Published in final edited form as:
Thromb Res. 2012 May ; 129(Suppl 2): S1–S4. doi:10.1016/j.thromres.2012.02.017.

Tissue factor and factor VIIa – Hemostasis and Beyond

Alisa S. Wolberg[1] and **Alan E. Mast**[2]

[1]Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[2]Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI

Abstract

Initiation of the coagulation cascade via exposure of active tissue factor (TF) to blood and formation of the factor VIIa/TF complex is essential for hemostasis and is an initial procoagulant signal in thrombosis. As of early 2012, over 20,000 articles listed on PubMed describe advances in the understanding of TF biology in the settings of hemostasis and thrombosis, as well as in signaling events in cancer, sickle cell anemia, hyperlipidemia, and a broad spectrum of inflammatory disorders. It is both inspiring and humbling, then, to consider not only what has been learned about TF regulation, but also the number of questions still remaining about its role in physiology. This supplement reviews both well-accepted and currently-controversial topics in coagulation and factor VIIa/TF biology, with particular foci on non-hemostatic roles of TF, innovative approaches for the treatment of hemophilia, and novel *in vivo* models of bleeding and thrombosis.

Overview of TF Biology

The presence of a “factor” within tissues that accelerates blood coagulation was described over 150 years ago. Mann and co-workers provide a concise summary of the history of TF discovery and isolation, and its structure and function. In addition, they review current controversies in the field of TF biology, including the presence and potential functions for circulating forms of TF, the expression of TF by hematopoietic cells and the mechanisms required for maintaining TF in an active conformation on the cell surface.

Regulation of TF activity

The fundamental component of most coagulation reactions is the cellular surface upon which the reaction takes place; however, the mechanisms by which coagulation proteins bind to membranes remain incompletely understood. Using nanoscale phospholipid bilayers (Nanodiscs), solid-state NMR, crystallography and computational studies, Morrissey and co-workers have probed the role of membrane composition in the activity of the catalytic

Corresponding Authors: Alisa S. Wolberg, PhD, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, 815 Brinkhous-Bullitt Building, CB #7525, Chapel Hill, NC 27599-7525, USA, Tel: 919-966-8430, Fax: 919-966-6718, alisa_wolberg@med.unc.edu. Alan Mast, MD, PhD, Blood Research Institute, 8727 Watertown Plank Road, PO Box 2178, Milwaukee, WI 53201-2178, Tel: 414-937-6310, Fax: 414-937-6284, alan.mast@bcw.edu.

Conflict of interest statement

Both ASW and AM have received research funding from Novo Nordisk A/S.

complex. Their work suggests membrane binding of factor VIIa occurs via a single "phospho-L-serine-specific" binding site plus 5–6 "phosphate-specific" interactions per γ-carboxyglutamic acid (Gla) domain, requiring bending of phospholipid headgroups to allow proper conformation for calcium interactions. These findings provide a rationale for the observation that phosphatidylcholine cannot synergize with phosphatidylserine to promote factor X activation by the factor VIIa/TF complex because it cannot "bend" to allow phosphate-calcium coordination.

Although the essential role of TF in coagulation and cellular signaling is well-appreciated, the mechanisms regulating the expression of these activities are still highly controversial. TF exists in both cryptic and active states. Prevailing theories suggest TF decryption involves cellular perturbations that expose procoagulant phosphatidylserine in the outer leaflet of the cell membrane and/or thiol-disulfide exchange reactions. Three review articles summarize current literature on this topic and present differing points of view regarding the physiological importance of different mechanisms. Ruf's review focuses on protein disulfide isomerase (PDI), a proposed regulator of cell surface TF thought to catalyze thiol exchange of the allosteric Cys186–Cys209 disulfide bond that results in TF decryption. Release of cellular TF-bearing microparticles does not occur when extracellular free thiols or PDI are blocked, leading to the conclusion that PDI is also part of a thiol exchange pathway that produces microparticles in the context of P2X7-dependent TF activation. The articles by Rao and co-workers and Butenas and Krudysz-Amblo cite evidence that draws into question the allosteric nature of the Cys186–Cys209 disulfide bond in modulating TF decryption. Studies of purified TF have not identified free sulfhydryl groups and unpaired cysteine residues have not been demonstrated to be present on the surface of cells containing cryptic TF. Thus, as yet there is no direct evidence for PDI-mediated modification of a TF disulfide bond. Potential causes of the controversial data include the use of different cell lines with nonstandard reagents, non-specific effects of the PDI inhibitors, and variable assay methods in different laboratories. All three articles emphasize the need for further studies in this area. Firm resolution of these controversies will likely require the development of new molecular probes that specifically identify different conformations of TF.

Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is the primary physiologically-relevant inhibitor of TF activity. TFPI is found in both plasma and cell-associated forms. There are at least two alternatively spliced human isoforms, TFPIα and TFPIβ, which appear to be differentially expressed by various cells and to differentially associate with the cofactor, protein S. These differences suggest these isoforms have distinct physiological functions. The review by Maroney and Mast focuses on the physiological role of platelet TFPI. In both humans and mice, all of the TFPI within platelets is TFPIα, the more evolutionarily-conserved of the two isoforms. Their article describes the characterization of TFPI synthesis and expression in megakaryocytes and the requirement for dual agonist stimulation of platelets to obtain TFPI expression on the platelet surface. Platelet TFPI limits thrombus growth following electrolytic vascular injury, indicating important, distinct roles for each pool of TFPI in hemostasis and thrombosis. Peraramelli and co-workers describe findings that further define the physiology of TFPI, focusing on the ability of the protein S/TFPI complex to inhibit TF. The protein S/TFPI complex effectively inhibits coagulation triggered by low TF concentrations, but when TF concentrations are above a threshold of 14 pM, the protein S/TFPI complex is a poor inhibitor of TF procoagulant activity, even when the TFPI concentration is in 10-fold excess over TF, corresponding to a concentration 10-fold higher than the reported Ki* for FXa/TFPI. It is hypothesized that the poor inhibition is a result of the slow initial interaction between TFPI and FXa. Peraramelli *et al.* also describe results from kinetic experiments demonstrating that the TFPI/protein S and the activated protein C/

protein S anticoagulant systems act synergistically to limit thrombin generation in plasma assays initiated with high (14 pM) amounts of TF, thereby explaining a plausible mechanism for how TFPI/protein S can dampen FXa generation at high TF concentration.

Tissue Factor and Microparticles

The search for means to promote or inhibit hemostasis or thrombosis, respectively, requires knowledge of the sources of procoagulant activity in health and disease. Owens and Mackman review findings on specific sources of TF that promote thrombosis following atherosclerotic plaque rupture. Hyperlipidemia leads to the formation of oxidized LDL capable of inducing TF in several places, including monocytes/macrophages, endothelial cells, and vascular smooth muscle cells. Elevated markers of coagulation activation (thrombin-antithrombin complexes) can be reduced with anti-TF inhibitory antibodies suggesting a central role for TF activity in hyperlipidemia-induced cardiovascular disease. In addition to reducing circulating plasma cholesterol, statins inhibit TF expression by circulating monocytes and macrophages and monocyte-derived microparticles/ microvesicles. Thus, at least some of the protective effects of statins in hyperlipidemia may result from their ability to reduce monocyte TF expression.

Microparticles, in particular, have become an area of major interest. As illustrated in hyperlipidemia, microparticles that express TF and phosphatidylserine have procoagulant activity *in vitro* and *in vivo*. Lacroix and Dignat-George paint a picture of microparticles as complete, miniature systems that carry multiple activities derived from their parent cell of origin, including procoagulant, anticoagulant, and profibrinolytic functions. In this way, microparticles are complex entities that may fine-tune coagulation and fibrinolysis at sites of vessel damage. Indeed, while increased circulating microparticles have been associated with several diseases, there is a lack of data demonstrating an etiologic role for microparticles in thrombosis or hemostasis. This gap may be due to technical limitations in measuring microparticles, or may reflect still naïve appreciation of microparticle contributions to the coagulation process. For example, overall activity reflects net factor Xa-generating capacity which is the product of an equilibrium between expression of TF and TFPI activities, but most assays measure TF activity of washed microparticles and do not incorporate plasma levels of TFPI in these assessments. Endothelial cell microparticles also carry the anticoagulant proteins thrombomodulin and the endothelial protein C receptor, as well as fibrinolytic activities including urokinase plasminogen activator, urokinase plasminogen activator receptor, and tissue plasminogen activator, suggesting they can both down-regulate thrombin generation and dissolve intravascular fibrin. In this case, expression of endothelial-derived microparticles may correlate with a lack of prothrombotic phenotype.

