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Tissue Factor Initiated Blood Coagulation

Keith Gomez and John H. McVey

Haemostasis and Thrombosis, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN

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1. ABSTRACT

Tissue factor (TF) is the cellular receptor and cofactor for blood coagulation factor (F) VII. Exposure of flowing blood to cells that express TF leads to the initiation of blood coagulation. Blood coagulation is tightly regulated to generate a local fibrin clot at the site of vascular injury without compromising blood flow in the vasculature. This chapter describes the initiation and propagation of the response and how it is ultimately down-regulated to prevent widespread inappropriate blood coagulation.

2. INTRODUCTION

The normal role of the vertebrate coagulation system is to rapidly prevent the loss of blood following vascular injury without compromising blood flow through either the uninjured or damaged blood vessels. Vertebrates have evolved a complex system to prevent blood loss that involves coordinate muscle contraction, cell aggregation (platelets) and the deposition of a clottable protein (fibrin). To achieve this a complex network of positive and negative regulated feed-forward and feedback reactions have evolved that result in controlled fibrin deposition and platelet activation only at the site of injury. The importance of such a system to multicellular organisms that possess a high-pressure vascular system is evidenced by the recent demonstration that the coagulation network is present in its entirety in all extant jawed vertebrates and must therefore have evolved over 430 million years ago (1-3).

Blood coagulation occurs when the enzyme thrombin is generated and proteolyses soluble fibrinogen, forming an insoluble fibrin polymer or clot. Platelets play an integral role in the formation of the stable thrombus and the processes of thrombin generation and platelet activation are intimately linked. Thrombin is a potent activator of platelets; conversely, activated platelets provide a phospholipid surface for the assembly of the coagulation complexes leading to thrombin generation. They release procoagulant molecules including, FV, FIX, FXI, von Willebrand factor (vWF), fibrinogen and plasminogen activator inhibitor -1, and the anticoagulant molecule tissue factor pathway inhibitor (TFPI) from storage granules. Blood coagulation is initiated by the exposure of coagulation FVII to cells that express the integral

membrane protein TF. This chapter will review our current understanding of how TF initiated blood coagulation is regulated and the pathophysiological consequences of aberrant expression of TF.

3. BLOOD COAGULATION NETWORK

Conventionally the coagulation system has been represented as a 'cascade' or 'waterfall' with 2 separate arms, the 'intrinsic' and 'extrinsic' pathways of blood coagulation (4,5). More recently it has become clear that whilst this view of coagulation is useful in understanding laboratory tests it does not accurately reflect the process of blood coagulation *in vivo*. In particular this scheme fails to explain the absence of a clinical bleeding tendency in deficiencies of FXII, prekallikrein or high molecular weight kininogen despite these deficiencies markedly prolonging surface-activated coagulation assays *in vitro*; it also fails to explain the less severe bleeding phenotype observed in FXI deficiency in contrast to the severe bleeding phenotype seen in deficiency of either FVIII or FIX; finally it fails to explain why deficiency of FVIII or FIX leads to a severe bleeding phenotype when the 'extrinsic pathway' should bypass the need for FVIII and FIX. The realisation that exposure of blood to cells expressing TF is necessary and sufficient to initiate coagulation *in vivo* has led to the current view of blood coagulation as an integrated network of interactions with positive and negative feedback loops (Figure 1). The intrinsic pathway (contact system) therefore does not appear to have an independent physiological role in haemostasis.

3.1. The resting state

In order to minimize blood loss from a site of injury, particularly from the high-pressure arterial circulation, an almost instantaneous response is required. The coagulation network therefore exists in a 'primed' state, however checks and balances have also evolved that ensure that inappropriate coagulation does not normally occur in the absence of vascular damage. Since blood coagulation *in vivo* is initiated by the exposure of FVII to cells that express the integral membrane protein TF, the primary control of haemostasis is the anatomical segregation of cells that express functional TF from other components of the coagulation network present in blood. TF is constitutively expressed at biological boundaries such as skin, organ surfaces, vascular adventitia and epithelial-mesenchymal surfaces. The TF expression pattern has been described as forming a 'haemostatic envelope', which ensures that following disruption of vascular integrity FVII/FVIIa in blood is exposed to cells that express TF, leading to the initiation of blood coagulation (6). Conversely it also ensures that inappropriate initiation of intravascular coagulation does not occur.

The endothelial cells that line the blood vessels of the vascular tree in the 'resting state' present an anticoagulant surface. Firstly, through expression of thrombomodulin (TM) and endothelial protein C receptor (EPCR), which play a pivotal role in the activation of the anticoagulant pathway; secondly through expression of glycosaminoglycans on their surface that promote the action of inhibitors of coagulation factors (antithrombin (AT) and TFPI); and lastly by the expression of the protease inhibitor, TFPI and protein S (PS) a cofactor in the anticoagulant pathway.

The serine proteases of the coagulation system (FVII, FIX, FX, XI, protein C (PC) and prothrombin) are all primarily synthesised in the liver and circulate in plasma as inactive zymogens that require proteolytic cleavage for activation. Cleavage generates a neo-N-terminus that then folds into a cleft in the protease domain creating a conformation essential for substrate binding and catalytic functions (7). The cofactors FV and FVIII are similarly synthesised primarily in the liver and also circulate as inactive forms that require limited proteolytic cleavage for activation.

