Neutrophil Extracellular Trap Impact on Deep Vein Thrombosis

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Abstract—Deep vein thrombosis (DVT) is a major health problem that requires improved prophylaxis and treatment. Inflammatory conditions such as infection, cancer, and autoimmune diseases are risk factors for DVT. We and others have recently shown that extracellular DNA fibers produced in inflammation and known as neutrophil extracellular traps (NETs) contribute to experimental DVT. NETs stimulate thrombus formation and coagulation and are abundant in thrombi in animal models of DVT. It appears that, in addition to fibrin and von Willebrand factor, NETs represent a third thrombus scaffold. Here, we review how NETs stimulate thrombosis and discuss known and potential interactions of NETs with endothelium, platelets, red blood cells, and coagulation factors and how NETs could influence thrombolysis. We propose that drugs that inhibit NET formation or facilitate NET degradation may prevent or treat DVT. (Arterioscler Thromb Vasc Biol. 2012;32:0-0.)

Key Words: deep vein thrombosis • deoxyribonuclease • inflammation • mouse model • neutrophil extracellular traps

Deep Vein Thrombosis: A Major Health Problem

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eep vein thrombosis (DVT) is a debilitating disease that may be complicated by pulmonary embolism (PE). Together DVT and PE are designated as venous thromboembolism. In the United States, venous thromboembolism develops in an estimated 900,000 patients each year, and PE is responsible for ≈300,000 deaths, which exceeds the mortality from myocardial infarction or stroke. DVT complications, in addition to PE, include postthrombotic syndrome caused by chronic venous stasis even in the absence of active thrombosis. DVT prophylaxis and treatment include anticoagulation by heparin, vitamin K antagonists, and thrombin and Factor Xa inhibitors. Surgical intervention for DVT treatment includes thrombectomy or catheter-based thrombolysis using urokinase, streptokinase, or tissue plasminogen activator (tPA). The success rate of pharmacological catheter-directed thrombolysis ranges from 59% to 100%. Morbidity from venous thromboembolism has not substantially changed within the last 2 decades, and contemporary prophylaxis is not always efficient.

DVT is epidemiologically associated with inflammatory diseases, such as infection, autoimmune disorders, and cancer. Acute infections10,11 and autoantibodies against prothrombin12 and phospholipids12 are risk factors for DVT and PE. Coincidence of venous thrombosis and malignancy was documented in the ranges from 59% to 100%. Morbidity from venous thromboembolism even in the absence of overt endothelial denudation20 and can be prevented by low molecular weight heparin.17 In contrast to arterial thrombosis, which usually results from atherosclerotic plaque erosion or rupture, the mechanisms of DVT development in intact vessels are poorly understood.

Venous thrombus development in humans often starts in the valve sinus where, because of complex fluid mechanics in the valve pockets, the blood flow pattern becomes abnormal leading to endothelial dysfunction. Stagnant blood flow (stasis) in the linear section of the blood vessel may have similar consequences.21,22 Stasis leads to hypoxia,23 which in turn may contribute to thrombogenesis and locally activate endothelium. We have recently demonstrated that 6 hours after inferior vena cava stenosis in mice, von Willebrand factor (VWF) is released.
from Weibel-Palade bodies and mediates platelet recruitment to endothelium (Figure 1A).19 This represents a key step in the initiation of DVT because neither VWF-deficient nor platelet-depleted mice form a thrombus.17,19 Lack of glycoprotein Ibα, a platelet receptor for VWF, prevents murine venous thrombosis17 and corroborates the importance of platelet adhesion in DVT. In addition to platelets, neutrophils are crucial for experimental DVT.16,17,24 P-selectin, exposed on activated endothelium or platelets, mediates initial recruitment of neutrophils (Figure 1A). Depletion of neutrophils or deficiency in P-selectin prevents DVT in mice.17 In baboons, pharmacological inhibition of P-selectin accelerated thrombolysis and restoration of blood flow in thrombosed veins.25

