies, including: age, gender (male>female), pregnancy, estrogen use, immobilization, surgery, trauma, neoplasia, hypercoagulability, smoking, cardiovascular disease, and obesity (17, 27-34).

4. ADHESION MOLECULES, INFLAMMATION, AND THROMBOSIS

4.1. Selectins

Selectins are glycoproteins that are expressed by leukocytes, activated endothelial cells and platelets. The role of selectins is to mediate the initial adhesion 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 (35), is up-regulated early during thrombosis promoting vein wall inflammation in multiple animal models (36).

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, termed P-selectin glycoprotein ligand-1 (PSGL-1), expressed on the surfaces of most hematopoietic cells (37) (Figure 2). Additionally, P-selectin:PSGL-1 interactions are responsible for thrombus amplification. The PSGL-1 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. P-selectin is up-regulated rapidly and down-regulated within minutes to hours. Additionally, the Src-family tyrosine kinases (SFK) have been shown to lead to thrombus amplification along with P-selectin. This is an intracellular signal that is activated following ligand- α M β 2-integrin binding on the surface of PMNs. This leads to firm adhesion between the PMNs and activated platelets. This interaction was not shown to be required for PSGL-1 mediated recruitment and rolling but was necessary for PMN-platelet adhesion at the site of endothelial injury (38).

We have defined the importance of P-selectin to thrombosis using a mouse model of stasis induced venous thrombosis. In mice, vein wall neutrophils were significantly elevated above sham control animals at day 2 after thrombosis and monocytes were significantly elevated above sham controls at day 6 after thrombosis while total inflammatory cell counts were 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 (36). Mice that are gene deleted in both P-selectin and E-selectin, show statistically decreased thrombus fibrin staining associated with decreased thrombus weights

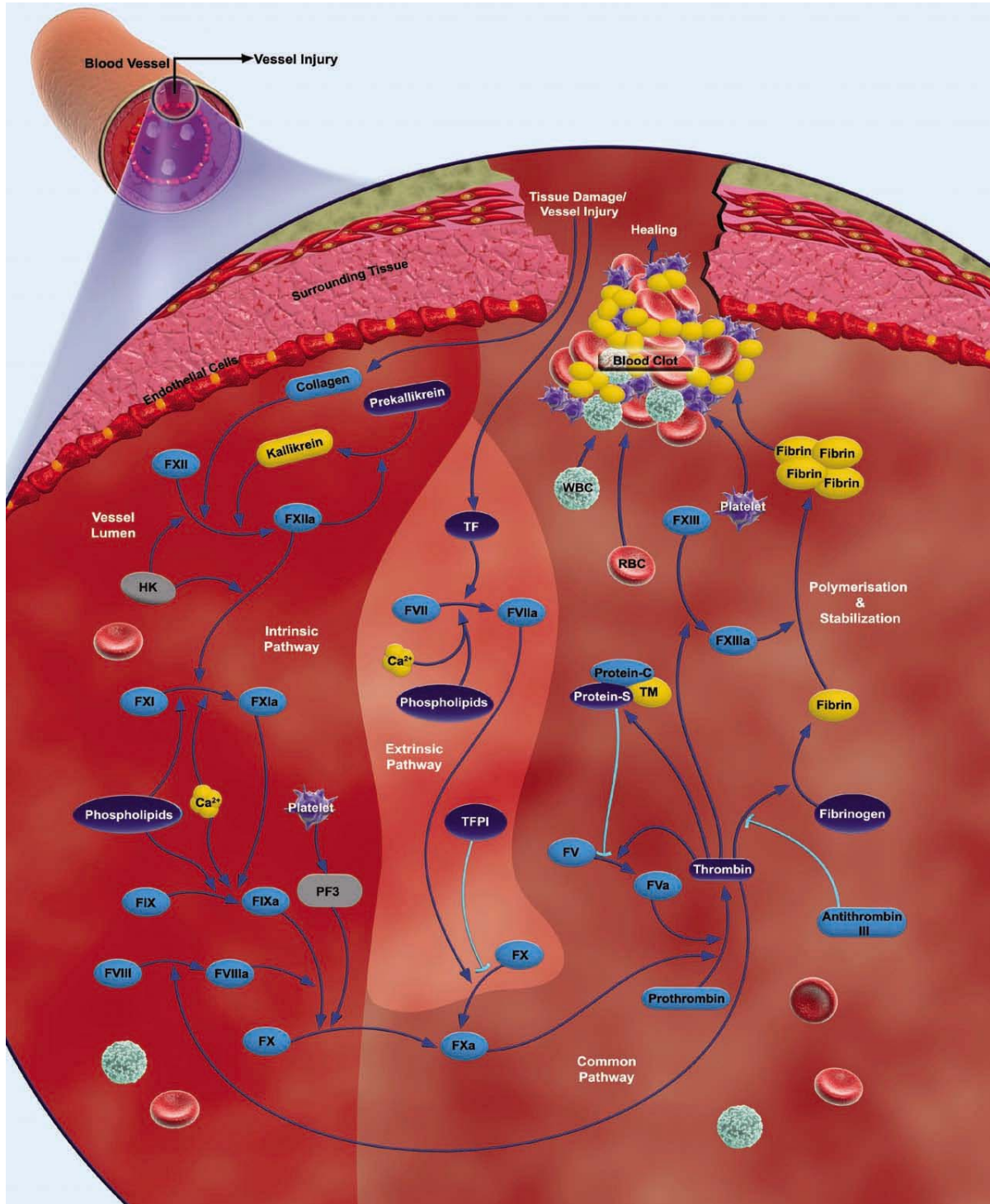


Figure 1. Secondary hemostasis: Coagulation factors that activate both the intrinsic and extrinsic pathways. Reproduced with permission from www.abcam.com, Cambridge, MA.

compared to controls evaluated 2 and 6 days post thrombosis in the same mouse model (37).

