New insights into the mechanisms of venous thrombosis

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Venous thrombosis is a leading cause of morbidity and mortality in industrialized countries, especially in the elderly. Many risk factors have been identified for venous thrombosis that alter blood flow, activate the endothelium, and increase blood coagulation. However, the precise mechanisms that trigger clotting in large veins have not been fully elucidated. The most common site for initiation of the thrombus appears to be the valve pocket sinus, due to its tendency to become hypoxic. Activation of endothelial cells by hypoxia or possibly inflammatory stimuli would lead to surface expression of adhesion receptors that facilitate the binding of circulating leukocytes and microvesicles. Subsequent activation of the leukocytes induces expression of the potent procoagulant protein tissue factor that triggers thrombosis. Understanding the mechanisms of venous thrombosis may lead to the development of new treatments.

Introduction

Thrombosis describes the formation of a clot within a blood vessel that reduces blood flow and may cause infarction of tissues supplied by that vessel. The most common forms of occlusive thrombosis occur in arteries and lead to myocardial infarction and stroke (1). Deep vein thrombosis (DVT) mostly occurs in the legs and is associated with pulmonary embolism (PE); collectively, these are termed venous thromboembolism (VTE) (2). The incidence of VTE in industrialized countries is 1–3 individuals per 1,000 per year (3–8). Importantly, there is a dramatic increase in the risk of VTE above the age of 50, and it reaches as high as 1 in every 100 individuals annually (3). These alarming statistics led the US Senate to designate March as “DVT Awareness Month” in 2005 and the Surgeon General’s call to action to prevent DVT and PE in 2008.

There are many genetic and acquired risk factors that are associated with VTE and recurrent VTE (reviewed in refs. 8–11). Strong genetic risk factors that lead to a hypercoagulable state include deficiencies in the anticoagulants antithrombin, protein C, and protein S. Moderate genetic risk factors include factor V (FV) Leiden, prothrombin G20210A, fibrinogen C10034T and non-type O blood. FV Leiden is present in approximately 5% of people of mixed European descent and is a variant of FV that is resistant to inactivation by activated protein C. Prothrombin G20210A is a single nucleotide polymorphism in the 3′ untranslated region of the prothrombin gene that leads to increased expression. Fibrinogen C10034T is a fibrinogen gamma-chain gene variant that leads to reduced levels of the alternatively spliced form of the fibrinogen gamma-chain that is associated with increased venous thrombosis (8). Finally, individuals with non-type O blood have increased clearance of von Willebrand factor (vWF). Since FVIII circulates in plasma bound to vWF, a reduction in plasma vWF is also associated with reduced levels of FVIII.

Acquired risk factors include age, surgery, obesity, cancer, pregnancy, hormone-based contraceptives, hormone replacement, antiphospholipid syndrome, acute infection, immobilization, paralysis, long-haul travel, smoking, hospitalization, reduced fibrinolysis, and acquired thrombophilia (increased levels of procoagulant factors and/or decreased levels of anticoagulant factors) (12–30). Obesity has a high prevalence in the US and Western countries (15, 25, 29), and one study showed that obesity (body mass index ≥ 30 kg/m²) increased the risk of thrombosis 2 fold (25). Another study analyzed the risk associated with oral contraceptives with or without FV Leiden and found that the incidence of thrombosis was increased 4 fold in individuals taking hormone contraceptives, 7 fold in those with FV Leiden, and 36 fold in individuals with both risk factors (24). This study demonstrated remarkable synergy of these risk factors. A VTE risk scoring model has been established for ambulatory patients with cancer based on 5 parameters (tumor site, leukocyte count, platelet count, body mass index, and either low hemoglobin and/or use of erythropoiesis-stimulating agents) (31). Symptomatic VTE was observed in 0.6% of patients with a score of 0 compared with 6.9% of patients with a score of 3 or higher. A recent study extended this scoring system to include the biomarkers D-dimer and P-selectin and found that patients with the highest score had a cumulative VTE probability after 6 months of 35% compared with a probability of 1% for those patients with the lowest score (32).

Clot formation

A blood clot contains a mixture of platelets and fibrin and in some cases red blood cells (1, 33). Importantly, the etiologies of arterial and venous clots are very different (1). Arterial clots are formed under high shear stress, typically after rupture of an atherosclerotic plaque or other damage to the blood vessel wall (34–36). They are platelet-rich (so called “white clots”) and are generally treated with antiplatelet drugs. In contrast, venous clots form under lower shear stress on the surface of a largely intact endothelium (36–39). They are fibrin-rich (so called “red clots” because they also contain red blood cells) and are treated with anticoagulant drugs.