Small amounts of activated factors: FIXa, FXa, FXIa and thrombin are continuously generated in normal individuals but these are rapidly inactivated by the protease inhibitor AT, a serpin (serine protease inhibitor), found at high concentrations in blood. The rate of inhibition by AT is substantially increased by binding glycosaminoglycans expressed on the surface of endothelial cells. FVIIa is not inhibited by AT and circulating levels of FVIIa average 0.4% of total FVII in blood (8). The levels of circulating FVIIa are influenced by both genetic and environmental factors. Triglyceride levels are a major determinant of FVIIa levels in blood and levels increase acutely in the post-prandial phase after a high fat meal (9). This increase is due to activation of zymogen rather than an increase in total FVII concentration and appears to be dependent on FIX since this rise is not observed in patients with FIX deficiency (haemophilia B)(10). FVIIa has little activity in the absence of its cofactor TF but this pool of FVIIa most likely serves to prime the system to respond upon exposure of TF.

Platelets actively participate in regulating thrombin production following injury to vessels. Thrombin is the most potent physiological activator of platelets and thrombin activated platelets release and/or recruit coagulation factors necessary both for accelerating thrombin generation and inhibiting prothrombin activation. Platelets store a number of procoagulant proteins within their α-granules, which include fibrinogen, FV, FIX, FXI and vWF that are rapidly released upon activation. Activated platelets also release TFPI and protease nexin II (PN-II; also known as beta-amyloid precursor protein, APP) an inhibitor of FXIa.

Thus in the absence of injury the balance of haemostasis is toward the maintenance of vascular blood flow by preventing or inhibiting blood coagulation, through the secretion or expression of anticoagulant molecules. However, all the factors necessary for a procoagulant response have been pre-synthesised and circulate in blood in inactive forms or are stored in secretory granules within endothelial cells or platelets ready for a rapid response following vascular damage. Furthermore the tissues that surround the vasculature express TF on their surface ready to trigger the initiation of coagulation upon exposure to blood.