Soluble P-selectin (sP-sel) is released from activated platelets and endothelial cells and levels rise significantly during pathologic conditions. Originally, the

function of sP-sel was unclear but now it has been shown to be an endogenous activator of coagulation via generation of plasma microparticles in addition to its ability to bind to PSGL-1 and therefore lead to leukocyte recruitment and rolling (39, 40).

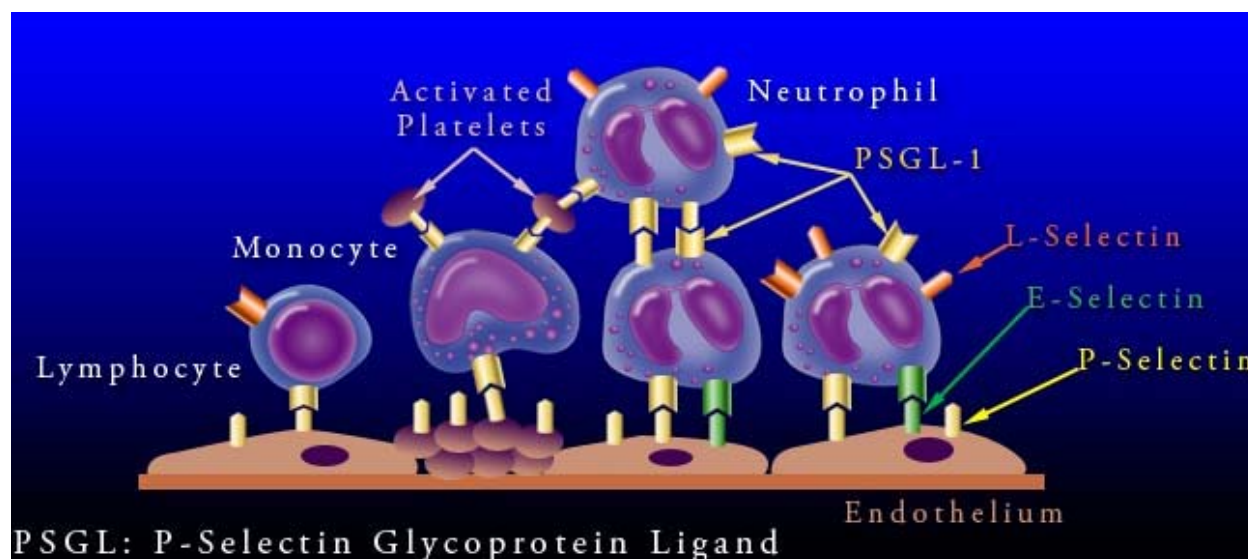


Figure 2. Leukocytes and activated endothelial cells express selectins. They recognize specific carbohydrate sequences and are responsible for the rolling and tethering of leukocytes onto stimulated vascular endothelium. Reproduced with permission from (161).

4.2. P-selectin inhibitors

We evaluated a novel oral P-selectin inhibitor (PSI-697) for its antithrombotic effects in a post two-day murine IVC ligation model. Daily administration of PSI-697 two days prior to ligation until IVC harvest, significantly decreased thrombus weight compared to non-treated IVC ligation controls (41). A follow up study was performed with PSI-697 using a rat stenosis model, which demonstrated its ability to decrease vein wall stiffness, decrease vein wall intimal thickening, and decrease inflammatory cytokine IL-13 compared to enoxaparin treated animals (42). In a similar study, baboons were treated with a single daily dose of PSI-697 three days prior to iliac vein balloon occlusion and daily for six days post occlusion. Animals treated with PSI-697 showed >80% vein lumen opening after complete occlusion compared to no opening in the control group (43). In the same study, PSI-697 significantly decreased venous wall inflammation at day six as determined by magnetic resonance venography compared to control with no change in coagulation function (43). Using the same baboon iliac vein occlusion model, we evaluated another novel P-selectin oral inhibitor, PSI-421, for its antithrombotic effects. Baboons received a daily oral dose two days prior and for six days post iliac vein occlusion. Animals treated with PSI-421 showed significantly greater percent iliac vein wall opening and decreased inflammation compared to controls and enoxaparin treated animals. Furthermore, animals in the PSI-421 group, had microparticle tissue factor activity (MPTA) that was significantly lower at the six hour time point on the day of thrombosis formation (44).

The use of aptamers is a recent therapeutic development that can be used to specifically target selectins. Aptamers are protein-binding oligonucleotides that are highly specific and can be designed to have short or

long half-lives depending upon whether an acute or chronic response is necessary. They have a low risk of toxicity and do not cause an immunologic response when administered (45). GM-1070, a pan-selectin antagonist, and an anti-P-selectin aptamer (ARC5690) have both been shown to effectively inhibit the adhesion of sickle RBCs and leukocytes to endothelial cells in a mouse model of sickle cell disease (46, 47). As this is the same interaction that can lead to thrombus amplification, they appear to be a promising future treatment option for VTE.

A recent meta-analysis comparing P-selectin/PSGL-1 inhibitors, demonstrated that P-selectin antagonism showed a significant difference in reducing venous inflammation or venous re-opening compared to saline controls (48). Anti-thrombotic, vein wall inflammation, and coagulation parameter results were similar to enoxaparin treated groups (48).