The blood coagulation cascade can be divided into three parts: the extrinsic, intrinsic, and common pathways (Figure 1 and reviewed in refs. 39–42). Under pathological conditions, tissue
factor (TF) is expressed on circulating leukocytes and possibly activated endothelial cells (40). In addition, TF is present on microvesicles (MVs), which are small membrane vesicles released from activated cells (43–45). These intravascular sources of TF may trigger the formation of venous clots. Recent studies have shown that FXII can be activated by extracellular RNA and polyphosphates and this activation of the intrinsic pathway may also contribute to venous thrombosis (46–49).

The coagulation cascade is regulated at several levels by different anticoagulant pathways (50). TF pathway inhibitor blocks the TF/FVIIa complex, whereas antithrombin inhibits all coagulation proteases, including thrombin (51, 52). Binding of thrombin to thrombomodulin on the surface of endothelial cells changes its substrate specificity from fibrinogen to protein C and therefore plays a key role in shutting down the clotting cascade (53). Binding of protein C to the endothelial cell protein C receptor enhances its conversion to activated protein C, which, in association with its cofactor protein S, cleaves and inactivates both FVa and FVIIIa (54). Importantly, loss of a single anticoagulant pathway leads to embryonic lethality (50). One explanation for this observation is that different tissues use distinct anticoagulant pathways to regulate clotting (50, 55). Clots in blood vessels are removed by proteolytic digestion of fibrin by plasmin (56). Levels of plasmin are regulated by plasminogen activators and inhibitors, particularly plasminogen activator inhibitor 1 (PAI-1) (57). This explains why elevated levels of PAI-1 are associated with thrombosis (8).

Traditionally, VTE is treated with anticoagulant drugs to prevent growth and embolization of the thrombus. Patients initially receive some form of injectable heparin, which acts rapidly, followed by a more prolonged course of an oral vitamin K antagonist (58–60). These drugs have been used for over 50 years. Heparins inhibit FXa and thrombin in an antithrombin-dependent manner, whereas vitamin K antagonists reduce the activity of vitamin K-dependent proteins, including FVIIa, FIXa, FXa, and thrombin. The limitations of these drugs have fueled the search for new anticoagulant therapies. Over the past 5 years, several new oral drugs have been developed, the two most advanced of which are rivaroxaban (Xarelto), which selectively inhibits FXa, and dabigatran etexilate (Pradaxa), which selectively inhibits thrombin (Figure 1 and refs. 61–65). Rivaroxaban was shown to be superior to the low-molecular-weight heparin enoxaparin in reducing VTE in four clinical trials involving total knee and hip replacement (65); in 2011, it was approved by the FDA for thrombosis prophylaxis to reduce the risk of DVT and PE following knee and hip replacement surgery. Dabigatran showed non-inferiority to enoxaparin in 3 out of 4 trials for high-risk orthopedic patients but has not been approved for thrombosis prophylaxis in this population (60).

Mechanisms of venous thrombosis

In the 19th century, the noted physician Virchow proposed a triad of physiological alterations that increase the risk of VTE: changes in blood flow, in the blood itself, and in the endothelial cells lining the blood vessel (66).

Reduced blood flow and stasis

Reduced blood flow and stasis allow the accumulation of procoagulant proteases, such as thrombin, that may overcome the local anticoagulant pathways and induce thrombosis. Reduced blood flow and stasis may explain the increased rate of VTE associated with surgery, hospitalization, paralysis, long-haul travel, cancer, obesity, age, and pregnancy (15, 18–20, 25, 28–30). Interestingly, there is a left-sided predominance of proximal thrombosis during pregnancy that is thought to be due to an acquired compression of the left common iliac vein by the presence of the fetus (67). Similarly, individuals with May-Thurner syndrome suffer from compression of left common iliac vein that increases the risk of DVT (68).