Hypercoagulability of blood is another important mechanism which contributes to DVT. Tissue factor (TF) is considered as the central coagulation-triggering molecule in DVT26 (Figure 1A and 1B). TF initiates the extrinsic coagulation pathway and is released in inflammation on microparticles originating from activated leukocytes. In murine DVT, flow restriction–induced fibrinogen deposition in the inferior vena cava of transgenic mice expressing low levels of human TF (<1%) and no mouse TF was substantially impaired in comparison with mice expressing normal levels of human TF.17 This finding indicates that TF is indispensable for thrombus growth. Another potential coagulation-dependent mechanism playing a role in DVT is that blood pooled in large veins (with relatively low endothelial surface-to-blood volume ratio) cannot be efficiently anticoagulated by the vessel wall because it would be in microcirculation where surface-to-volume ratio is much higher.27 Thus, the mechanisms of flow disturbance–triggered DVT involve endothelial activation, procoagulant shift in blood, and recruitment of platelets and leukocytes. We and others have recently uncovered that neutrophils are recruited and release neutrophil extracellular traps (NETs) in experimental DVT (Figure 1B). NETs may be a new target for therapeutic development, and their implications for DVT will be the main topic of this review.

Neutrophil Extracellular Traps
Neutrophils are the first leukocytes recruited to sites of infection, where they phagocytose invading bacteria.28 Within the phagosome, microbes are killed by locally high concentrations of antimicrobial proteins and reactive oxygen species (ROS). NETs are produced to allow neutrophils to trap and disarm microbes in the extracellular environment.29 NETs are scaffolds of intact chromatin fibers with antimicrobial proteins, ideal to retain large quantities of microbes (Figure 2A). Therefore, some pathogenic bacteria have evolved to express an extracellular deoxyribonuclease (DNase), which dismantles NETs and promotes virulence.30,31 Extracellular traps are formed in humans, animals, and even plants,32 indicating that NETs provide an evolutionary conserved protective mechanism. Indeed, extracellular trap formation is not restricted to neutrophils,33–35 and different cell types use different cellular mechanisms to release extracellular trap. One mechanism used by human neutrophils is NETosis.36 NETosis is a multistep cell death program (Figure 2C). On activation, certain enzymes translocate from the granules to the nucleus. Histones are degraded by neutrophil elastase (NE)17 and citrullinated by peptidylarginine deiminase 438 to unwind chromatin. Further hallmarks are the breakdown of granular and nuclear membranes and cytolyis as the final step in NETosis.36
NETosis involves signaling pathways which lead to ROS production and upregulation of antiapoptotic proteins. It is distinct from apoptosis, where nuclear condensation and DNA fragmentation occur, and necrosis, where the plasma membrane breaks before the nuclear envelope. In vitro, the kinetics of NETosis varies from <30 to 240 minutes, likely depending on the type and concentration of the stimulus, isolation procedure of neutrophils, and sensitivity of the detection method. Alternatively, NETs may be released from viable neutrophils by ejecting mitochondrial DNA, nuclear contents, or DNA-containing vesicles, but the underlying signaling pathways of these processes are not well defined.

NETs Link Innate Immunity With Thrombosis

NETs provide a new link between innate immunity and thrombosis. NETs stimulate platelet adhesion (Figure 2B) and coagulation and are abundant in experimental deep vein thrombi in baboons and mice, where they colocalize with VWF. Treatment of mice with DNase1 prevents thrombus formation underscoring the importance of NETs for DVT. NETs interact with endothelium, platelets, and coagulation factors and may be able to influence thrombolysis.

NETs and Endothelium

Activation of endothelium and Weibel-Palade release play a crucial role in the initiation of DVT. Cocultures of activated endothelial cells and neutrophils promote NET formation, which is dependent on platelets* or interleukin-8 and ROS released from endothelium. NETs in turn induce endothelial cell death, an effect likely mediated by NET-associated proteases or cationic proteins, such as defensins and histones. Histones display a high affinity for phospholipids, and their binding to membranes results in pore formation and an influx of ions. Interactions of histones with endothelium could promote thrombosis by exacerbating endothelial activation and Weibel-Palade release through an increase of intracellular calcium levels. We have observed that plasma VWF increases, and DVT is aggravated in mice infused with purified histones.