4.3. Plasminogen activator inhibitor-1 (PAI-1)

The fibrinolytic system acts as a balance to the coagulation cascade to prevent vascular thrombosis. Plasminogen is activated by the proteolytic cleavage of a single arginine-valine peptide bond to form plasmin by similar serine proteases known as plasminogen activators. Plasmin, a serine protease inhibitor, is the primary enzyme responsible for cleaving fibrin and fibrinogen during the process of fibrinolysis. The end result of this process is formation of fragment E and 2 molecules of fragment D, which exist as a covalently linked dimer (d-dimer) (49). D-dimer levels can be measured in the blood to determine if clot formation and fibrinolysis is actively occurring. It has been shown in human patients that elevated d-dimer levels may be useful as a measure for risk of recurrent VTE (50). In the plasma, PAI-1 is the main inhibitor of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Alterations in PAI-1 concentrations or its

activity may influence coagulation and fibrinolysis processes. We hypothesized that the aging process may influence circulating factors contributing to thrombogenesis and venous inflammation. Using a two-day post mouse IVC ligation model, we demonstrated that older C57BL/6J mice (11-month-old) had significantly greater thrombin mass, leukocyte microparticle fractions, and active plasma plasminogen activator inhibitor-1 (PAI-1) concentrations compared to young C57BL/6J mice (2-month-old) (32). Our results demonstrated the importance of PAI-1 in pathologic venous thrombosis and that further research is needed to characterize its role in venous thrombogenesis.

Studies in humans with acute myocardial infarction that received either rt-PA or streptokinase showed increased PAI-1 levels post-treatment that was more pronounced in the streptokinase group (51). This study also demonstrated a relationship between higher PAI-1 levels and poor patency post-treatment. Therefore, use of a PAI-1 inhibitor at the time of thrombolytic therapy may lead to improved thrombus resolution and a more favorable clinical outcome.

Tiplaxtinin (PAI-039), an oral small molecule inhibitor of PAI-1, has been shown to delay thrombus formation in a canine electrolytic injury model of coronary artery occlusion (52). We were the first group to evaluate PAI-039 for its venous thrombosis inhibitory properties in a rodent IVC stenosis model. PAI-039 was administered orally at both high (10mg/kg) and low (1 mg/kg) daily doses starting 24 hours after stenosis and continued for four days. Results demonstrated a significant 52% (low dose group, $p < 0.05$) and 23% (high dose group, $p < 0.05$) reduction in thrombus weight compared to controls (53). Although enoxaparin treated animals showed similar thrombus weight reductions of 39% compared to controls, coagulation parameters, aPTT and TCT were significantly different than the PAI-039 treated group (53). Results of this study lend credence that PAI-1 inhibition may be a useful therapy for treatment of venous thrombosis with minimal direct effects upon coagulation. Further dose response and efficacy studies are warranted.

4.4. Von willebrand factor

von Willebrand factor (vWF), a glycoprotein, mediates platelet adhesion and stabilizes procoagulant Factor VIII to promote initiation and formation of a stable thrombus at the site of vascular injury (54). As with P-selectin, vWF is stored in Weibel-Palade bodies within vascular endothelium cells (55) and is synthesized in megakaryocytes. Defects in vWF have been linked to the inheritable disorder, von Willebrand's disease (56). In plasma, multimers of vWF ranging from 500 – 20,000 kDa are regulated and cleaved under shear stress into less active multimers by the protease ADAMS13 (57-60). vWF is a ligand for glycoprotein Iba (GPIb) within the GPIb-IX-V complex and integrin α Ib β 3 which mediates platelet adhesion and thrombus formation (57, 61). Using a ferric chloride venous injury model, Chauhan *et al.*, reported that occlusive thrombus formation is dependent upon vWF and not GPIb indicating that vWF uses other adhesion molecules under venous flow conditions (58).

Furthermore, arterial thrombosis appears to be more dependent upon GPIb interactions while venous thrombosis is highly dependent on interactions with vWF (57). A recent *in-vitro* study evaluating the role of platelets in microscopic fibrin formation under coagulating conditions and low shear rates demonstrated that fibrin formation was reduced and delayed when binding of vWF to GPIb-V-IX was blocked or with plasma deficient in vWF (62). Results of these studies are promising and provide evidence that future pharmacologic therapies aimed at modulating vWF activity may be useful for treatment or prevention of venous thrombosis.

5. MEDIATORS OF INFLAMMATION

Inflammation and thrombosis have been shown to interact and have mechanisms in common. P and E-selectin are cell adhesion molecules with critical roles in thrombogenesis. Animal studies using rat and mouse thrombosis models demonstrated upregulation of P-selectin and E-selectin in the vein wall 6 hours and 6 days after thrombus induction, respectively (63).

A study by Wagner *et al.* showed that the increase in the number of P-selectin molecules present on the endothelial cell surface is due to the release from the Weibel-Palade Body (WPB). WPBs are the endothelial specific storage organelle for regulated secretion of von Willebrand factor (vWF) and P-selectin onto its membrane (64). Thus the exocytosis of WPB initiates a rapid translocation of P-selectin to the endothelial surface resulting in augmented endothelial adhesiveness for leukocytes and platelets.

Recently, a pathway linking IL-6, a well-studied inflammatory cytokine, and fibrosis was found in the context of venous thrombosis in a mouse model of DVT (65). The biological effect of neutralizing IL-6 was demonstrated to occur via chemokine ligand 2 (CCL-2) at both the gene expression and protein level at early time points during DVT. These early events lead to significantly decreased fibrosis at later time points in DVT (65).

Other components of the innate immune system have also been shown to play a role in the progression of thrombosis. Complement activation generates the C5b9 complex, which if deposited on cells causes exposure of phosphatidylserine on the surface of cells, thus initiating activation of the coagulation cascade (66). 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 (67).

