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Recently Identified Factors that Regulate Hemostasis and Thrombosis

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Abstract

The blood coagulation cascade is essential for hemostasis but excessive activation can cause thrombosis. Importantly, recent studies have identified factors that contribute to thrombosis but not hemostasis. These include factor XII (FXII), tissue factor-positive microparticles (MPs) and neutrophil extracellular traps (NETs). Recent studies have shown that FXII plays a role in thrombosis but not hemostasis. FXII is activated in vivo by a variety of negatively-charged polyphosphates, which include extracellular RNA, DNA and inorganic polyphosphate (PolyP) that are released during cell damage and infection. These findings have triggered the development of nucleic acid-binding polymers as a new class of anticoagulant drug. Other studies have analyzed the role of MPs in experimental thrombosis. MPs are small membrane vesicles released from activated or apoptotic cells. We and others have found that tissue factor-positive MPs enhance thrombosis in mouse models and are elevated in the plasma of pancreatic cancer patients. Finally, NETs have been shown to contribute to experimental venous thrombosis in mouse models and are present in human thrombi. NETs are composed of chromatin fibers that are released from neutrophils undergoing cell death. NETs can capture platelets and increase fibrin deposition. The recent advances in our understanding of the factors contributing to thrombosis in animal models provide new opportunities for the development of safer anticoagulant drugs.

This review will discuss recent advances in our understanding of the role of inorganic polyphosphates (PolyP) in hemostasis and thrombosis, and factors that contribute to thrombosis but not hemostasis. We will summarize recent studies on three new players: FXII, tissue factor (TF)-positive microparticles (MPs) and neutrophil extracellular traps (NETs).

The coagulation protease cascade

The coagulation cascade is essential for hemostasis and has been studied for many years. However, we still have a limited number of drugs that are used clinically to prevent and treat

thrombosis. Importantly, all of these drugs are associated with bleeding side effects because they target key proteases in the coagulation cascade. This includes the new oral anticoagulant drugs (NOACs), which target either factor Xa or thrombin.

The coagulation cascade can be divided into the extrinsic, intrinsic and common pathways. The extrinsic pathway produces small amounts of thrombin that activates a variety of components in the cascade that allows amplification of the cascade via the intrinsic pathway to produce large amounts of thrombin (Figure). Thrombin cleaves fibrinogen to fibrin resulting clot formation (Figure). The extrinsic pathway of the coagulation cascade is initiated upon the exposure of extravascular TF to blood. Formation of the TF/FVIIa complex triggers the coagulation cascade by activating both FX and FXI (1). This pathway is “extrinsic” to blood since significant levels of TF are not present in blood in healthy individuals. The extrinsic pathway is essential for hemostasis. The intrinsic pathway of the coagulation cascade is comprised of three proteases, FXIIa, FXIa, FIXa and the cofactor FVIIIa. Under physiologic conditions, this pathway is activated by thrombin cleavage of FXI (2). Deficiencies in FIX and VIII lead to mild to severe bleeding in humans (hemophilia B and A, respectively) while FXI deficiency results in only a minor increase in bleeding with injury (hemophilia C) (3). The intrinsic pathway can be activated ex vivo by negatively-charged compounds, such as kaolin, that activates FXII. FXII activation has been showed to directly modify fibrin clot structure by increasing fibrin fiber density (4). The common pathway consists of the proteases FXa, thrombin and the cofactor FVa. Proteases in the common pathway are the major targets of current anticoagulant therapy (Figure).

Renne and colleagues were the first to show that FXII deficient mice exhibit reduced thrombosis in several different arterial thrombosis models without any increase in tail vein bleeding time (5). This observation was important because it suggested that thrombosis could be separated from hemostasis and suggested that FX could be a new target for the development of safe anticoagulant drugs. FXI deficient mice have no apparent hemostatic defects and humans with FXI deficiency have a small increase in bleeding after injury (refs). These observations suggest that inhibition of FXIa might also reduce thrombosis with minimal effects on hemostasis.

Despite the knowledge that FXII is activated by negatively-charged substances it was unclear what was activating FXII in vivo. Several candidates have been proposed as FXII activators over the years. Preissner and colleagues reported that extracellular RNA bound to both FXII and FXI and suggested that this was the “long sought after natural foreign surface” for the activation of the intrinsic pathway (6). There is also evidence that DNA-rich NETS and PolyP activate FXII and initiate the intrinsic pathway of the coagulation cascade (7, 8).