NETs and Platelets

NETs can also contribute to thrombus formation through interaction with platelets (Figures 1B and 2B). NETs are large structures and may promote thrombus stability similarly to VWF and fibrin. When perfused with blood, NETs bind platelets and support their aggregation indicating that they are substrates for platelet adhesion and also provide a stimulus for platelet activation. Platelets may bind to NETs directly and indirectly. Purified histones associate with the platelet surface in vitro, presumably via electrostatic interactions with phospholipids or carbohydrates or via toll-like receptors on platelets. Platelets also bind double- and single-stranded DNA in vitro. Interestingly, platelets from patients with systemic lupus erythematosus have immune complexes of DNA and anti-DNA antibodies on their surface, which can be released by incubation with DNase. NET degradation is impaired in patients with systemic lupus erythematosus attributable to a reduced DNase1 activity in serum, and future studies may address whether the inability to degrade NETs correlates with the increased risk for venous thrombosis in these patients. Platelet–NET interactions could also be mediated by adhesion molecules, such as VWF, fibrinogen, or fibronectin. These molecules bind to NETs presumably because of their affinity for histones or DNA. Activation of platelets by NETs might be triggered by histones or neutrophil proteases in NETs. Purified histones stimulate influx of calcium into platelets and promote activation and aggregation in vitro. When infused into mice, histones colocalize with platelets and
induce thrombocytopenia and thrombosis.\textsuperscript{16,51,54} NETs contain enzymatically active NE and cathepsin G,\textsuperscript{29} and these proteases potentiate platelet aggregation through proteolytically activating platelet receptors.\textsuperscript{65,66} Interactions of NETs with platelets may result in a vicious cycle of NET formation and platelet activation, because platelets prestimulated with lipopolysaccharide or collagen trigger neutrophils to release NETs.\textsuperscript{43,45}

**NETs and RBCs**

Red thrombi are typical for DVT. But unlike platelets, the role of RBCs in thrombus formation is not well defined, and they are frequently considered passively entrapped. However, RBCs may promote coagulation by exposing phosphatidylserine and altering blood viscosity.\textsuperscript{57} We found that in addition to platelets, RBCs avidly bind to NETs after perfusion of whole blood.\textsuperscript{15} Activated neutrophils or platelets can also recruit RBCs at low venous shear in vitro.\textsuperscript{68} Similar to platelets, RBC may interact with NETs directly or indirectly. DNA was eluted from the surface of isolated RBCs from cancer patients\textsuperscript{69} indicating that RBCs can bind DNA. Interestingly, in experimental DVT in mice, NETs are predominantly found in the red, RBC-rich part of the thrombus,\textsuperscript{16} suggesting that NETs could be important for RBC recruitment to venous thrombi (Figure 1B).

**NETs and Coagulation**

Fibrin is abundantly present in venous thrombi. In vitro, NETs stimulate fibrin formation and deposition, and fibrin colocalizes with NETs in blood clots.\textsuperscript{15,17,45} NETs stimulate the extrinsic and intrinsic coagulation pathways.\textsuperscript{15} NE is known to cleave TF pathway inhibitor and enhance Factor Xa activity.\textsuperscript{70} NETs contain NE and bind TF pathway inhibitor and therefore facilitate proteolytic inactivation of TF pathway inhibitor by NE.\textsuperscript{45} NETs also bind Factor XII and stimulate fibrin formation via the intrinsic coagulation pathway.\textsuperscript{71} DNA and histones in NETs may play an important role in stimulating coagulation as well. Nucleic acids enhance the activity of coagulation serine proteases,\textsuperscript{71} and histones promote coagulation indirectly by activating platelets and stimulating release of procoagulant polyphosphates from platelet granules.\textsuperscript{56,72} In addition, histones inhibit antiglucans in plasma. Histones interact with thrombomodulin and protein C and inhibit thrombomodulin-mediated protein C activation.\textsuperscript{73} As a consequence, histones dose dependently increase plasma thrombin generation in vitro. Histones in NETs may exhibit similar functions and thus promote fibrin deposition in DVT (Figure 1B).