Certain mediators have demonstrated an anti-inflammatory effect during thrombosis. A study by Henke *et al.* established that IL-10 can modulate the inflammatory response in a rat ligation model of venous thrombosis (68). The rats that received viral IL-10 gene transfer had fewer leukocytes in their vein walls, most notably affected was the number of PMNs. There was also a significant decrease in cell adhesion molecule (CAM) expression but

no effect on local procoagulant activity (specifically TF or vWF). There was a trend toward decreased levels of TNF- α in the transfected rats which is notable as this cytokine serves as a stimulus for CAM up-regulation.

6. TISSUE FACTOR

Tissue factor (TF) is a membrane-bound protein (46-kDa) that triggers thrombin generation by forming a complex with factor VIIa which triggers the activation of the coagulation cascade (69, 70). Tissue factor pathway inhibitor (TFPI) is the endogenous polypeptide that acts to reversibly inhibit formation of both thrombin and factor Xa (71). Therefore this may be investigated as a potential target for treatment of venous thrombosis. 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 (72). It has been hypothesized that the dynamics of thrombus propagation support a role for circulating TF as the diffusion distance from the vessel wall to the luminal surface of the thrombus increases (73). Although the cellular origin of circulating TF is unknown, there is evidence to suggest that leukocytes and leukocyte-derived microparticles are the major source (74). 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 (75). 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*. Furthermore, TF can be transferred from leukocytes to platelets *in vitro* via an interaction involving CD15-expressing membrane microparticles and P-selectin (76). 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 (26, 77-79). Additionally, it is also unknown whether these leukocytes merely transport TF or actually express it (67). 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 not TF from leukocytes was more important for thrombus formation (80). The expression of TF circulating on monocytes has been shown to be important in acute thrombotic events (11, 81). 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 TNF, IL-1, MCP-1, or IL-6 to express TF on their cell surfaces (82, 83).

Currently, there is much debate in the scientific community regarding the exact distribution of TF and which source is most significant in promoting a prothrombotic environment. In fact, both sources may be important and the effect may depend on the nature of the stimulus. Vein wall tissue factor may be most important with vein wall injury or vein wall dilatation, while

leukocyte tissue factor may be most important when there is no vein wall injury. Recent work by Pawlinski *et al.* demonstrated that gene deletion of TF in myeloid cells but not endothelial cells significantly reduced coagulation activation upon induction of endotoxemia in a mouse model (84). Monocytes have been shown to be capable of producing TF and TF has been detected in the α -granules of quiescent platelets (Figure 3) (85, 86). Additional studies will need to be conducted in order to determine the exact role played by each source of TF during episodes of venous thrombosis in order to determine which area would be most beneficial as a therapeutic target.

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 (87, 88), 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 (89). Procoagulant MPs, derived more from activated leukocytes and less from activated platelets produced are recruited to the area of thrombosis (90), where they amplify coagulation via tissue factor and factor VIIa (Figure 4; Figure 5) (37, 39, 76, 85, 88, 91-93). The co-localization of fibrin, platelets, and leukocytes in the developing thrombus (88, 94) and the contribution of P-selectin to leukocyte-platelet interactions to generate tissue factor (95) support their central role of inflammation in thrombogenesis.

Platelet-derived MPs are involved in venous thrombosis in the syndrome of heparin-induced thrombocytopenia (96). Less is known regarding leukocyte-derived MPs, although two studies suggest that these MPs are associated with endothelial cell activation and cytokine gene induction as indicated by elevations in IL-6, MCP-1, TNF, and a factor Xa procoagulant response mediated by JNK1 signaling pathway (97, 98). Microparticles have been found to impair endothelial cell nitric oxide transduction from endothelial cells (99). 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 (100). The procoagulant nature of microparticles induced by P-selectin has been demonstrated in a recent paper by Hrachovinová *et al.*, (35). 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 (35).

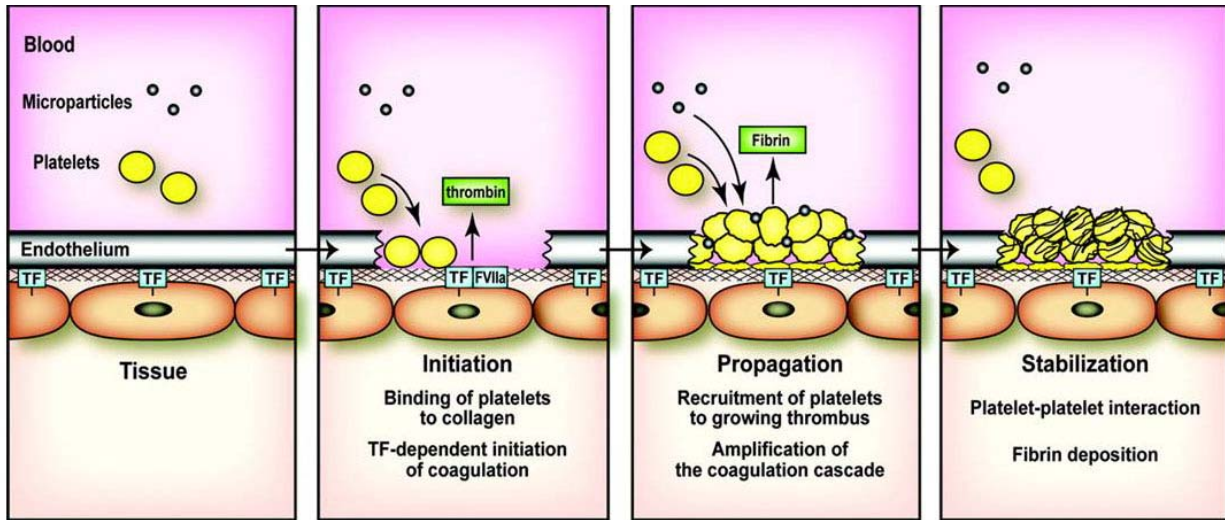


Figure 3. Formation of a clot at the site of blood vessel injury. In a healthy individual, TF expressed by vascular smooth muscle cells, pericytes, and adventitial fibroblasts in the vessel wall is physically separated from its ligand FVII/FVIIa by the endothelium. Vessel injury leads to the rapid binding of platelets to the subendothelium and activation of the coagulation cascade by TF. Propagation of the thrombus involves recruitment of additional platelets and amplification of the coagulation cascade by the intrinsic pathway, and possibly by TF-positive MPs and TF stored in platelets. Finally, fibrin deposition stabilizes the clot. De novo synthesis of TF by platelets may also play a role in stabilization of the clot. Reproduced with permission from (162).