Non-hemostatic roles of tissue factor

In addition to its role as the primary initiator of blood coagulation, several non-hemostatic roles for TF have been identified. TF is an alternatively-spliced protein made in two isoforms: the typical full-length, transmembrane TF and alternatively spliced TF (asTF) that lacks exon 5 and has a unique C-terminal region that permits secretion by the cell. asTF has very low procoagulant activity, and identification of its physiological function has been an area of active research. The review article by Srinivasan and Bogdanov summarizes how asTF stimulates angiogenesis in a process that does not involve proteolytic activity, but rather is a consequence of direct interaction between asTF and endothelial cell β1 integrins. Interestingly, it appears that asTF proangiogenic activity is totally independent of PAR activation. Instead, it acts as a ligand for β1 integrins, with binding producing activation of the PI3K/Akt/NFkB signaling pathway. Thus, the two alternatively-spliced forms of TF

activate cellular signaling through separate pathways and therefore may produce distinct physiological and/or pathological outcomes.

The contribution of TF to the development and progression of disease is discussed in two articles focusing on cardiac fibrosis and atherothrombosis. Ten Cate presents data clearly indicating TF present within atherosclerotic plaque contributes to atherothrombosis, yet TF does not seem to play a significant role in driving the progression of atherosclerosis. In contrast, inhibiting TF activity with anti-TF antibody, TFPI, or active site inactivated fVIIa in animal models of inflammatory disease suggests a role for the pro-inflammatory activity of TF mediating disease. These concepts are further described by Rauch, who reviews the role of TF in maintaining cardiac function. Mice with low amounts of TF have cardiac fibrosis. Although this finding initially suggested that myocardial hemorrhage causes fibrosis, mice with low TF and no TFPI have improved hemostasis, but still suffer from cardiac fibrosis. These findings led to additional studies to identify non-hemostatic functions for myocardial TF. These studies have found that TF plays a role in maintaining the structural integrity of cardiac muscle and that asTF, as well as full-length TF, appears to reduce TNF-α driven apoptosis of primary cardiomyocytes.

Novel approaches for the treatment of hemophilia

Standard therapy for hemophilic patients is replacement factor; however, hemophilic patients with inhibitors necessitate alternate therapeutic strategies. Four papers in this series review recent developments in this search for safe, new, faster-acting hemostatic agents.

The first of these articles reviews an approach that unites information from studies on TF regulation with findings on hemophilia biology, suggesting TFPI inhibition may improve factor Xa generation and activity in hemophilic patients. Petersen and colleagues discuss current strategies to inhibit the anticoagulant activity of TFPI via polyclonal and humanized monoclonal antibodies, non-anticoagulated sulfated polysaccharides (fucoidan), RNA aptamers, and inhibitory peptides. Each of these molecules have shown hemostatic efficacy in a variety of hemophilia models, including mice, rabbits, dogs, and non-human primates, and the humanized monoclonal antibody mAb2021 has entered clinical trials in humans.

Perhaps the most sought treatment and potential “cure” for hemophilia is gene therapy. Montgomery and Shi review several novel approaches for gene therapy, including attempts at platelet and endothelial cell-specific expression of factor VIII or factor IX. Endothelial cells are normally capable of both vWF and factor VIII expression, and platelets are adept at transporting hemostatic proteins to sites of injury, making these cells attractive targets for factor VIII and IX expression. Indeed, hemophilic mice with megakaryocyte-directed expression of factor VIII show factor VIII expression in platelets that is hemostatically effective even in the presence of inhibitory antibodies. Unfortunately, though megakaryocyte-specific expression of factor IX also shows hemostatic efficacy in hemophilic mice, platelet-derived factor IX is blocked in the presence of inhibitory antibodies. Likewise, endothelial cell-directed expression of factor VIII shows hemostatic efficacy in the absence, but not presence, of anti-factor VIII inhibitory antibodies. Though promising, clearly additional work is necessary to develop viable gene therapy approaches for hemophilia patients without and with inhibitors.