**Implications of NETs in Thrombolysis**

To degrade and solubilize thrombi to restore blood flow, fibrin and VWF as the main scaffolds need to be proteolytically fragmented by the proteases plasmin and a disintegrin and metalloproteinase with a thrombospondin domain 1 motif, member 13, respectively. NETs are newly recognized third scaffolds that need to be undone during thrombolysis (Figure 1C).

NETs were seen to colocalize with fibrin in clots\textsuperscript{15} and with VWF in venous thrombi.\textsuperscript{15,16} In vitro, we could show that NETs provide a scaffold for blood clots that is resistant to tPA-induced thrombolysis.\textsuperscript{15} We incubated recalcified blood with neutrophils which were prestimulated to release NETs. As shown in Figure 3, after filtration, blood clots appeared in control samples and tPA- or DNase-treated blood but not in blood treated with the combination of tPA and DNase (Figure 3A). Immunostainings revealed that in the presence of tPA, blood clots lacked fibrin and were held together by a scaffold of extracellular DNA (Figure 3B).

DNase1 is the predominant nuclease in plasma. Interestingly, the plasminogen system cooperates with DNase1 during chromatin degradation. DNase1 has only limited activity to degrade chromatin because it preferentially degrades protein-free DNA. Plasminogen, activated by either tPA or urokinase-type plasminogen activator, degrades histones and therefore allows for degradation of DNA by DNase1.\textsuperscript{74} Monocytes/macrophages may also support the DNA degradation because their lysosomes contain DNase2, which is important for the removal of apoptotic cells (Figure 1C).\textsuperscript{75} NETs and fibrin degradation by plasmin and DNase could result in the simultaneous release of DNA and fibrin fragments. In baboon DVT, plasma DNA increases\textsuperscript{15} with similar kinetics to the fibrin degradation product D-dimers.\textsuperscript{76} Recently, in collaboration with Thomas Wakefield’s group, we found increased levels of DNA in plasma from patients with DVT compared with healthy controls and symptomatic patients who did not have DVT. Here also, plasma DNA concentrations correlated with D-dimers (unpublished data; Diaz and Fuchs, 2012). Therefore, it is plausible that circulating DNA may reflect the degradation of NETs within a thrombus.

**Figure 3. Neutrophil extracellular traps (NETs) are tissue plasminogen activator (tPA)-resistant scaffolds of blood clots.**

A, Photographs of blood clots. Citrated blood is mixed with neutrophils, which are prestimulated to release NETs. Blood is supplemented with deoxyribonuclease (DNase) and tPA as indicated, and clotting is induced by recalcification. Controls (Ctrl) do not receive DNase or tPA. tPA in combination with DNase dissolves blood clot.

Bar: 1 cm. B, Fluorescence images of DNA (light blue), fibrinogen (green), and von Willebrand factor (VWF; red) in blood clots shown in A. Extracellular DNA colocalizes with fibrin in untreated blood clots (Ctrl). DNase degrades extracellular DNA but not nuclei and fibrin in blood clots. tPA induces degradation of fibrin but not extracellular DNA. The remaining extracellular DNA scaffold is sufficient to retain RBCs and platelets (indicated by punctuate VWF staining). In this in vitro experiment, ultralarge VWF multimers were not present, and their role in the thrombus scaffold could not be evaluated. Bar: 50 μm. Reproduced and modified from Fuchs et al.\textsuperscript{15}
NETs may also promote thrombolysis. In vitro studies have shown that NE and cathepsin G can degrade fibrin,77 and these proteases are present on NETs and could enhance fibrinolysis in DVT. In addition, NETs may also recruit plasminogen from the plasma. Histone H2B can serve as a receptor for plasminogen on the surface of human monocytes/macrophages32 and perhaps could do so in NETs.