A recent study reported that microparticles from pericardial blood of cardiac surgery patients were found to be highly thrombogenic in a rodent IVC thrombosis model (101). Ramacciotti, *et al.* showed that older microparticles are prothrombotic in a murine IVC model (102). In this study, microparticles were obtained from C57BL/6J

mice at two hours (young MPs) and two days (older MPs) after IVC ligation. When re-injected into wild-type C57BL/6J mice, in which the IVC was previously ligated two days prior, there was a trend towards higher thrombus weights in the mice that received older MPs compared to those that received re-injections of young MPs. Furthermore, tissue factor associated with microparticles showed a significant correlation to total microparticle concentrations ($R=0.99$) (102).

In addition to their pro-thrombotic effects, recent literature demonstrated that microparticles contain surface proteins derived from their parent cell (platelet or leukocyte) that modulate the thrombotic process. Abdullah *et al.* evaluated plasma samples obtained on day two of a baboon IVC ligation model for unique surface proteins associated with microparticles (103). In this study, both fibrinogen gamma-chain isoform 2 and alpha-1-antichymotrypsin were upregulated when compared to baseline (103). The fibrinogen gamma chain contains binding domains, which allow interactions with growth factors, integrins, and platelet aggregation suggesting that these domains may assist in anchoring microparticles to the site of injury (104, 105). Alpha-1-antichymotrypsin, a serine protease inhibitor, may act to inhibit neutrophil adhesion to fibronectin (106, 107).

The role of microparticle associated surface proteins was further elucidated in a complimentary study in human patients. Galectin-3 binding protein precursor

(Gal3BP) and alpha-2 macroglobulin were found to be upregulated in microparticles collected from human patients diagnosed with deep venous thrombosis (108). Gal3BP is a member of the lectin family, associated with integrin mediated cell adhesion (109). A detailed review of galactins and their role in venous thrombogenesis, inflammation, and fibrosis is summarized by Diaz *et al.* (110). Alpha-2 macroglobulin modulates coagulation through inhibition of plasmin, kalikrenin, and thrombin. The results of these studies provide evidence that microparticle associated peptides may be potential biomarkers for venous thrombosis and further research is needed to understand their role in vascular inflammation and thromboogenesis.

Of interest and importance, MPs have also been found to be present in normal healthy individuals and in the normal situation. 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 (111). 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 (112). 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 (113, 114).

8. INFLAMMATION AND ARTERIAL THROMBOSIS

Arterial thrombosis, whether acute or chronic, is initiated by damage to the vascular endothelium, which

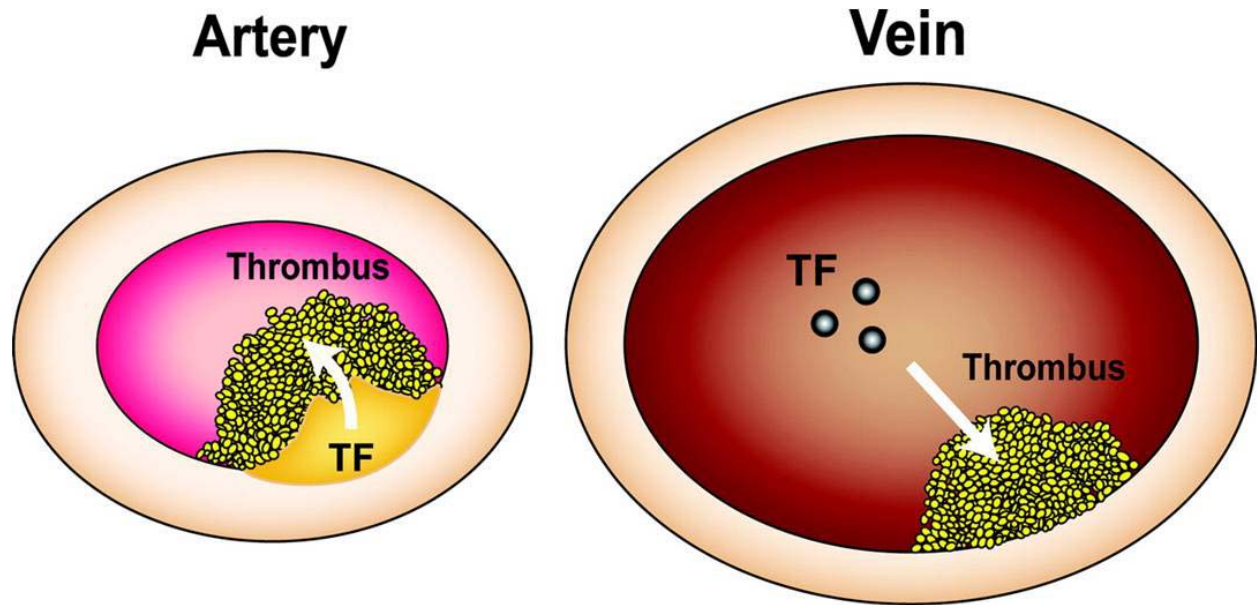


Figure 4. Contribution of vessel wall and MP TF to arterial and venous thrombosis. Arterial thrombosis, particularly after rupture of an atherosclerotic plaque, exposes large amounts of TF to blood and leads to the formation of an occlusive thrombus. The gold area in the arterial wall represents an atherosclerotic plaque. Venous thrombosis is not associated with disruption of the vessel wall. This suggests that MP TF plays a more important role than vessel wall TF in venous thrombosis. Reproduced with permission from (162).