The demonstrated success of the bypassing agent recombinant factor VIIa for hemophilia patients with inhibitors has fueled investigations to develop similar, but faster-acting molecules. Given findings that recombinant factor VIIa has TF-independent procoagulant activity on the platelet surface, a number of molecules with enhanced TF-independent activity and/or enhanced lipid binding have been developed. Hoffman reviews one molecule in particular, NN1731 (V158D/E296V/M298Q-FVIIa, vatreptacog alpha), that has

demonstrated enhanced TF-independent activity in *in vitro* and *in vivo* models of hemophilia. This molecule has now completed a Phase II clinical trial. Perhaps equally importantly, studies with NN1731 have revealed surprising information about the nature of Gla-containing protein binding to the platelet surface. First, although NN1731 only differs from recombinant factor VIIa in three amino acids in the catalytic domain, it binds to a greater number of sites on activated platelets than does recombinant factor VIIa. Second, although NN1731 increases factor Xa generation on activated platelets, it does not increase thrombin generation to the same proportion. These findings suggest as yet unidentified mechanisms regulate platelet procoagulant activity in hemophilia and other hemostatic and thrombotic disorders.

Clearly, the field of hemophilia research is ripe for the development of novel hemostatic agents. In addition to the approaches discussed above, Persson reviews several additional molecules currently in development, including a recombinant factor VIIa molecule with Gla domain mutations that enhance phospholipid binding, and two protein fusion approaches that increase the association of factor VIIa to platelets. In one these, factor VIIa was targeted to the activated platelet surface by fusion to a single chain Fv fragment that recognized platelet GPIIb/IIIa. In the other, investigators took an indirect approach designed to increase factor VIIa on the platelet surface by localizing annexin V-conjugated TF to the phospholipid exposed during platelet activation. As Persson notes, the continued development of these and other molecules that exhibit increased activity and circulating half-life is an actively growing area of investigation. It will be fascinating to monitor this field to see which approaches proceed to clinical use over the next decade.

Animal models of bleeding and thrombosis

Although *in vitro* cellular and biochemical studies have yielded considerable information on the role of TF in hemostasis and thrombosis, studies in animal models have enabled a more complete understanding of coagulation *in vivo*. Two aspects of these investigations are critical: 1) the choice of a model that appropriately recapitulates hemorrhagic and thrombotic coagulopathies in humans, and 2) the choice of evaluative methods that reveal essential information about the operant pathophysiologic mechanisms. Four articles are presented that address these points in a variety of animal models, including mice, dogs, and non-human primates. Cooley's review of murine thrombosis models highlights limitations associated with current murine models, with particular focus on the caveats of using the models to understand the etiologic differences between arterial and venous thrombosis. The common models – ferric chloride application, light-activated Rose Bengal, electrolytic injury, inferior vena cava ligation, and laser injury may not simulate the pathological triggering factor for either arterial or venous thrombosis. Nonetheless, these models demonstrate sensitivity to certain pharmacologic interventions, suggesting reasonable biologic relevance in appropriate conditions. Noting obstacles in imaging thrombotic processes in large vessels, Cooley highlights a novel methodology from his laboratory that enables the collection of fluorescence imaging data from the femoral vein of mice. Because this system can be used to image thrombi in both small and large vessels, it may permit comparative understanding of thrombodynamic processes in these different vessels. Ivanciu and colleagues review advances using state-of-the-art videomicroscopy from studies to image hemostasis in mice following laser injury to the mouse cremaster arteriole. Their manuscript highlights strengths of this method, including the ability to monitor the dynamics of thrombus development in real time and produce 3-dimensional images of intravascular thrombi that show the spatial localization of protein and cellular components of the clot. The use of labeled, non-inhibitory antibodies and potentially recombinant variants that allow for direct labeling, may allow direct, quantitative assessments of the accumulation of thrombus components and demonstrate the complex mechanisms that occur during this process.

Although the mouse offers significant strengths in terms of cost, size, and amenability to genetic manipulation, the close phylogenetic, anatomical, and physiological similarities of non-human primates to humans make these models essential for understanding pathophysiologic mechanisms promoting thrombosis in humans. Myers reviews technical aspects of existing non-human primate models of arterial and venous thrombosis, several of which resemble current murine models (e.g., photochemical induction and inferior vena cava occlusion) and may bridge the translational gap between murine models and human pathology. Obvious ethical concerns about the use of non-human primates have given rise to strict guidelines regarding their use. Thus, models in other animal species are likely to remain the predominant approach for most studies of hemostasis, thrombosis, and thrombus resolution.