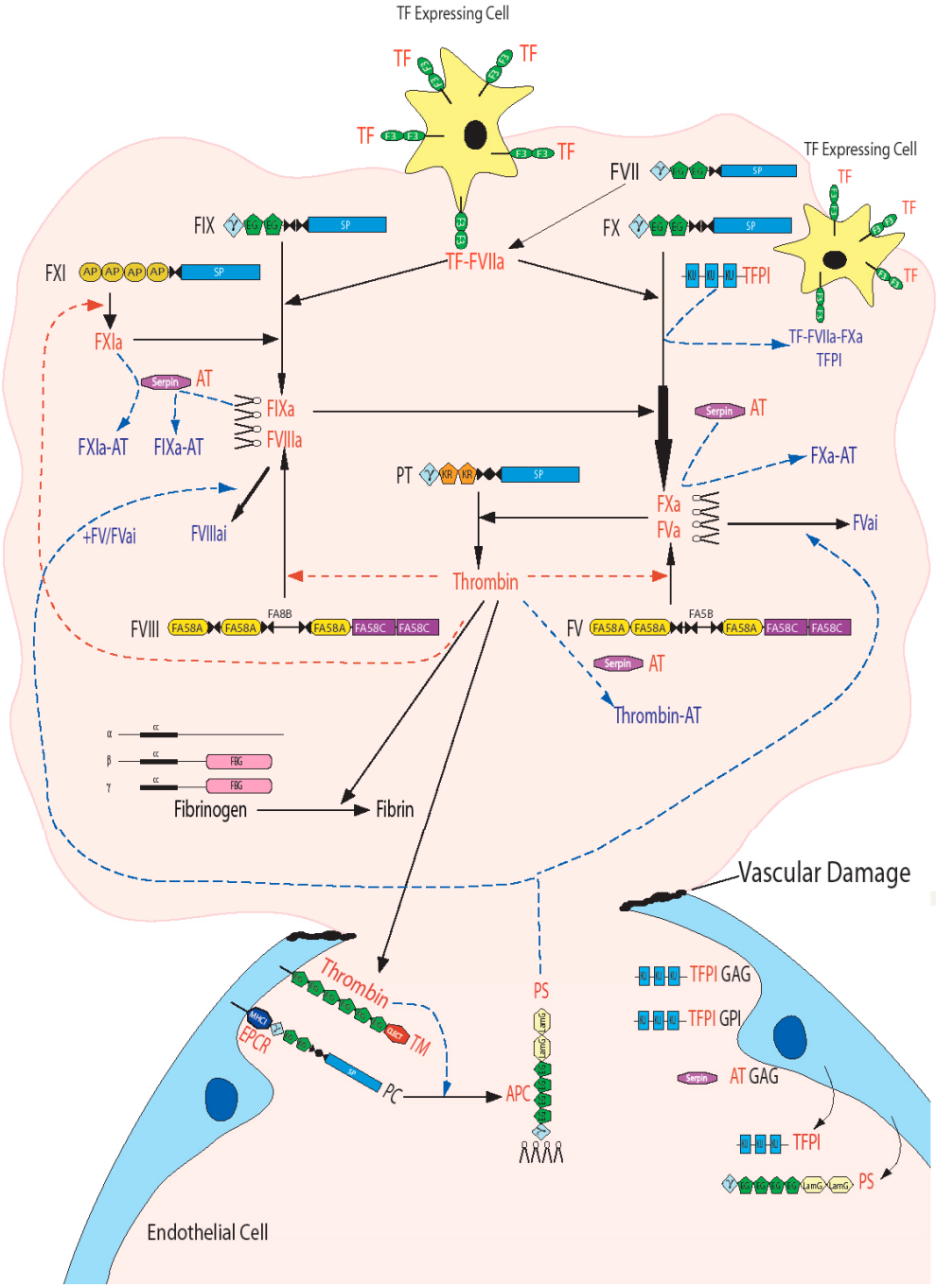

Figure 1. TF initiated blood coagulation following vascular damage. Following vascular damage blood is exposed to cells expressing TF on their surfaces. Formation of the TF-FVII/FVIIa complex initiates coagulation by activating FIX and FX. The trace amounts of thrombin generated in the initiation phase of coagulation are insufficient to initiate significant fibrin polymerisation but sufficient to activate FV and FVIII by limited proteolysis in a positive feedback loop (dashed red arrows). In the 'propagation phase' of coagulation, FVIIIa forms a complex with FIXa (tenase) and FXa forms a complex with FVa (prothrombinase) leading to the explosive generation of thrombin and a fibrin clot. Thrombin also activates FXI in a further positive feedback loop (dashed red arrow). FXIa is then able to activate further FIX independent of the TF-FVIIa complex. The FIXa-FVIIIa and the FXa-FVa complexes assemble on phospholipid surfaces. The initiation of blood coagulation is shut down by the action of TFPI, which forms a quaternary complex with TF-FVIIa-FXa. The serine proteases FIXa, FXa, FXIa and thrombin are all inhibited by AT. Thrombin bound to TM on endothelial cell surfaces activates PC bound to its receptor EPCR. APC in complex with its cofactor PS inactivates FVa and FVIIIa by further proteolytic cleavages. Dashed blue arrows indicate negative feedback loops. Domain organisation of proteins is indicated (see Structure Function) and all abbreviations are given in the text. Protein names are coloured: zymogens or pro-cofactors, black; functionally active proteins, red; inactived proteins, blue.

3.2. Initiation of blood coagulation

Following disruption of vascular integrity blood is immediately exposed to cells expressing TF, leading to the initiation of blood coagulation (Figure 1). The formation of the TF-FVII complex promotes the activation of FVII. Activation is catalysed by many proteases *in vitro*, but the most efficient catalyst *in vivo* is most probably autoactivation by either FVIIa or the TF-FVIIa complex formed from the pool of FVIIa within the circulation. The formation of the TF-FVIIa complex results in the activation of FIX and FX. FXa then activates prothrombin to thrombin, however in the absence of its cofactor (FVa), FXa generates only trace amounts of thrombin.

Mutations in the gene encoding TF have been predicted to lead to either a bleeding (loss of function) or a prothrombotic (gain of function) phenotype, however no congenital abnormalities have been described to date. Targeted disruption of the mouse TF gene (*f3*) results in embryonic lethality of $f3^{-/-}$ embryos at embryonic days 9.5-10.5, most probably due to a failure of vasculogenesis (11-13). Hence loss in early pregnancy most probably accounts for the lack of TF null individuals in clinical practice. Mice that express low levels of TF have recently provided some novel insights into the importance of TF and FVII in maintaining adequate haemostasis in particular vascular beds and tissues (reviewed in 14). Hereditary FVII deficiency (OMIM: 227500) is a rare autosomal recessive bleeding disorder, which in severe cases is often associated with life threatening gastrointestinal and central nervous system bleeds, reflecting the pivotal role of FVII in the initiation of coagulation. FVII deficiency associated with a bleeding phenotype is rare, however because novel cases are often identified in patients presenting with a prolonged prothrombin time without bleeding it is now the most commonly reported blood coagulation gene 'disorder'(15).

3.3. Amplification of the procoagulant response

Although insufficient to initiate significant fibrin polymerisation, trace amounts of thrombin formed in the 'initiation' stage of coagulation are able to activate FV and FVIII by limited proteolysis in a positive feedback loop. In the 'amplification' phase of coagulation, FVIIIa forms a complex with FIXa (the tenase complex) and generates further FXa, which in complex with FVa (the prothrombinase complex) leads to the explosive generation of thrombin that ultimately leads to the formation of a fibrin clot. Thrombin also activates FXI in a further positive feedback loop, resulting in further generation of FIXa independent of TF-FVIIa.

Deficiency of FVIII (haemophilia A; OMIM: 306700) and FIX (haemophilia B; OMIM: 306900) share an indistinguishable clinical phenotype, characterised by bleeding into muscles, joints and other organs with consequent damage, which if untreated leads to progressive arthropathy, musculo-skeletal deformity and early death. The severity of the bleeding in haemophilia A and B supports a key role for the FIXa-FVIIIa complex in the amplification phase of blood coagulation. In contrast, the relatively mild bleeding associated with FXI deficiency (OMIM: 264900) suggests that the back-activation of FXI by thrombin may only be required in severe trauma.

A key feature of these processes is the assembly of multiprotein complexes on a negatively charged phospholipid surface (Figure 2). Each of these complexes consists of a cofactor (TF, FVa, FVIIIa), an enzyme (FVIIa, FIXa, FXa) and a substrate that is a zymogen (FIX, FX and prothrombin) of a serine protease. The product of one reaction becomes the enzyme in the next complex. Platelets activated at sites of vascular injury play key roles in normal haemostasis. By adhering to the exposed subendothelium and aggregating, they create a physical barrier that limits blood loss. In addition, platelets accelerate thrombin generation by providing a negatively charged phospholipid surface that promotes the activation of FX and prothrombin. Furthermore, they release procoagulant factors that contribute to the local coagulation response. The importance of providing a negatively charged phospholipid surface for the assembly of the procoagulant response is seen in the extremely rare bleeding disorder, Scott syndrome (OMIM: 262890), which is characterised by a failure to expose phosphatidylserine on the outer leaflet of the plasma membrane and is associated with a moderate bleeding tendency (16,17).