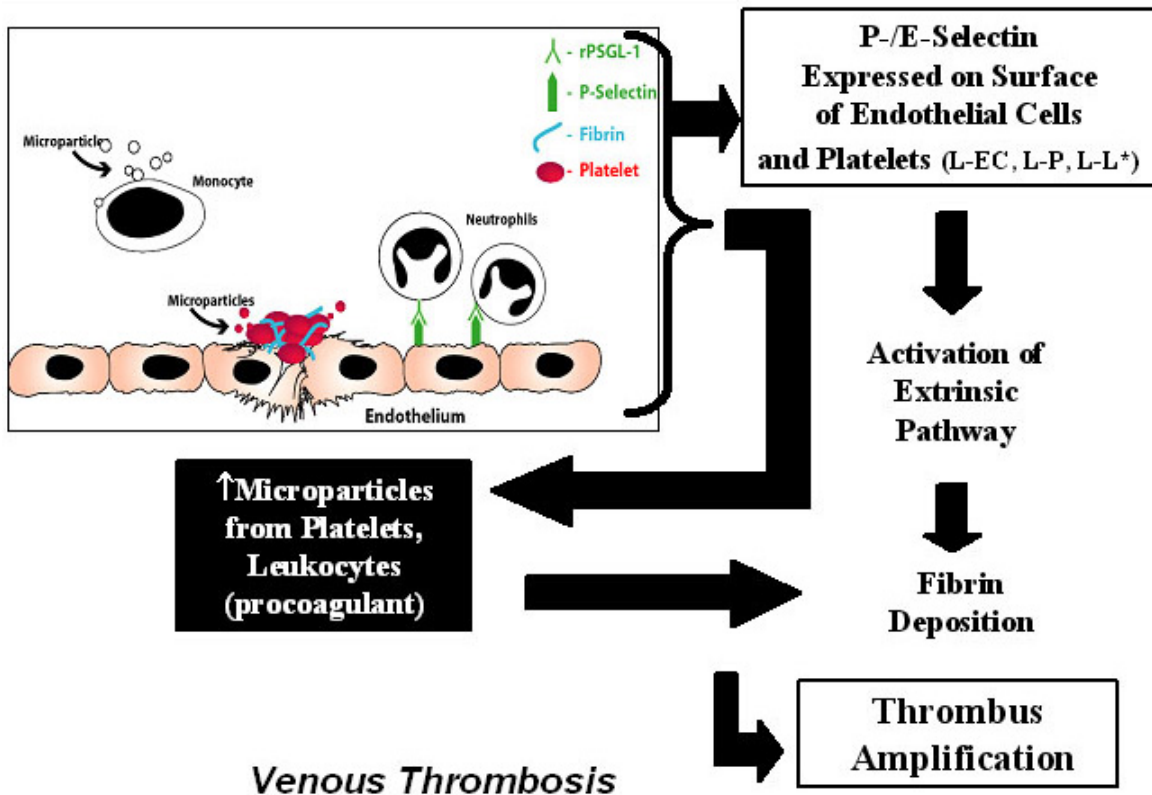


Figure 5. Proposed mechanism of venous thrombosis involving the amplification of thrombus formation by the elaboration of microparticles and the thrombogenic nature of inflammatory cells (especially monocytes). Reproduced with permission from (37).

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) (115). 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 (116).

9. INFLAMMATION AND VENOUS THROMBOSIS

Inflammation and thrombosis are closely linked events. A study using a rat IVC ligation model showed a chronological relationship between thrombosis and inflammation. At day 1 post-ligation, neutrophils were the predominant cell type present in the vein wall while this shifted to predominantly monocytes and lymphocytes as the thrombus matured (day 6) (117). This same study showed a significant increase in cytokine levels (TNF- α , IL-6, MIP1- α , MCP-1) over the 6 day time period while this was not observed in the sham operated controls. Thus it was demonstrated that a significant inflammatory response occurs during venous thrombosis.

Venous thrombosis may result 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 postthrombotic syndrome is lessened. If this does not occur, the thrombus may incompletely resorb, 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 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 (116). Neutropenic cancer patients are not protected from DVT, and multiple neutropenic episodes are significantly

associated with recurrent VTE in patients with malignant disease who require filter placement due to a failure of, or contraindication to, anticoagulation (118). 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).

Neutrophils also seem to be important both for early thrombus resolution in a rat model of thrombosis (119) but not in a mouse model (120). This apparent discrepancy may suggest the heterogeneity in the neutrophil compartment (121).

The presence of monocytes and uPA has found to be important in later thrombus organization and recanalization. Injection of monocyte chemotactic protein-1 into thrombi in a rat DVT model resulted in enhanced thrombus resolution (122). Thrombi have been found to contain increasing amounts of both tPA and uPA activity as they resolve (101) and this activity are expressed by invading monocytes (123). 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 (124, 125). Finally, a pathway between inflammation and fibrosis post-venous thrombosis was recently discovered (65). In this work, neutralizing IL-6 significantly decreased fibrosis *in vivo*, using a mouse model of venous thrombosis. This effect was observed via CCL-2. Thus a pathway involving IL-6, CCL2, monocyte recruitment, and fibrosis was clearly established in the context of venous thrombosis (65). 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.