Recently, investigators have developed a new mouse model of venous thrombosis that involves stenosis rather than complete ligation of the inferior vena cava (69–71). In this model, the lumen of the vessel is reduced by 80%–90%, but the procedure does not denude the endothelium; however, the endothelium is activated and releases vWF and P-selectin from Weibel-Palade bodies that capture leukocytes and platelets (69, 70). P-selectin glycoprotein ligand-1 (PSGL-1) that is expressed on leukocytes binds to P-selectin.
The endothelial lining of blood vessels plays a critical role in preventing thrombosis by providing a surface that prevents attachment of cells and proteins required for clotting (75). An intact and healthy endothelium expresses various anticoagulants, such as TF pathway inhibitor, thrombomodulin, endothelial protein C receptor, and heparin-like proteoglycans (76). In addition, endothelial cells express the ectonucleotidase CD39/NTPDase1, which metabolizes the platelet agonist ADP. Finally, endothelial cells release the platelet inhibitors nitric oxide and prostacyclin (75, 77, 78). However, activated endothelial cells downregulate expression of the anticoagulant protein thrombomodulin and upregulate expression of the procoagulant protein TF (79). Activation also leads to the expression of various adhesion molecules on the surface of the endothelium, such as P-selectin, E-selectin, and vWF, that capture leukocytes, platelets, and MVs (80, 81). Hypoxia has been shown to promote the release of vWF from Weibel-Palade bodies in endothelial cells (82).

**Procoagulant changes in the blood**

Thrombophilia describes a disorder in which the blood has a tendency to clot. Thrombophilia can be caused by increases in procoagulant proteins, the presence of variant clotting proteins that are more procoagulant, decreases in anticoagulant proteins, and/or decreased fibrinolysis. In addition to the genetic risk factors described above, age, major surgery, cancer, pregnancy, hormone contraceptives, and obesity also confer susceptibility (3, 7, 13–16, 19–23, 25). Pregnancy produces a transient hormone-induced hypercoagulable state that probably evolved to protect women from hemorrhage at childbirth or in the event of miscarriage (20). During a normal pregnancy, levels of FVII, FVIII, FX, fibrinogen, vWF, and PAI-1 are increased and do not return to baseline until 8 weeks postpartum (21). Similarly, one study analyzed the use of oral hormone contraceptives and found increased levels of FVII, FVIII, FX, prothrombin and fibrinogen (23). Obese individuals have elevated levels of FVIII, FIX, and PAI-1 that likely contribute to the increased risk of VTE (29). Interestingly, monocyte TF expression was found to be increased 1 day after surgery to remove tumors (17). Similarly, TF expression on peripheral blood mononuclear cells has been shown to be increased 1 day after total knee arthroplasty (18). Levels of FVIII and fibrinogen were also increased 2–3 days after surgery (17), which appears to be secondary to inflammation. Importantly, these procoagulant changes in the blood preceded the peak of VTE that was observed 7 days after surgery (19).

**Activation of the endothelium**

The endothelial lining of blood vessels plays a critical role in preventing thrombosis by providing a surface that prevents attachment of cells and proteins required for clotting (75). An intact and healthy endothelium expresses various anticoagulants, such as TF pathway inhibitor, thrombomodulin, endothelial protein C receptor, and heparin-like proteoglycans (76). In addition, endothelial cells express the ectonucleotidase CD39/NTPDase1, which metabolizes the platelet agonist ADP. Finally, endothelial cells release the platelet inhibitors nitric oxide and prostacyclin (75, 77, 78). However, activated endothelial cells downregulate expression of the anticoagulant protein thrombomodulin and upregulate expression of the procoagulant protein TF (79). Activation also leads to the expression of various adhesion molecules on the surface of the endothelium, such as P-selectin, E-selectin, and vWF, that capture leukocytes, platelets, and MVs (80, 81). Hypoxia has been shown to promote the release of vWF from Weibel-Palade bodies in endothelial cells (82).

**Valve pockets as a site of thrombus initiation**

Blood is returned from the venous system of the lower limbs to the heart by the calf muscles in the legs acting as pumps. In addition, valves in the large veins prevent reflux of the blood. However, the valve sinus is prone to thrombosis because of the irregular patterns of blood flow and the potential for a low oxygen tension, especially during immobilization or long-haul travel (74). Experimental stasis has been shown to result in a significant decline in oxygen tension in the sinus (83). Interestingly, the number of valves in individuals can vary, and those with more valves have a higher frequency of DVT (84). Finally, studies that analyzed non-trauma-related venous thrombi by autopsy and phlebography concluded that they originated in the valves and sinuses of the calf veins (74). To protect against thrombosis, endothelial cells lining the valve sinus express higher levels of the anticoagulant proteins thrombomodulin and endothelial cell protein C receptor and lower levels of vWF compared with those of venous endothelial cells (85). My group believes that this protective pathway becomes overwhelmed under pathological conditions.