3.4. Initiation of the anticoagulant response

Following the initiation of coagulation various inhibitory mechanisms prevent extension of the coagulation process beyond the site of vascular injury that might otherwise result in unnecessary occlusion of the blood vessel. TFPI associated with the endothelial cell surface rapidly inactivates the initiation complex by forming a quaternary inhibited complex (TF-FVIIa-FXa-TFPI) (Figure 3). Thrombin stimulates both endothelial cells and platelets to release further TFPI. TFPI inhibition of the TF-FVIIa complex is FXa-dependent, and kinetic analyses support the suggestion that TFPI likely reacts directly with FXa and FVIIa within the TF-FVIIa-FXa complex, however a two step mechanism in which TFPI firstly binds FXa and then interacts with the TF-FVIIa complex is also possible (18). Importantly, a consequence of this mode of action is that TFPI can only inhibit the initiation complex once FXa generation by the TF-FVIIa complex has occurred. It does not prevent initiation of blood coagulation but provides negative feedback thus preventing further generation of FXIa/FXa by TF-FVIIa. The relative local concentration of TF and TFPI will therefore be important in determining the efficiency of this process and this may be regulated in a tissue specific manner (19). This may explain the different bleeding phenotype seen in FVII deficiency in comparison with the classic haemophilias (A and B). Spontaneous muscle haematoma or haemarthroses are the typical features of haemophilia A and B but are not common in patients with FVII deficiency. The most frequent bleeding phenotype in FVII deficiency is mucosal bleeding including easy bruising, epistaxis, and menorrhagia. In severe cases life threatening CNS bleeding is common.

Endothelial cells constitutively express TM, which binds thrombin and by an allosteric mechanism alters its substrate specificity. The procoagulant substrates

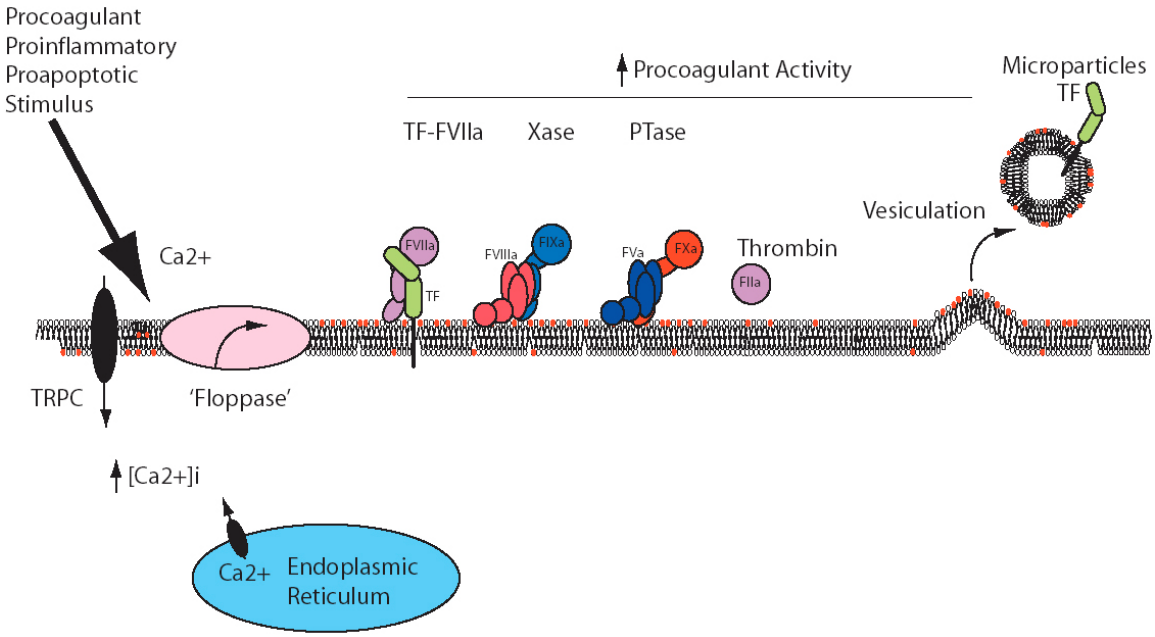

Figure 2. Phospholipid mediated procoagulant responses. Procoagulant, pro-inflammatory or pro-apoptotic stimuli lead to release of Ca^{2+} from intracellular stores. Calcium release induces transient receptor potential channel (TRPC) activation and calcium entry leading to redistribution of phosphatidylserine in the plasma membrane. The nature of the transporter (Floppase) mediating phosphatidylserine redistribution is unknown. Phosphatidylserine on the outer leaflet of the plasma membrane increases the activity of TF-FVIIa, the FIXa-FVIIIa (tenase) and FXa-FVa (prothrombinase) complexes, leading to increased thrombin generation. The transverse migration of phosphatidylserine is coincident with membrane vesiculation. The microparticles generated may contain TF. , represent phosphatidylserine and , represent neutral phospholipids.

of thrombin, including FV, FVIII and fibrinogen are no longer efficiently proteolysed. The preferred substrate of the thrombin-TM complex is PC. Thrombin converts PC to activated PC (APC) by a single proteolytic cleavage, which is enhanced by PC binding to its receptor, EPCR, present on endothelial cells. It should be noted that EPCR is not present on the surface of all endothelial cells that express TM (20, 21). This differential expression pattern may subtly alter rates of APC generation in different parts of the vascular tree. APC in complex with its cofactor PS rapidly inactivates the procoagulant cofactors FVa and FVIIIa by specific proteolytic cleavages in a negative feedback loop.