9.1. Neutrophil extracellular traps (NETs)

Of recent interest is the role of Neutrophil Extracellular Traps (NETs) in acute inflammation. In addition to their phagocytic and bactericidal functions, activated neutrophils have been shown to release chromatin DNA and histone containing granular antimicrobial proteins which form extracellular matrices or traps (126). The mechanisms regarding neutrophil cell death and the formation of NETs appear to be separate from apoptosis and necrosis and appear to rely upon the formation of intracellular reactive oxygen species (127). NETs have been shown to kill bacteria, fungi, and parasites while forming a microbial containment barrier (128, 129). NETs have been reported to form in the vasculature during sepsis and in inflammatory non-infectious disease states such as small-vessel vasculitis (130, 131).

In collaboration with Fuchs *et al.* we demonstrated that neutrophil extracellular DNA traps (NETS) were associated with deep venous thrombosis

when using an experimental baboon model of occlusive balloon induced iliac deep vein thrombosis (132). Plasma DNA samples collected at baseline were significantly lower than plasma DNA samples collected at two and six days post-DVT ($p < 0.01$) (132). Furthermore, the rise in plasma DNA kinetics was similar to our previous findings of plasma D-dimers in the same experimental model (44). In addition to plasma DNA, histologic samples of iliac veins (6 days post-DVT) and controls were stained using an antibody directed towards DNA histone complexes (nuclear origin) and evaluated for the presence of DNA nuclei and extracellular DNA or NETs. Affected vessels demonstrated punctate staining of nuclear DNA and diffuse staining of extracellular DNA compared to controls. Furthermore, immunocolocalization of VWF strings were dispersed within the DNA core and between the DNA core and the vessel wall (132). These are the first reported results demonstrating that markers of NETs were present in both the plasma and thrombus using a baboon model of deep venous thrombosis. These results provide early evidence regarding the interactions and roles of NETs (DNA and histones), vWF, and platelet binding in venous thrombosis.

10. BIOMARKERS FOR DEEP VENOUS THROMBOSIS

Clinical signs and duplex ultrasound are currently utilized to diagnose deep venous thrombosis. At this time there is no single biomarker that has been shown to definitively verify the presence of a venous thromboembolism. D-dimers are a byproduct of fibrin degradation upon activation of the fibrinolytic pathway. Due to their high sensitivity, D-dimer assays have been utilized as an adjunct to current diagnostic therapy to rule out the presence of venous thromboembolism (133, 134). Unlike their high sensitivity (96%) and negative predictive value (95%), D-dimers were shown to have a poor specificity (40%) and low positive predictive value (48%) for deep venous thrombosis compared to venography (135). As a result, D-dimer assays, when used alone, are not likely to replace the current diagnostic standard of duplex ultrasound evaluation to confirm deep venous thrombosis. A logistic regression dichotomous model showed a sensitivity of 73%, specificity of 81%, and accuracy of 77% when D-dimers, P-selectin, and microparticle levels were evaluated simultaneously to distinguish DVT (confirmed by duplex ultrasound) from symptomatic non-DVT patients (negative on duplex ultrasound) (136).

Increased levels of vWF are a known risk factor for VTE (137) and a study by Smith *et al.* showed that the syntaxin binding protein 5 (STXBP5) gene, which is thought to be associated with vWF:Ag levels, was associated with VTE risk in a population of human patients and may be useful in the future as a potential screening tool (138).

C-reactive protein (CRP) is an acute phase protein that has been shown to influence various factors associated with the development of vascular disease, including cytokine levels and leukocyte adhesion (40).

Soluble P-selectin (sP-sel) has been shown to increase microparticle production as well as leukocyte adhesion (39, 40). Finally, a study by Wang *et al.* in a mouse model of endotoxemia revealed that TF-positive microparticle levels were predictive for the risk for the development of disseminated intravascular coagulation, and therefore possibly for other vascular disorders (139). These soluble factors have potential future use as biomarkers for vascular disease but require additional research effort to document their efficacy.

Human patients with stable angina were shown to have up-regulation of platelet P-selectin (CD62P), PM-Agg, and monocyte CD11b and that these markers were correlated with degree of coronary artery stenosis (140). Therefore, further investigation of their predictive role in other vascular diseases is warranted.

Additional biomarkers and proteins such as thrombin, interleukins, and fibrin monomers and their utility as biomarkers for venous thromboembolism are summarized by Barnes *et al.* (141). Another avenue worth exploring is assessing the proportion of total neutrophil-derived microparticles expressing activated Mac-1 (integrin) as a potential biomarker for pathological inflammation and potential VTE occurrence (142).

11. THROMBOLYTICS

Thrombolytic agents work on a different arm of the coagulation cascade than the traditional anticoagulation medications (e.g. warfarin, LMWH). They act on the fibrinolytic system by promoting the activation of plasminogen to plasmin which in turn breaks down fibrin and fibrinogen. The currently available thrombolytic agents include streptokinase, tissue plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA). These medications may be administered systemically or directly at the site of the thrombus via a catheter, although the latter administration route is the most common as systemic administration may not allow therapeutic levels to be reached at the site of the thrombus and may lead to an increased bleeding risk (143).

Catheter directed therapy involves placement of a catheter directly at the site of the thrombus followed by infusion of the thrombolytic agent (144). This allows for thrombus dissolution by both a mechanical and pharmacological means. The use of the procedure can be helpful to prevent postthrombotic syndrome and improve patient outcome, when compared to traditional anticoagulants, as it helps restore vessel patency as well as removing the thrombus from the valves of the vein (145).