Besides methodologies to detect intravascular thrombus formation, improved methodologies are also needed to detect bleeding in hemorrhagic scenarios. Current plasma-based clotting assays do not directly measure hemostasis *in vivo*. Scola *et al*. present their experience with a novel technology for assessing hemostasis *in vivo*. This methodology, Acoustic Radiation Force Impulse (ARFI), uses ultrasound to monitor the bleeding rate and time to hemostasis. ARFI can detect bleeding in canine models of hemophilia and von Willebrand Disease, and has demonstrated the ability to assess hemostasis at femoral arteriotomy following diagnostic percutaneous coronary catheterization in humans. These exciting preliminary data suggest ARFI will be useful in assessing bleeding in patients on platelet antagonists and anticoagulant therapy in the clinic, with additional applications for detecting active gastrointestinal and intra-cerebral hemorrhage on the horizon.

Activation of coagulation in disease

Abnormal expression of TF has been well-documented and strongly-implicated in the pathogenesis of prothrombotic and proinflammatory diseases. Chantrathammachart and Pawlinski highlight the contribution of TF to the vascular pathology seen in sickle cell anemia. Patients with sickle cell disease (SCD) exhibit high plasma levels of procoagulant microparticles and markers of increased thrombin generation, platelet activation, and fibrinolysis. Data showing that inhibitory anti-TF antibody abrogates activation of coagulation in a murine model of SCD strongly implicate TF as a central mediator of the pathology and as a potential therapeutic target. Ongoing investigations to identify the cellular source of procoagulant TF in SCD, and its proinflammatory effects on the vasculature, are expected to reveal important information on the mechanism by which antithrombotic therapeutics attenuate not only coagulation, but also vascular inflammation and organ damage in SCD patients.

Besides the obvious contribution of the extrinsic pathway in initiating coagulation, the ability of contact system activation to promote fibrin formation implicates this pathway as well in thrombotic disease. Notably, the absence of bleeding in patients deficient in contact factors make this pathway an attractive potential target for anticoagulation approaches. Maas and Renne discuss mechanisms that regulate the plasma contact system, including factor XII autoactivation and plasma kallikrein-mediated factor XII activation. Though artificial agents are well known to activate the contact pathway *in vitro*, negatively-charged compounds, including extracellular RNA, collagen, and polyphosphate, have recently been shown to activate factor XII *in vivo*, and represent exciting new targets for antithrombotic therapy.

The traditionally distinct lines between soluble clotting factors and cellular activities have been blurred by modern appreciation of the cross-talk between these contributors to thrombosis and bleeding. Amid this series of reviews on TF, Bagoly *et al*. focus on factor XIII, a zymogen of a transglutaminase found in plasma and tissues that cross-links fibrin

chains and other clot-associated proteins. Enzymes released from polymorphonuclear neutrophils, including human neutrophil elastase may activate factor XIII and/or proteolytically degrade factor XIIIa, representing additional mechanisms by which cells implicated in thrombosis could further modulate fibrin stability. Factor XIIIa activity is also found in platelets, monocytes, and macrophages where its gene expression both regulates, and is regulated by, cellular processes. Cellular factor XIII may also participate in organization and remodeling of extracellular matrix proteins, macrophage phagocytosis, cellular locomotion of dendritic cells, and regulation of angiotensin receptor 1 in hypertensive patients and apoE-deficient mice. These provocative findings promise increasingly complex pathways on the horizon to understanding mechanisms in hemostasis and thrombosis.

Conclusions

This collection of reviews illustrates the complexity and diversity of coagulation pathways and their newly-described roles in inflammatory disease. Molecules once described with simple terminology (*e.g.*, "TF" and "TFPI") are now known to be generated in multiple cells, each producing specific and functionally-unique isoforms subject to independent regulatory mechanisms that promote or inhibit coagulation. Findings on the roles of these proteins cannot necessarily automatically be extrapolated to all diseases, but will need to be experimentally tested in physiologically-relevant models *in vitro* and *in vivo*. Ultimately, these studies will lead to the development of safe, effective treatments to prevent bleeding and thrombosis in a variety of diseases.