The activated coagulation proteases FIXa, FXa, FXIa and thrombin are all inhibited by AT. The rate of inhibition by AT is substantially increased by binding glycosaminoglycans on the surface of endothelial cells. In contrast, the inhibition of the TF-FVIIa complex by AT is too slow to be of physiological relevance.

TFPI is primarily located on the endothelial cell surface; APC is only generated at the endothelial cell surface and AT functions more efficiently at the endothelial cell surface suggesting the primary role of these anticoagulant molecules is to prevent extension of the procoagulant response beyond the vicinity of vascular injury thus preventing intravascular coagulation and maintaining blood vessel patency. The endothelium should not be regarded as a simple homogeneous cell type and relative expression of these anticoagulant molecules may vary between segments of the vascular tree and at any given location. Therefore the contribution of these molecules to the haemostatic balance may vary between different organs, within the vascular loop of a given organ and even between neighbouring endothelial cells of a single vessel.

Mutations in the gene encoding TFPI would be predicted to lead to a prothrombotic phenotype; however no congenital abnormalities have been described to date. Targeted disruption of the mouse TFPI gene results in embryonic lethality of 60% of *tfpi*-/- embryos at embryonic days 9.5-11.5, none of the remaining embryos survive to the neonatal period (22). Hence loss in early pregnancy most probably accounts for the lack of TFPI null individuals in clinical practice. Mutations in the gene encoding AT are associated with venous thrombosis (OMIM: 107300). The importance of AT is also demonstrated by the success of heparin anticoagulant therapy, which is mediated principally through the activation of AT. The relevance of PC activation by thrombin-TM/PC-EPCR complexes is evident from the severe hypercoagulable condition associated with homozygous deficiencies of PC (OMIM: 176860) or PS (OMIM: 176880), and the strong risk of venous thrombosis in individuals homozygous for the FV Leiden polymorphism (FV R506Q, OMIM: 227400.0001), which renders FVa resistant to inactivation by APC.

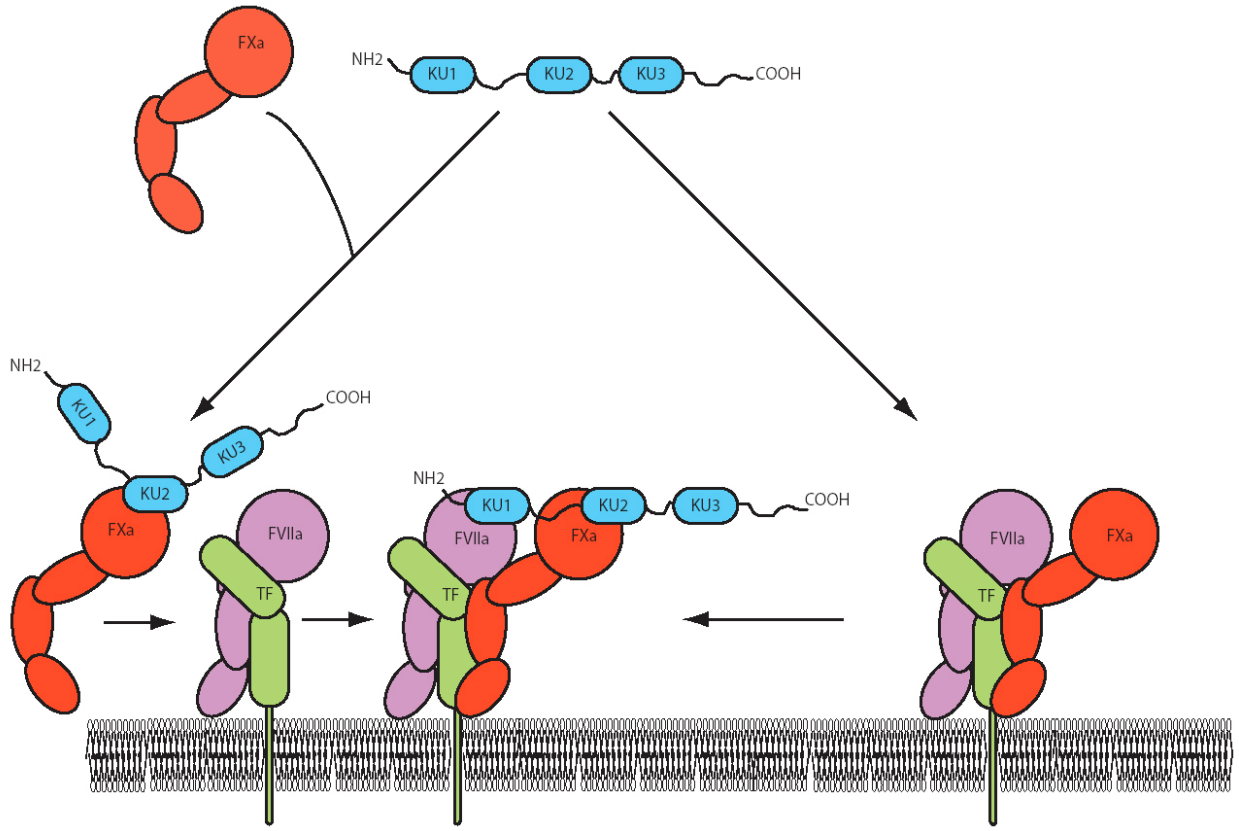

Figure 3. TFPI inhibition of TF-FVIIa-FXa. TFPI can either directly inhibit the transient TF-FVIIa-FXa complex or form a complex with free FXa: this binary complex then forms a quaternary complex with TF-FVIIa. The first Kunitz (KU1) domain of TFPI binds to FVIIa, KU2 binds to FXa. The role of the third Kunitz domain (KU3) is not known but is required for full anti-coagulant activity. The positively charged carboxy-teminal tail is believed to interact with glycosaminoglycans (GAGs) on the endothelial cell surface. TFPI-alpha is associated with a GPI anchored protein on the cell surface, TFPI-beta is GPI anchored on the cell surface (see section 4).