A multicenter study evaluated the outcome of catheter-directed thrombolysis using urokinase in patients with documented lower-limb DVT (144). Major bleeding complications occurred in 11% of patients with the most common location being the site of catheter insertion. Marked lysis (50-100%) occurred in 83% of patients with a 59.7% cumulative patency rate at 12 months post-infusion. This study demonstrated the effectiveness of catheter-

directed thrombolysis in a large population of human patients. Another human study evaluated the effectiveness of urokinase infusion via catheter in patients with documented iliofemoral DVT (33). The overall technical success rate was 79% and it was noted that efficacy of thrombolytic therapy decreased as thrombus age increased. Sillesen *et al.* investigated the results of rt-PA infusion via catheter in patients with acute iliofemoral DVT (146). Thrombus lysis occurred in 93% of patients and none of these individuals experienced restenosis by 24 months post-infusion. Only 4% of treated patients developed valvular reflux so it appeared that this therapy was effective in preserving valvular function post-DVT.

Konstantinides *et al.* evaluated outcome after infusion of either rt-PA, streptokinase, or uPA in patients with confirmed pulmonary embolism (PE) (147). Patients that received thrombolytic therapy had a lower mortality rate (4.7% vs. 11.1%) and lower rate of PE recurrence (7.7% vs. 18.7%) than patients who received heparin therapy. However, patients that received thrombolytic therapy did have a higher occurrence of major bleeding episodes (21.9% vs. 7.8%).

These studies demonstrate the efficacy of thrombolytic therapy in dissolution of thrombi along improved clinical outcome in carefully selected patient populations with episodes of DVT or PE.

12. NOVEL MOUSE MODELS OF DEEP VENOUS THROMBOSIS

The mouse possesses unique physiological and genetic characteristics that make it an extremely useful tool to evaluate venous thrombosis. Currently there are no reported mouse models that spontaneously develop deep venous thrombosis, yet several experimental models exist for DVT research including: photochemical (148), stasis (36, 149, 150), electrolytic stasis (151), IVC stenosis (152), and mechanical trauma (153, 154). Day *et al.* provides a review of the current options (155).

The IVC photochemical injury model uses Rose Bengal dye activated by a green light laser (540 nm) for 15 minutes (155, 156). This technique produces a subtle endothelial injury that activates the vascular endothelium, but produces inconsistent thrombosis (authors' personal communication, 9-1-09 DDM).

The majority of our research is performed using the stasis (ligation) mouse model of DVT in which the inferior vena cava is ligated with a non-reactive suture directly below the renal veins to produce complete blood stasis (36, 41, 149, 155, 157). In this model, the IVC yields quantifiable amounts of vein wall tissue and thrombus. In addition, the ligation model can be utilized to evaluate interactions between the vein wall and the occlusive thrombus and to assess the progression from acute to chronic inflammation. The major disadvantage with the stasis model is that the lack of blood flow inhibits the maximal effect of administered systemic therapeutic agents at the site of the thrombus and vein wall.

Venous thrombogenesis can be evaluated in a temporal fashion using a murine femoral vein electrical stimulation thrombus model (151). Thrombosis is induced at the site of the electrode and grows in a sequential fashion. Cooley *et al.* (151) speculated that thrombus formation in their model is the result of either direct electrical injury (resistance heating) or a free radical induced injury to the vein wall. When compared to IVC thrombosis models, the main limitation of this model is the small yield of thrombus and vein wall mass available for protein and gene expression analysis.

In the mouse stenosis model a silk ligature is placed around the IVC and tied down on a piece of 5-0 Prolene® which is then removed to reduce blood flow. This reduction in blood flow when combined with temporary endothelial compression with hemoclips, produces a laminar thrombus allowing the study of cellular kinetics and effects of therapeutic agents (152, 158). Although technically simple, easily reproducible and delivers a large sample, the degree of stenosis is inconsistent with constant variations in thrombi sizes.

The mechanical injury model measures the kinetics of temporal thrombus growth and resolution in the femoral vein. This model uses an external mechanical force to damage the endothelium while fiber optic technology is used to trans-illuminate the vein to visualize and record thrombosis. One disadvantage is that this model only allows studying of early stages of thrombosis. In addition, this model is expensive since specialized optical equipment is required to visualize thrombus generation and resolution (153, 154, 159).

The Electrolytic Inferior Vena Cava Model (EIM) is a new mouse model of DVT recently published by Diaz *et al.* (160). In this model, a 25G stainless-steel needle, attached to a silver coated copper wire (KY-30-1-GRN, Electrospec, Dover, NJ), is inserted into the exposed caudal IVC, and positioned against the anterior wall (anode). Another wire is implanted subcutaneously completing the circuit (cathode). A current of 250 μ Amps over 15 minutes was applied using a Grass S48 square wave stimulator and a constant current unit (Grass Technologies, An Astro-Med, Inc., West Warwick, RI). The direct current results in the formation of toxic products of electrolysis that activates the endothelial surface of the IVC promoting a thrombogenic environment and subsequent thrombus formation. This new model is the first IVC thrombosis mouse model that consistently produces thrombosis in a venous thrombus formation fashion, giving enough amount of sample per mice to study thrombogenesis, thrombus resolution and pharmacologic applications in the field of venous thrombosis. Importantly, our lab has demonstrated that heat does not participate in thrombus formation in the EIM model. This is important information because heat applied to the vein wall will inevitably lead to protein denaturation. Thus, while Cooley's model induces DVT in the femoral vein (151), our EIM model induces DVT formation in the IVC with the expected larger thrombus size, which ultimately allows obtaining larger samples (vein wall and thrombus) for

research assays minimizing the number of animals required per study.

13. 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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