**NETs in DVT: Questions and Challenges for the Future**

Now that a new polymeric scaffold, NETs, has been identified in thrombi of deep veins,15 what should be explored to make the best use of this observation to progress toward the generation of new approaches to prevention and treatment of DVT? First, it will be important to learn more about what triggers NET formation in DVT. If the trigger(s) was identified, their generation could be inhibited. The trigger could be the interaction of neutrophils with activated cells, such as platelets or endothelium.33,35,46 This could be confirmed if the neutrophil surface receptors involved were identified and their inhibition tested in DVT. Alternatively, environmental factors such as hypoxia, ROS, cytokines, or possibly coagulation proteases generated early in thrombosis could induce NETosis. In vitro, ROS is a common denominator of extracellular trap formation used by different types of leukocytes.34–36 Platelets may potentiate ROS production in platelet-neutrophil complexes,37 and endothelial ROS was shown to trigger NET formation by neutrophils in vitro.46 Protease-activated receptors are present in neutrophils80 but whether or how they respond in thrombosis is an open question. Perhaps, studies should move from purified neutrophils to more complex in vitro systems, which may better model DVT and involve endothelium, platelets, and neutrophils under hypoxic and other experimental conditions.

After the neutrophil is triggered to NETosis, much needs to be clarified about the cell biology of NETs formation. Again, if the cellular processes leading to nuclear dissolution and NET ejection were elucidated, their inhibition in DVT could be envisioned. At least 2 enzymes, NE37 and peptidylarginine deiminase 438 are implicated in chromatin decondensation and NET generation. Their sequence of action, substrates, and processes through which they access the nucleus are not clear. The inhibition effects of these enzymes on DVT should be evaluated using available inhibitors and knockout mice. Whether the final discharge of NETs (Figure 2C) from the neutrophil is produced by cellular lysis36 or a secretory process42,43 that could be inhibited is also an important question.

The time of onset and duration of NET formation in venous thrombogenesis need to be determined to better understand their function in the process. The challenge will be to capture single neutrophils forming NETs in DVT by using intravital microscopy in mice. If NETs are produced rapidly after flow restriction, they may facilitate thrombus initiation. This is supported by the absence of visible thrombi in the most DNase-treated mice.16,17 The observation that NETs are located at the interface between thrombus and vascular wall15 may suggest that they help anchor the thrombus to the vessel. NETs were observed in mature thrombi, but whether these were remnants or whether NETs are formed continuously during venous thrombosis is not known. In an aged thrombus, NETs may participate in thrombus remodeling, for example by recruiting endothelial progenitor cells for neovessel formation. Whether NETs may recruit TF-containing microparticles that are important components of DVT is also not known.

An important challenge will be to determine the effect of NET on thrombolysis. In vitro and in vivo observations indicate that chromatin, fibrin, and VWF form a colocalized network within the thrombus that is similar to extracellular matrix.15–17 It is likely that each of the components will need to be cleaved by their own appropriate enzyme (Figure 1C) and also that the presence of one component may influence the degradation or stability of the other. Fibrin is cross-linked/stabilized by Factor XIII transglutaminase. Whether NET components provide a substrate(s) to Factor XIII and could be cross-linked to fibrin is not known. Peptidylarginine deiminase 4 is eventually secreted from neutrophils during NET formation and was shown to citrullinate fibrin in rheumatoid arthritis.81 Whether this occurs in DVT and how it may affect fibrin degradation by plasmin is unknown. As part of thrombolysis, monocytes are recruited to the thrombus. Interestingly, they are equipped to degrade fibrin and DNA.75,76 Whether NETs promote or interfere with monocyte recruitment to the thrombus has not been investigated.

In summary, in vitro and in vivo observations indicate that NETs could influence initiation, growth, and resolution of DVT. NETs may nucleate thrombus formation by enabling locally high concentrations of platelets, RBCs, and coagulation factors. NETs together with fibrin and VWF could cooperatively provide thrombus stability. Future studies should determine whether NETs formation in DVT can be prevented and most importantly from a clinical perspective whether DNase could be developed as a new, hopefully safer, therapeutic drug for thrombolysis.

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**Disclosures**

None.

**References**


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