3.5. Intravascular tissue factor

Cells within the vasculature do not normally express TF; however induced expression of TF in cells within the vasculature is implicated in the pathogenesis of thrombosis in atherosclerosis, disseminated intravascular coagulation, malignancy and hyperacute rejection of xenografts (23-26). *In vitro* TF expression can be induced in endothelial cells and monocytes by a variety of agonists including endotoxin, cytokines and phorbol esters. TF gene expression is primarily controlled at a transcriptional level although post-transcriptional stabilisation of TF mRNA also contributes to its rapid induction. The proximal promoter has been extensively studied and in endothelial and monocyte derived cells the cis- and trans-acting factors required for appropriate TF gene regulation *in vitro* have been characterised (27). Induction of TF mRNA following stimulation occurs rapidly and peaks at 2h. TF protein levels on the cell surface peak at 4h and these remain high for up to 24h. *In vivo* expression of TF by endothelial cells however remains controversial. In an experimental model of Gram negative sepsis in baboons endothelial expression of TF could only be demonstrated in the splenic vasculature (28). Furthermore, there are conflicting data concerning the expression of TF on endothelial cells in tumour vasculature (29,30). In contrast, *in vivo* expression of TF on monocytes is clearly associated with inflammation, infection and atherosclerosis. Rupture of atherosclerotic plaques exposes a large amount of TF expressed on macrophage-derived foam cells leading to thrombosis and myocardial infarction. In endotoxemic animals and humans monocytes express TF leading to disseminated intravascular coagulation (31).

Recently, it has been proposed that TF in blood, so-called 'blood-borne' TF contributes to the propagation of the thrombus (32). Low but readily detectable levels of TF antigen are found in normal plasma (100-150pg/ml) (33) and these levels increase dramatically during disseminated intravascular coagulation, in patients with certain leukaemias, and in other conditions associated with increased thrombotic risk. This blood-borne TF is associated with microparticles (Figure 2) and the recruitment of these microparticles to thrombi appears to be dependent on P-selectin and PSGL-1 (34-37). These observations have led to a revised hypothesis of blood coagulation following vascular injury. In this model, vascular injury leads not only to the exposure of vessel wall TF, but also the expression of P-selectin on activated

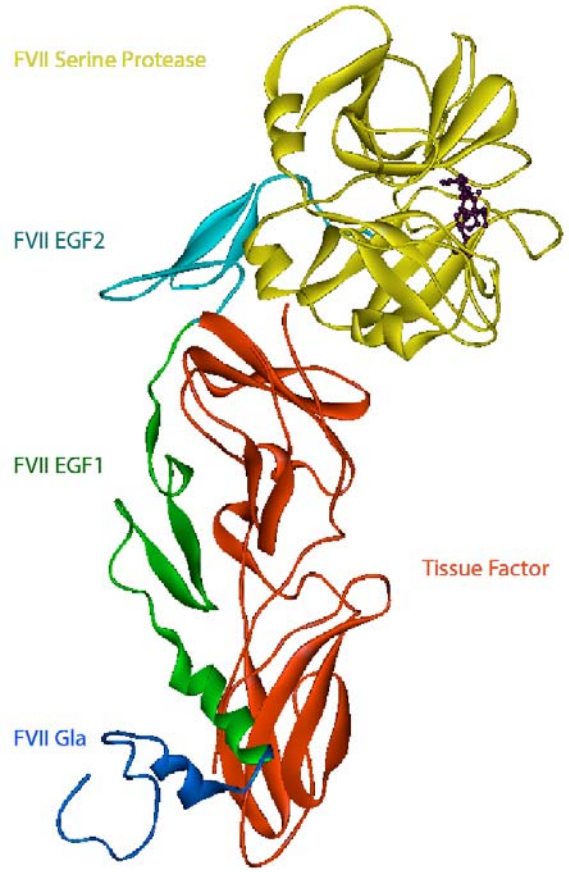

Figure 4. TF-FVIIa structure. Ribbon diagram of the TF-FVIIa structure. TF is shown in red; FVII Gla in dark blue ; FVII EGF1 in green; FVII EGF2 in light blue ; FVII SP in yellow. The active site inhibitor is represented by ball and stick. The coordinates are from the file 1DAN deposited in the Protein Data Bank (http://www.rcsb.org/pdb/). The figure was prepared using the program WebLab ViewerLite 3.3 (http://www.accelrys.com).

endothelial cells and adherent platelets. The vessel wall TF initiates coagulation leading to thrombin generation and platelet accumulation. Subsequent recruitment of TF-positive microparticles to the growing thrombus via the interaction of P-selectin and PSGL-1 initiates generation of a fibrin meshwork and contributes to further platelet accumulation. At present there are still a number of unanswered questions. What is the source of this blood-borne TF? How are these microparticles formed? Is this process regulated? Furthermore, since blood remains fluid in the absence of vascular injury TF-microparticles must normally circulate in an inactive form but become activated when incorporated into the developing thrombus.