There is a large literature on the effect of different FXII and FXI inhibitors on thrombosis in various animal models (Figure). FXII inhibitors have been shown to reduce both venous and arterial thrombosis in multiple animal models without an associated increase in bleeding (9-11). These inhibitors include H-D-Pro-Phe-Arg-chloromethylketine and infestin 4, which was cloned from the midgut of the blood-sucking insect *Triatoma infestans*. Inhibitory anti-FXI antibodies and small molecules have been shown to reduce both venous and arterial

thrombosis in mice, rats, rabbits, and baboons without an associated increase in bleeding (12-16). Antisense oligonucleotides (ASOs) have also been used to reduce levels of different coagulation factors and determine the effect on thrombosis. ASOs target complementary mRNA resulting in RNA degradation and a reduction in protein expression. They are particularly promising for the targeting of genes expressing coagulation proteins because of the high level of sensitivity of liver tissue to ASOs (17). FXII and FXI ASOs have been shown to reduce arterial and venous thrombosis in various animal models without increasing bleeding (9, 11, 17, 18).

Role of PolyP in hemostasis and thrombosis

PolyP is a highly anionic linear polymer that is synthesized from ATP (19). In humans, this molecule is secreted by platelets after activation (19). Morrissey and colleagues were the first to report a role for PolyP in blood coagulation (20). PolyP was shown to affect numerous steps in the coagulation cascade, including activating FXII, enhancing the activation of FV, increasing the activity of thrombin-activated fibrinolysis inhibitor (TAFI), and inhibiting tissue factor pathway inhibitor (TFPI) (20). Other studies have extended this early observation and have also demonstrated that PolyP enhances fibrin clot structure stability (7, 21, 22). These results led to the suggestion that PolyP may be considered for use as a general procoagulant agent in the treatment of patients with hemostatic defects (23).

Microorganisms store PolyP in subcellular organelles termed acidocalcisomes. In mammalian platelets PolyP is stored in dense granules. There are important differences between bacterial and platelet PolyP. Bacteria produce long-chain PolyP (up to several thousand phosphate units) whereas platelets contain short-chain PolyP (60-100 phosphates units)(19). PolyP of different size have different activities in the coagulation cascade (22). Long chain PolyP polymers (>250-500mers) have been shown to be a strong activator of FXII and affect fibrin clot structure (22). On the other hand, shorter polymers (<100mers) increase FV activation and inhibit TFPI (22). Polyphosphate in both size categories also serve as a cofactor for thrombin activation of FXI (24). Renne and colleagues showed that human platelet PolyP induced pulmonary embolism and increased vascular permeability in mice in a FXII-dependent manner (7). The study concluded that "PolyP links platelet plug formation (primary hemostasis) and fibrin generation (secondary hemostasis). If platelet PolyP is playing a central role in blood coagulation by activating FXII then one would expect FXII deficient humans and mice to have a major hemostatic defect. However, FXII deficient humans and mice demonstrate normal hemostasis (3). Importantly, a second group could not reproduce the in vivo results produced by Renne and colleagues using the mouse pulmonary embolism model and concluded that platelet PolyP is a weak activator of FXII (25). Drs. Renne and Morrissey provided separate rebuttals to the Faxalv study and demonstrated that the negative in vivo results may be due to the extended storage of PolyP resulting in degradation and loss of activity (26, 27). The role of platelet PolyP as a weak activator as opposed to a strong activator of FXII is supported by previous studies (22). Clearly, more studies are needed to resolve this controversy and determine the role of platelet PolyP in regulating the coagulation cascade.

Does platelet PolyP play a role in hemostasis? This question was addressed in a recent study that generated mice deficient in inositol hexakisphosphate 6 (IP₆) kinase, which is an enzyme required for the synthesis of platelet PolyP (28). Platelets from these mice had a 3-fold reduction in phosphate levels. These IP₆ kinase knockout mice had slower platelet aggregation, increased plasma clotting times, and altered fibrin structure. Furthermore, the mice had prolonged tail vein bleeding times and were resistant to thromboembolism (28). Since the mice had defects in both platelet aggregation and clotting it is unclear if the *in vivo* hemostatic defect were due to a primary effect on platelets and/or coagulation. Nevertheless, this genetic approach supports the notion that PolyP is a general procoagulant agent.

