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# Factor VIIa and tissue factor – from cell biology to animal models

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Binding of factor VIIa (FVIIa) to tissue factor (TF) and the subsequent initiation of the clotting cascade is an essential for hemostasis. However, the aberrant expression of FVIIa-TF contributes to thrombosis. Despite the tremendous progress made in the past 25 years in understanding the molecular mechanisms involved in the interaction between FVIIa and TF, there is less known about the cell biology of these proteins. Availability of hemophilic mice (by specific knock-out of FVIII or FIX genes) and novel TF transgenic mice has allowed us in recent years to investigate the importance of TF-FVIIa-induced coagulation from wound healing to sepsis. This supplement explores new aspects of TF-FVIIa biology, with a particular focus on structural biology, cell biology and animal models.

## I. Factor VIIa structure and function

In the first review, Hedner provides her personal experience on how FVIIa was conceived as a novel therapeutic agent in treating hemophiliacs with inhibitors, development of rFVIIa, and how rFVIIa became a reality in successfully treating hemophiliacs with inhibitors. This review coupled with a recent article by Kisiel on the same subject (J Thromb Haemost 7: 1053-56, 2009) provides a fascinating story of the discovery and development of rFVIIa as an effective therapeutic option for restoring hemostasis in inhibitor patients. The next two reviews discuss recent developments in FVIIa structure and function. Lee and colleagues describe models of the solution structure of the FVIIa-TF-FXa ternary complex based on their recent refinements of two independently-derived docking models generated earlier. These models should be useful for comparative purposes and for designing new experiments aimed at further refinement of the TF-FVIIa-FXa structure. FVIIa binding to TF is necessary for its enhanced procoagulant activity since free FVIIa exhibits zymogen-like behavior, i.e., low intrinsic activity. Persson and Olsen's review provides a brief summary of our current knowledge of the allosteric mechanism by which TF induces and stabilizes the active conformation of FVIIa. The crosstalk between the N-terminal domain of TF and the protease domain of FVIIa, in particular Met 306, provides the starting point for the allosteric activation of FVIIa.

## II. FVIIa activity and interaction with cells

Until recently, the only known cofactor/receptor for FVIIa was TF. However recent studies from several laboratories provide evidence that FVIIa may also interact with other receptors present on a variety of cells. The importance of these findings and their potential



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pathophysiological significance are the topic of three reviews in this section. Lisman and de Groot discuss the interaction of FVIIa with glycoprotein Ib on the surface of activated platelets and how this new finding adds to our current understanding of the mechanism of rFVIIa in treating patients with bleeding disorders. Hoffman and Monroe discuss the importance of rFVIIa binding to activated platelets in restoring thrombin generation in hemophiliacs and provide a hint that the platelet surface binding and activity of FVIIa can be enhanced by relatively minor changes in FVIIa structure. Pendurthi and Rao provide a brief review of recent studies of FVIIa interaction with endothelial cell protein C receptor and discuss the potential significance of this interaction in hemostasis, FVIIa treatment of bleeding disorders and FVIIa transport and clearance. Cumulatively, understanding how FVIIa interacts with receptors on platelets and endothelial cells and how these interactions modify FVIIa activity would be helpful in improving the use of rFVIIa in the treatment of various bleeding disorders.

## III. Regulation of tissue factor: Factor VIIa activity

For FVIIa to function efficiently, it not only has to form a complex with TF but this complex must be formed on a suitable phospholipid surface. Importantly, TF-FVIIa activity is modulated by phospholipids, the cellular source of TF, post-translational modifications of TF and blood flow conditions. Three reviews in this section focus on various factors that regulate TF-FVIIa activity. In the first review, Morrissey and colleagues describe how structural insights into clotting protein and membrane interactions can be obtained using a combination of approaches that include nanoscale membrane bilayers, solid-state NMR, and large-scale molecular dynamics simulations. In the second review, Butenas and colleagues discuss how natural and recombinant TF proteins differ in their posttranslational modifications and how these differences translate into different cofactor activity. They also highlight the importance of exercising caution in using recombinant TF as a surrogate for the natural TF, especially in diagnostic and biological experiments. The lack of standardization of recombinant TF remains a major concern when comparing studies from different laboratories. In the third review, Diamond describes how flow conditions regulate the growth of TF-dependent thrombus formation and the contribution of surface TF and blood-borne TF to this process. It appears that traces of blood-borne TF present in healthy donors would not significantly contribute to clotting events triggered by surface TF (i.e. cellular TF that comes from extravascular cells or is from vascular cells). However, elevated levels of circulating TF that may present in disease states, such as patients with acute coronary syndrome or cancer, could increase the risk for thrombosis.