4. STRUCTURE/FUNCTION OF TF, FVII and TFPI

4.1. Tissue factor

TF is structurally organized in three domains: an extracellular domain extends from the mature N-terminus to residue 219, a 23-residue hydrophobic sequence which follows represents the transmembrane spanning segment and a cytoplasmic domain of 21 residues contains a cysteine (Cys245) which may be acylated to palmitate or stearate on the inner leaflet of the membrane. The extracellular region consists of two-fibronectin type III modules orientated at an angle of 125˚ to each other (38). The extracellular domain of TF is necessary and sufficient for procoagulant activity since recombinant variants lacking either the intracellular domain or both the intracellular and transmembrane domains retain full procoagulant activities (39). TF functions as a cellular receptor and cofactor for FVII/FVIIa, enhancing the proteolytic activity of the bound protease several thousand fold. It achieves this in several different ways: binding of FVII to TF makes it exquisitely sensitive to proteolytic activation; through an allosteric effect on the active site it enhances the proteolytic activity of the enzyme and lastly it localises FVIIa on the cell surface at an appropriate distance from the membrane, enabling its macromolecular substrates FIX and FX to dock with the TF-FVIIa complex, thereby allowing efficient cleavage (40).

TF has been implicated in a number of coagulation-independent functions, including inflammation, angiogenesis and tumour metastasis, and it has been proposed to have a role in cell signalling (41-43). It is still unclear how formation of the TF-FVIIa complex influences various biological processes, however, emerging evidence suggests that TF-FVIIa participates in cell signalling through its proteolytic activity either directly by activating receptors or indirectly by generating FXa and/or thrombin that then activate cellular receptors. The receptors responsible for transducing the extracellular proteolytic activity are the protease-activated receptors (PARs) that belong to the family of seven transmembrane domain, G-protein-coupled receptors (44, 45). The PARs, of which there are 4 known members: PAR 1, 2, 3 and 4, are activated by proteolytic cleavage leading to the exposure of a neo-amino terminus that folds back and activates the receptor. Thrombin activates PAR-1, PAR-3 and PAR-4, whereas FXa and TF-FVIIa activate PAR-2. It is likely that the failure of TF-dependent generation of coagulation proteases and PAR signalling is responsible for the death *in utero* of *f3*$^{-/-}$ mice (11-13) rather than the failure to generate fibrin to control haemostasis since mice deficient in fibrinogen develop normally but die shortly after birth due to a severe haemostatic defect (46). In addition, mice deficient in PAR-4 that are unable to thrombin-activate their platelets also develop normally but die in the perinatal period due to severe haemorrhage further suggesting that the role of TF in early embryogenesis is non-haemostatic (47).

TF is structurally related to the cytokine receptor family (38). Cell signalling via these receptors usually involves participation of the cytoplasmic domain; however, although the 21-amino acid cytoplasmic domain of TF contains serine residues (Ser253 and Ser258) that are phosphorylated when cells are stimulated with phorbol esters, evidence for a direct role in signal transmission was limited. Mice that have been engineered to only express TF lacking the cytoplasmic domain develop normally and are

fit and healthy (48). Interestingly, they appear to be more resistant to endotoxemia due to a failure to recruit and activate leucocytes (49). Recently a functional role for phosphorylation of the TF cytoplasmic domain was shown in tumour metastasis (50). The TF-FVIIa complex was shown to promote tumour and developmental angiogenesis through PAR-2 signalling and the TF cytoplasmic domain was shown to regulate this process. The cytoplasmic domain is primarily unphosphorylated and palmitoylation of Cys245 suppresses agonist-induced phosphorylation of Ser258. PAR-2 signalling leads to phosphorylation of the cytoplasmic tail, which is thought to release the negative regulatory control of the TF cytoplasmic tail on PAR-2 mediated angiogenesis (51). Thus loss of palmitoylation in conjunction with up regulation of PAR-2 determines the degree of phosphorylation of the TF cytoplasmic domain and PAR-2 mediated angiogenesis.

4.2. Factor VII

FVII is synthesized primarily in the liver and circulates in blood (10nM) as a single chain molecule of 416 amino acids (molecular mass: 50kDa), with a half-life of approximately 3h. FVII shares a common protein structure with FIX, FX and PC: they are all zymogens of vitamin K-dependent serine proteases comprised of a Gla (gamma-carboxylated glutamic acid)-aromatic stack-EGF1 (epidermal growth factor-like)-EGF2-SP (serine protease) domain structure (Figure 4). Although they have distinct functional properties within the coagulation network, analysis of the gene organizations, protein structures and sequence identities suggest they have resulted from gene duplication events. Indeed FVII and FX are tandemly linked on chromosome 13 (q34) suggesting they have arisen through tandem gene duplication.