The above polyphosphate studies have triggered the development of new anticoagulant drugs that can inactivate these polyphosphates. Sullenger and colleagues screened a variety of nucleic acid binding polymers and identified a cationic poly (amido amine) (PAMAM) dendrimer called PAMAM-G3 that prevented thrombosis without increasing tail vein bleeding in mice (29). Similarly, Morrissey and colleagues found that the cationic compounds 1.0 dendrimer and polymyxin B reduce both venous and arterial thrombosis in mice (30).

Microparticles in hemostasis and thrombosis

MPs are small (0.1-1.0 μ m) membrane vesicles released from activated or apoptotic cells and cells that have undergone oncogenesis (31-33). These vesicles were originally described as “platelet dust” that was observed being released from activated platelets by microscopy (34). MPs are formed by the outward blebbing of the plasma membrane with subsequent release after proteolytic cleavage of the cytoskeleton (32, 35). The majority of MPs have phosphatidylserine (PS) exposed on their surface, although PS-negative MPs can be formed after platelet activation (36). They contain a selective group of cell surface proteins, which includes TF (32, 37). MPs are procoagulant due to the presence of negatively-charged phospholipids, such as PS, and TF on the surface (38-40). TF binds FVII/FVIIa and PS facilitates the assembly of positively-charged coagulation factor complexes (41-43). There is indirect evidence for a role of MPs in hemostasis (44). Injection of soluble P-selectin-Ig into hemophilia A mice resulted in an increase in the number of circulating MPs, including TF-positive MPs, and a correction of the tail-vein bleeding time (44). Similarly, infusion of red blood cell-derived MPs into thrombocytopenic rabbits corrected ear bleeding time in a dose-dependent manner (45). However, there is no evidence that circulating MPs contribute to hemostasis in healthy individuals. The majority of MPs in blood in healthy individuals are derived from platelets and it is difficult to separate the role of platelet-derived MPs in hemostasis from the role of the platelets themselves (46).

MP enhancement of experimental thrombosis is well documented. For instance, injection of MPs isolated from cardiac bypass surgery patient plasma was shown to enhance thrombosis in a TF dependent manner using a rat IVC stenosis model of venous thrombosis (47). One study examined the docking of tumor-derived MPs to sites of ferric chloride-induced mesenteric vessel injury and laser-induced cremaster arteriole injury *in vivo* (48). MPs derived from PANC02 mouse pancreatic adenocarcinoma tumors accumulated at sites of vascular injury and thrombosis (48). This MP accumulation was reduced by treatment of the

mice with an inhibitory P-selectin antibody (48). Mouse pancreatic tumor-derived MPs were also found to enhance ferric chloride-induced thrombosis in mice (48). However, cleavage of surface ligands on these tumor MPs by trypsinization or treatment of mice with an inhibitory P-selectin antibody prior to MP infusion attenuated the MP-induced enhancement of thrombosis (48). We found that growth of human pancreatic tumors expressing TF was associated with release of TF-positive MPs into the circulation and activation of coagulation. In addition, injection of TF-positive tumor-derived MPs enhances thrombosis in an inferior vena cava (IVC) stenosis model of venous thrombosis (49).

Statin treatment is associated with a reduction in venous thrombosis (50, 51). The Jupiter trial demonstrated a reduction in VTE in patients with high levels of C-reactive protein but normal blood lipids that were prophylactically treated with rosuvastatin (50). Our laboratory has shown that hypercholesterolemia can induce monocyte TF expression and release of TF-positive MPs in vivo (52). Moreover, we found that simvastatin treatment attenuated this increase in TF-positive MPs in both hypercholesterolemic mice and in monkeys (52). Patients with a variety of thrombotic disease often have elevated levels of TF-positive MPs suggesting that they may contribute to the prothrombotic state and thrombosis (33, 35, 53).

We hypothesize that TF-positive MPs could represent a new target for anticoagulant therapy that would have minimal effect on hemostasis. A concern of this therapy, however, would be that it would only be effective in prothrombotic disease states that are shown to be dependent on MPs. While there is evidence for a role of MPs in thrombosis in vivo in some diseases such as pancreatic cancer associated thrombosis, definitive evidence for a reduction in thrombosis associated with targeting MPs in patients does not yet exist. Potential strategies would include blocking the formation, increasing the clearance and/or preventing the docking of the MPs to the activated endothelium. P-selectin inhibitors are already in development for use as anti-thrombotic agents (54-56). However, these inhibitors block platelet aggregation which would affect hemostasis, potentially resulting in bleeding side effects.