## IV. Tissue factor expression and cell biology

It is highly controversial as to which circulating blood cells are capable of synthesizing TF. In the first review, Osterud describes data generated in his own laboratory and from other groups on the ability of various blood cell types to express TF in health and disease. It seems that the only blood cells capable of synthesizing TF in humans are monocytes. Very low levels of cell surface TF expression may be seen in a few circulating monocytes in healthy individuals. A variety of disease conditions activate monocytes to express TF. In contrast, TF present on activated platelets and granulocytes probably comes from fusion of microvesicles shed by activated monocytes. In the next review, Wolberg and Aleman discuss the role of TF procoagulant activity in fibrin formation and stability of the fibrin network. This review also describes the contribution of cells, plasma and blood flow on thrombin generation and fibrin formation, structure, and stability. Understanding the specific mechanisms by which cells, plasma and blood flow regulate fibrin structure and stability may not only help in understanding hemostasis in finer details, but also be helpful in identifying effective targets for hemostatic and antithrombotic therapies. In general, only a small fraction of the TF found on the cell surface is coagulantly active whereas the vast majority is non-functional, which often is referred

to as cryptic TF. How cryptic TF differs from active TF and which mechanisms are responsible for the conversion of cryptic TF to active TF (decryption) has been hotly debated. It has been proposed recently that the cryptic form of TF contains unpaired cysteine thiols at Cys 186 and Cys 209 and that decryption of TF involves the oxidation of Cys186 and Cys209 to form the disulfide bond. Protein disulfide isomerase (PDI) was proposed to be responsible for the oxidation step. However, others have either challenged this hypothesis or found theoretical loopholes in it (Bach and Monroe, Arterioscler Thromb Vasc Biol 29: 1997-8, 2009). Here, Popescu and colleagues summarize these controversies and add one more controversy to this subject. They suggest that PDI could regulate TF activity and binding of coagulation proteins at least in part through changes in lipid asymmetry of the plasma membrane.

## V. Tissue factor and microparticles

TF microparticles (MPs) have become a hot topic. Several studies have found that circulating TF-positive MPs are elevated in a variety of diseases, including cancer, and that they may trigger venous thrombosis. This has led to the proposal that TF-positive MPs may be a useful biomarker to identify patients at risk for thrombosis. The first review by Key points out that MPs are heterogeneous in both size and procoagulant properties. Indeed, MPs are generated by different cells within the vasculature, particularly platelets. The procoagulant activity of MPs is increased with the exposure of phosphatidylserine (PS) and the presence of TF. However, a major challenge in studies with MPs is how to measure TF. Flow cytometry measured levels of TF antigen but the MPs must be > 1μm in diameter and this approach gives no information about TF activity. Other physical methods for measuring MPs are discussed. PS-positive MPs can be captured using annexin V but not all MPs have PS exposed on their cell surface. Finally, high speed centrifugation can be used to isolate the MPs from plasma followed by measurement of TF activity in a two-stage clotting assay with or without an anti-TF antibody. Freyssinet and Toti describe the process of MP formation and their different properties. It is proposed that the hemorrhagic phenotype of Scott syndrome is due to defect in the generation of PS-positive MPs. Interestingly, stimulation of the same cell type with different stimuli can lead to the formation of MPs with distinct antigens and properties. In addition, MPs can be transferred from one cell to another. As discussed earlier, leukocyte-derived, TF-positive MPs may bind to various cell types, such as platelets, granulocytes and endothelial cells. The authors also emphasize the need for standardization of MP determination. The final review in this section by Nieuwland discusses the role of MPs in disease. It is proposed that MPs allow cell-cell communication but also may provide a means for a cell to unload unwanted proteins. In cancer, MPs can promote tumorogenesis by facilitating transfer of the oncogenic growth factor receptor EGFRvIII from one cell to another.

## VI. Tissue factor pathway inhibitor

A discussion of the TF-FVIIa complex would not be complete without reviews on the physiological inhibitor of the complex, namely tissue factor pathway inhibitor (TFPI). Importantly, the major role of TFPI is to counter-balance the procoagulant activity of the TF-FVIIa complex. Indeed, knock out of the TFPI gene in mice is embryonic lethal. TFPI contains 3 Kunitz domains and an acidic C-terminus. It forms a quaternary complex with TF-FVIIa-FXa. The review by Maroney and colleagues describes the different isoforms of TFPI. In humans the α isoform is the major isoform whereas in mice the β isoform predominates. The primary site of expression is the vascular endothelium. Here, the majority of TFPIα is associated with a glycosyl-phosphatidylinositol anchored protein. A second minor pool is bound to glycosaminoglycans and is released by treatment with heparin. Small amount of TFPI are also found in the circulation. The presence of TFPI on endothelial cells likely prevents activation of the coagulation system by low levels of TF in the blood. In addition, TFPI is present in platelets (8-10% of total TFPI in plasma) and is exposed on the cell surface after

activation. It would be interesting to know if this TFPI is sufficient to inhibit the low levels of TF in platelets. The second review by Holroyd and Simari discusses the potential roles of TFPI beyond anti-coagulation. They propose that TFPI contributes to inflammation, angiogenesis and lipid metabolism. Of these, lipid metabolism is the most intriguing. TFPIα circulates in plasma bound the lipoproteins via its acidic C-terminus. Unexpectedly, when mice overexpressing TFPI in their vasculature were crossed with Apo $E^{-/-}$ mice there was a decrease in atherosclerosis and in total plasma cholesterol. This suggested a role of TFPI in systemic lipid metabolism.