In FVII the Gla domain contains ten glutamic acid residues that are post-translationally modified by the addition of a carboxyl group to the gamma-carbon by a vitamin K-dependent carboxylase. Blocking this post-translational modification by coumarin derivatives such as warfarin represents the current therapy of choice for the long-term treatment and prevention of thromboembolic events. The Gla domain forms a Ca^{2+} dependent fold that confers affinity to negatively charged phospholipid membranes, promoting the assembly of functional complexes on these surfaces. The EGF domains are widely dispersed in nature and are often involved in protein-protein interaction. The typical structure is a beta-pleated sheet maintained by a characteristic 1-3, 2-4, 5-6 arrangement of three disulphide bonds. The aspartate at position 63 is post-translationally modified by beta-hydroxylation. There are two O-linked glycosylation sites at Ser52 and Ser60. The serine protease domain is homologous to that of chymotrypsin and contains the archetypal catalytic triad: His193, Asp242 and Ser344 that is critical for catalytic activity of these enzymes. In this family of proteases the structural elements responsible for the chymotrypsin-fold are highly conserved: amino acid substitutions to surface loops that border the active site and the substrate recognition pocket confer the diverse functional properties of these proteases. FVII is activated by a single proteolytic cleavage between residues Arg152 and Ile153 yielding a two-chain disulphide-linked molecule. Cleavage generates a neo-N-terminus that then folds into a cleft in the protease domain creating a conformation essential for substrate binding and catalytic functions. FVIIa has little activity in the absence of its cellular receptor and cofactor TF.

4.3. TF-FVIIa complex

Extensive site-directed mutagenesis of TF and FVII in conjunction with the structures of TF, TF-FVIIa and 'zymogen' FVIIa has provided detailed insight into the allosteric activation of FVIIa and the function of the TF-FVIIa complex (reviewed in 52). The structure of the complex of active site-inhibited FVIIa and the extracellular domain of TF (Figure 4) revealed the main sites of contact on FVIIa for TF are located in EGF1 and the protease domain, with additional points of contact involving the aromatic stack/Gla region and EGF2 domain (53). The main sites of contact on TF for FVIIa are located on both fibronectin type III domains and the interface region between them.

The activation of FVII requires cleavage of the peptide bond between Arg152 and Ile153. However, whereas for chymotrypsin and the other blood coagulation proteases this produces a fully formed active site for FVIIa there is an additional requirement of association with TF. FVIIa without TF has been described as zymogen-like. Indeed the insertion of the neo-N-terminus essential for activity seems incomplete, thus FVIIa exists in equilibrium between partially and fully active forms that is driven to the active enzyme under the influence of TF. The solution of the structure of a truncated 'zymogen' FVIIa has provided further insight into the mechanism of TF activation of FVIIa (54). It has been proposed that when FVIIa binds TF there is a re-registration of a beta-strand in the protease domain of FVIIa that results in loss of H-bonds that normally form an impediment to insertion of the neo-N-terminus required for the formation of a competent active site.

Cells that express TF have much lower procoagulant activity at rest than following treatment with a calcium ionophore. Although TF is present on the surface of the cell it only becomes fully active when the properties of the membrane are altered. This 'encrytpted' TF is most likely due to changes in the phospholipid environment. In resting mammalian cells phosphatidylserine and phosphatidylethanolamine are sequestered in the inner leaflet of the plasma membrane under the control of an inward aminophospholipid translocase. When subjected to stimulation cells of the vascular compartment relax this membrane asymmetry (Figure 2). The procoagulant activity of TF is highly sensitive to the availability of phosphatidylserine, thus TF de-encryption may be the result of phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane. The migration of phosphatidylserine to the outer leaflet is coincident with membrane blebbing and consequent shedding of microvesicles that may contain TF.

4.4. TFPI

TFPI is synthesised by endothelial cells and megakaryocytes, and two different forms are produced

through alternative mRNA splicing: TFPI-alpha and TFPI-beta (55, 56). TFPI-α is a 276 amino acid, 46kDa glycoprotein with an acidic N-terminal sequence followed by 3 tandem domains with homology to the Kunitz-type protease inhibitors (KU) and a basic C-terminal region. TFPI-beta is a 225 amino acid protein in which the Ku3 and the basic C-terminal domain of TFPI-alpha are replaced with an alternative C-terminal domain that directs the attachment of a glycosyl phosphatidylinositol (GPI) anchor. The KU2 domain binds and inhibits FXa and thus TFPI can act as a direct protease inhibitor of FXa. The KU1 domain binds and inhibits TF-FVIIa in a manner dependent on FXa generated by the initiation complex. The KU3 domain has no known protease substrate but is necessary for full anticoagulant activity. The basic C-terminus is required for rapid inhibition of FXa by KU2 and interaction with the cell surface. Circulating concentrations of TFPI are approximately 2.5nM, but this pool of TFPI is largely truncated at the C-terminus and has poor anticoagulant activity. The largest pool of TFPI is associated with the endothelial cell surface. Heparin infusion induces a large increase (2- to 4-fold) in the plasma concentration of TFPI-α (57) suggesting that TFPI-alpha binding to ECs is mediated by surface glycosaminoglycans, however recent evidence indicates that a significant portion of TFPI-alpha associates with the cell surface through interaction with a GPI-anchored protein (58, 59). *In vitro* PI-specific phospolipase C which cleaves GPI membrane anchors releases 80% of TFPI from the surface of cultured endothelial cells and the remaining 20% is released by heparin treatment. The majority of cell-surface bound TFPI is therefore either GPI-anchored or is tightly bound to a surface GPI linked protein. A consequence of the attachment of TFPI through a GPI-anchor is its localization into caveolae/lipid raft microdomains on the cell surface. Although the functional significance of this is not clear as membrane localization to lipid rafts does not appear to enhance inhibition of TF-FVIIa by TFPI. TFPI is also found in platelets and is released in response to stimulation by thrombin.

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Send correspondence to: Dr J.H. McVey, Haemostasis and Thrombosis, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK, Tel: 44-20 8383 8253, Fax: 44-870 131 3540, E-mail: john.mcvey@csc.mrc.ac.uk

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