NETs and thrombosis

NETs are primarily released by activated neutrophils via a cell death program called NETosis that is distinct from either apoptosis or necrosis (57). NETS consist of nucleic acids decorated with histones and other proteins that are involved in the entrapment and killing of both Gram-negative and Gram-positive bacteria as well as fungi in innate immunity (58, 59). NETosis involves chromatin decondensation followed by the fragmentation of the nuclear envelope and neutrophil granules allowing for the mixing of components within the cell before plasma membrane lysis and NET release (57). Non-suicidal pathways of NETosis have also been identified which involve the release of nuclear or mitochondrial DNA by living cells through the budding of NET-filled vesicles (60-62). Of note, simply assaying for free DNA or histones in the plasma is not sufficient evidence for NET release as free DNA can also be released in other forms of lytic cell death (59).

NETs have been shown to enhance experimental venous and arterial thrombosis through multiple mechanisms. As mentioned above, they provide a negatively-charged surface for

the activation of FXII (63). Histones, a major component of NETs, have also been shown to induce activation and aggregation of platelets (64, 65). Neutrophils that have been stimulated to release NETs activate FXII in vitro in a histone H2A and H2B dependent manner (63). NETs enhance activation of the extrinsic pathway of the coagulation cascade through the inactivation of TFPI (66). They also increase venous thrombosis by binding both platelets and red blood cells (64). These structures have been imaged within venous thrombi in both mice and primates (63, 64, 67, 68). Importantly, mice with impaired NET formation have a decreased incidence of thrombosis in the IVC stenosis model (67). Two studies have demonstrated protection from venous thrombosis in mice that have been treated with DNase 1, which degrades cell free DNA and NETs (63, 68). The anticoagulant heparin has been shown to displace histones from NETs resulting in their degradation and a reduction in venous thrombosis (63, 64). Similarly, carotid artery thrombosis is reduced in mice that are deficient in two neutrophil serine proteases that are present on NETs (66). Infusion of purified histones into mice has also been shown to enhance venous thrombosis (68). Polysialic acids have been shown to neutralize histones resulting in a reduction in NET-mediated cytotoxicity, although these agents have not yet been tested in a thrombosis model (69).

At present, there is little evidence that NETs are required for hemostasis. The tail vein bleeding time was prolonged two-fold in mice that were deficient in two neutrophil serine proteases that are known to be present on NETs (66). Also, while histones are only one component of NETs, it is of note that infusion of histones into mice causes thrombocytopenia that is associated with a profound increase in tail vein bleeding time (65).

Recent studies provide evidence for an association between NET release and thrombosis in humans (70-73). Patients diagnosed with acute VTE have been shown to have increased plasma nucleosomes and activated neutrophils (72). Further, both acute VTE and thrombotic microangiopathies are associated with increased plasma DNA and myeloperoxidase after diagnosis (70, 71). Finally, neutrophils and NETs were present in thrombi isolated from patients with acute myocardial infarction (73).

Conclusion

There are many people that have contributed to our increased understanding of the pathways involved in hemostasis and thrombosis. The discovery that PolyP affects many aspects of the clotting cascades suggests that it may be a useful hemostatic agent for the treatment of a variety of bleeding disorders. In contrast, several factors have been identified that appear to contribute to pathologic thrombosis but not hemostasis. These include FXII, TF-positive MPs and NETs. Inhibition of FXII activators with nucleic acid polymers may provide a safe way to reduce thrombosis. However, these drugs may only be effective in patients in which thrombosis is triggered via the intrinsic pathway and are likely to be less efficacious than current anticoagulant drugs that target the common pathway of coagulation. At present, it is unclear if interfering with NET formation will be a viable approach to reducing arterial and venous thrombosis in patients.

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Figure.

Separating thrombosis and hemostasis in the coagulation cascade.

Current anticoagulant therapies target the common pathways and, as a result, have significant bleeding side effects. Targeting thrombotic triggers as opposed to targeting the pathways involved in hemostasis could potentially allow for the creation of novel antithrombotic agents with minimal bleeding side effects.