## VII. FVIIa in animal models

The section contains a review by Margaritis on the long-term expression of FVIIa in the treatment of hemophilia dogs. As discussed above, rFVIIa is used to treat bleeding in inhibitor patients. However, this is expensive and rFVIIa has a short half-life. Therefore, there would be significant advantages of using long-term expression FVIIa as a means to treat hemophilia. Margaritis and his colleagues in the High group had previously shown that expression of FVIIa in mice restores hemostasis to hemophilic mice and noted that higher levels of FVIIa led to thrombosis. This is important work but mice are not the best pre-clinical model of hemophilia. In contrast, dogs have been used as a pre-clinical model of hemophilia as demonstrated with the early studies with rFVIIa. A colony of hemophilic dogs is maintained by Tim Nichols at the University of North Carolina at Chapel Hill for these types of study. Margaritis and colleagues found that continuous expression of FVIIa from a transgene introduced into the dogs using gene transfer led to phenotypic correction in hemophilia dogs over a period of up to 45 months. Expression of canine FVIIa in the dogs at levels of 1.3-2.6 μg/ml provided safe and effective long-term hemostasis. The challenge of using this approach in human patients remains the immunological response to viral vector. Indeed, in humans a cytotoxic T lymphocyte response destroyed transduced hepatocytes and prevented long-term expression of FIX in hemophilia B subjects. Overcoming this obstacle could pave the way to gene therapy-based approaches to hemophilia treatment.

## VIII. Tissue factor in animal models

The final section in this series describes studies of TF in mouse models. The mouse is used because it is relatively easy to genetically manipulate the mouse genome and to create mouse lines with altered TF expression. However, it should be noted that mice are often not the best model of human disease and results need to be repeated in other animal models before clinical studies can begin. Gross and Vaezzadeh discuss the use of TF-positive MPs as a novel approach to treat hemophilia. The idea was first proposed by Wagner and colleagues who showed in 2003 that an increase in endogenous MPs restored hemostasis in hemophilia A mice. It should be noted that the role of TF-positive versus TF-negative MPs was not determined in this study. Gross and Vaezzadeh generate leukocyte-derived MPs *in vitro* and then analyze their ability to reduce bleeding in hemophilic mice. The PSGL-1-positive, TF-positive MPs are proposed to home to the site of vascular damage by binding to P-selectin on the activated platelets. This is an interesting approach. In hemophilia patients, it may be quite difficult to determine a dose of MPs that restores hemostasis without increasing the risk of thrombosis. Pawlinski and Mackman summarize our current knowledge on the different cell types that express TF in endotoxemia and sepsis. They also describe a series of experiments that analyze the cellular sources of TF that contribute to the activation of coagulation in endotoxemic mice. Surprisingly, TF expression by both bone marrow and non-bone marrow cells drives coagulation. Using a cell type-specific deletion approach, they found that myeloid cells (most likely monocytes) was the major source of TF amongst bone marrow cells, whereas the TF source of the non-bone marrow cell was not identified. TF expression by endothelial cells did not contribute to activation of coagulation in the model suggesting that the source of TF was

extravascular cells that are exposed to blood due the increased vascular permeability. The final review is by Monroe and colleagues. They describe their recent studies on wound healing in hemophilia B mice, which have delayed wound healing. These studies showed that adequate thrombin generation was required at the time of wounding and throughout the healing phase to fully restore wound healing in hemophilia B mice. Preliminary studies demonstrated that mice expressing low levels of TF also had a delay in wound healing. Future studies will compare and contrast wound healing in mice deficient in the intrinsic and extrinsic pathways. Marcus Carr from Novo Nordisk closes the issue with a review on the future directions of hemostasis: normalizing the lives of patients with hemophilia. Long-acting versions of rFVIIa, rFVIII and rFIX are being developed and may be available to patients in the near future.

## Conclusion

The TF-FVIIa complex is essential for hemostasis. Indeed, high doses of rFVIIa can be used as a bypass agent to restore hemostasis in hemophilia patients with inhibitors. However, the mechanism of action of rFVIIa is still debated with both TF-dependent and TF-independent pathways proposed. The regulation of TF-FVIIa activity remains an area of active research. Future studies should elucidate the roles of PS and PDI in the decryption process. A new hot area is TF-positive MPs, although standardization of the methods for determining levels of TF-positive MPs in plasma is urgently needed. It will be interesting to see if they can be used as a biomarker to identify patients at risk for thrombosis, particularly cancer patients. Although current mouse model systems yielded wealth of information in understanding the mechanisms of FVIIa and TF function in hemostasis and thrombosis, there are number of limitations exist with these model systems. Continual refinement of these models and development of new models in mouse along the use of other animal models are needed to broaden our understanding of the mechanisms of hemostasis and thrombosis.