**TF as a trigger for venous thrombosis**

At present, the triggers for venous thrombosis are unknown. However, it is tempting to speculate that the potent procoagulant TF...
plays a key role in some forms of VTE because under pathological conditions it is present on circulating monocytes, MVs, and possibly activated endothelium (40). Activated monocytes and tumor cells are the primary sources of TF-positive MVs in the circulation (43). One may propose that the first step in venous thrombosis is activation of the endothelium and expression of the adhesion receptors P-selectin and E-selectin as well as vWF (Figure 2). The activated endothelium then captures circulating leukocytes, TF-positive MVs, and platelets. Finally, induction of TF expressed by the bound leukocytes together with TF on MVs triggers thrombosis. This hypothesized sequence of events is supported by recent studies using a mouse inferior cava stenosis model (70). It was found that a genetic deficiency of TF in either hematopoietic cells or myeloid cells dramatically reduced venous thrombosis, which indicates that TF expression by leukocytes and possibly leukocyte-derived MVs initiated thrombosis in this model (70). Monocytes—and to a lesser extent neutrophils—in the thrombus expressed TF. This study also demonstrated a role for FXII and platelets in the propagation of the thrombus (70).

Importantly, major surgery is associated with an induction of TF expression by circulating monocytes (18). In addition, studies have shown that patients with cancer and mice containing tumors have high levels of tumor-derived, TF-positive MVs (86–91). One study demonstrated binding of tumor-derived MVs to an injured blood vessel and increased thrombosis in mice with tumors (92). In addition, levels of TF-positive MVs increased prior to VTE in two patients with pancreatic cancer in a small prospective study (93). However, TF is not the only factor that may trigger thrombosis; recent studies have also shown roles for vWF, platelets, extracellular chromatin from neutrophils, and even red blood cells in venous thrombosis in animal models (Figure 2 and refs. 69, 70, 94–96).

**Receptor-mediated binding of leukocytes and MVs to activated endothelium**

P-selectin appears to be a key endothelial cell receptor that captures circulating leukocytes and leukocyte-derived MVs expressing PSGL-1 (Figure 2) (72). In a mouse microvascular thrombosis model, docking of leukocyte-derived MVs to the site of thrombus was shown to require P-selectin, and thrombosis was reduced in mice deficient in either P-selectin or PSGL-1 (33, 97). Hematopoietic cell–derived, TF-positive MVs have been shown to play an important role in this microvascular thrombosis model (98). Importantly, inhibition of platelet P-selectin also blocked the recruitment of leukocytes and reduced fibrin deposition in a baboon model of thrombosis (99). More recently, it was shown that an oral P-selectin inhibitor reduced thrombosis in a baboon stasis model (100). Inhibition of P-selectin also reduced thrombosis in tumor-bearing mice (93). A pan-selectin inhibitor that has primary activity against E-selectin reduced thrombosis in an electrolytic inferior vena cava mouse model (101). In addition, neutrophils promote thrombosis by releasing serine proteases that inactivate the anticoagulant TF pathway inhibitor (102). These studies suggest that blocking the binding of leukocytes and MVs to the activated endothelium may represent a novel strategy to reduce VTE. However, it is important to note that leukocytes also play a role in the resolution of venous thrombi, which may limit this therapeutic approach to prevention rather than treatment of venous thrombosis (103, 104).

**Statins and VTE**

Statins are commonly used to treat hyperlipidemic patients and reduce the incidence of arterial thrombosis. Interestingly, recent studies found that statins also reduce VTE (105–108). A meta-analysis of 8 observational studies concluded that statins reduce the risk of VTE but cautioned that additional randomized controlled trials should be performed (109). One study randomized a group of over 17,000 healthy men and women with normal low-density lipoprotein cholesterol levels but high inflammation and treated them with rosuvastatin or placebo (110). The statin group exhibited a 43% reduction in the rate of VTE compared with that of the control group. At present, the mechanism by which statins reduce VTE is unclear, but the authors speculated that one mechanism may be the reduction of monocyte TF expression. Indeed, statins have been shown to inhibit TF expression in monocytes in vitro and in vivo (111–115). My group recently found that simvastatin reduced peripheral blood mononuclear cell TF expression and TF-positive MVs in hyperlipidemic monkeys, without affecting plasma cholesterol levels (115). Taken together, these results suggest that the anticoagulant activity of statins is mediated, in part, by their ability to inhibit monocyte TF expression.

**Conclusions and future studies**

Changes in blood flow, in the blood itself, and in the endothelium all increase the risk of VTE. New studies indicate roles for leukocytes, platelets, and MVs in the initiation and propagation of the thrombus and suggest that inhibition of the binding of leukocytes and MVs to the activated endothelium may represent a new therapeutic strategy to reduce the risk of VTE. These approaches, along further study of the antithrombotic activity of statins, suggest that improved therapies for this common disease may soon be available.

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