The role of the Tissue factor pathway in hemostasis and beyond

John H. McVey

School of Bioscience & Medicine
Faculty of Health & Medical Sciences
University of Surrey
Guildford
GU2 7XH UK

Correspondence to John H. McVey:
Department of Biochemical Sciences
Faculty of Health & Medical Sciences
University of Surrey
Guildford
GU2 7XH UK

Tel: +44 1483 686390; e-mail: j.mcvey@surrey.ac.uk

Tissue factor pathway in haemostasis and beyond McVey

Purpose of review:

The role of tissue factor (TF) in the initiation of blood coagulation network leading to the generation of a fibrin clot has been well defined over the past 50 years. Although much is known about this sequence of events and its regulation many important questions remain unresolved. More recently, a complex role for TF in cellular processes independent of fibrin generation has emerged. This review summarizes some of the advances in this field.

Recent findings:

TF is the cellular receptor and cofactor for factor VII/VIIa however controversy still surrounds expression of TF within the vasculature, the role of circulating microvesicle pools of TF and mechanisms of 'encryption' of TF activity. However, there have been significant advances in the role of TF initiated cell signalling. Lastly, an alternatively spliced TF transcript has been identified and some insights into its role in cancer cell metastasis/proliferation have been elucidated.

Summary:

Understanding of TF structure-function has increased substantially however multiple controversies still surround some aspects of its regulation. TF has emerged as a pivotal player in orchestrating not only fibrin generation but in wound repair. Derangement of these repair processes contributes significantly to the pathophysiology of a number of disease processes.

Keywords

TF, FIII, CD142, thromboplastin

Introduction

The initiation of blood coagulation and subsequent wound repair is a fundamental defence mechanism conserved in all extant vertebrates [1,2]. The high blood pressure generated on the arterial side of the vertebrate circulation requires a powerful almost instantaneous but strictly localized procoagulant response in order to minimize blood loss from sites of vascular injury without

compromising blood flow. In addition it interacts with other defence mechanisms, such as the immune system and the inflammatory response. At the heart of this complex network of interacting pathways is the cellular receptor tissue factor.

Tissue Factor: structure and function

Tissue factor (TF) also known as thromboplastin or factor (F) III is a 47kDa type 1 transmembrane glycoprotein that is the cellular receptor and cofactor for blood coagulation factor VII/VIIa (FVII/VIIa). The gene encoding human TF (*F3*) is located on chromosome 1 (1p22-p21) and spans 12640 nucleotides and consists of 6 exons. The 2351 nucleotide mRNA transcript encodes a 295 amino acid primary translation product that is processed to a mature TF protein of 263 amino acids in length after cleavage of the 32 amino acid signal peptide. The 219-residue extracellular region consists of two-fibronectin type III domains orientated at an angle of 125° (Figure 1) anchored into the cell membrane by a 23-residue transmembrane sequence followed by a 21 amino acid cytoplasmic tail. There are 3 N-linked glycosylation sites (N43, N156 & N169), 2 disulfide bridges (C81-C89 & C275-C295), an S-palmitoyl cysteine (C277) and a phosphorylated serine at position 290 (all residues are numbered according to Goodeve et al (2011) [4] using HGVS nomenclature numbering the initiation methionine (+1) of the reference protein sequence (NP_001984) and may differ from previous publications by legacy numbering +32). These potential post-translational modification sites with the exception of the N-linked glycosylation site N156 are all highly conserved (Figure 1).

Systematic site-directed mutagenesis [5-10] and x-ray crystallographic analysis [11-13] of TF and TF-FVIIa complexes have identified the molecular determinants of FVII binding to TF. The extracellular region of TF binds tightly to FVII/FVIIa to form a highly active procoagulant complex. The extracellular region of TF is both necessary and sufficient for activity; as variants lacking either the 21-residue cytoplasmic domain or both the cytoplasmic and transmembrane domains retain full procoagulant activities [14,15]. The substrates of the TF/FVIIa complex are FIX and FX. FVII, IX & X share a common protein structure, they are zymogens of vitamin K-dependent serine proteases with the domain architecture: γ -carboxylated (Gla) domain, epidermal growth factor (EGF) like domain 1, EGF like domain 2 and serine protease domain (Gla-EGF1-EGF2-SP; Figure 1). The Gla domain contains a number of glutamic acid residues that are post-translationally modified by addition of a γ -carboxyl group in a vitamin K-dependent process. It forms a Ca^{2+} dependent fold that confers affinity to negatively charged phospholipid membranes. EGF-like domains are widely dispersed in nature and are often involved in protein-protein interactions. The serine protease domain is homologous to that of chymotrypsin. Although the structural elements responsible for the trypsin fold and archetypal catalytic triad serine, histidine and aspartate are highly conserved, amino acid substitutions to surface loops that border the active site and substrate recognition pocket confer diverse functional properties to the individual proteases [16]. FVII/VIIa interacts with the extracellular domain of TF primarily through the Gla and EGF-1 domains although the EGF2 and serine protease domains also contact TF [12]. Similarly, Gla and EGF-1 domains of the substrates, FIX and FX, represent the primary determinants in binding to TF-FVIIa and formation of the ternary complex [17,18]. A putative substrate-binding region (S195 G196 K197 K198) [7,19-21] that is highly conserved (Figure 1) in TF has been identified in binding FIX/FX through their Gla domains. The importance of Ca^{2+} ions in generating the characteristic loop structure of the Gla domain is well documented, however recently it has been shown that at physiologic concentrations of divalent

cations Mg^{2+} occupies 2 or 3 of the divalent ion binding sites in the Gla domain. These results suggest that Mg^{2+} plays an important role in physiologic blood coagulation. Furthermore residues in the TF substrate-binding region have been implicated in binding the FX Gla domain in a Mg^{2+} -dependent manner [13,22,23].

Conversion of FVII to FVIIa is the result of a single proteolytic cleavage at Arg212–Ile213 (nomenclature according to Goodeve et al [4], legacy numbering +60) peptide bond producing a disulfide-linked two-chain molecule. Unlike other members of the trypsin superfamily the neo N-terminus generated upon activation of FVII fails to insert into the activation pocket leading to a non-optimal alignment of the catalytic machinery, rendering FVIIa ‘zymogen like’ with significantly reduced catalytic activity. Binding of FVIIa to TF allosterically corrects this defect, transforming FVIIa into a catalytically competent enzyme. In addition, TF ensures optimal orientation and positioning of the FVIIa catalytic domain above the membrane surface for optimal interaction with its substrates (FIX & FX), thereby enhancing the proteolytic activity by 10^6 -fold [24,25].

Tissue factor initiates blood coagulation

Exposure of blood to cells expressing TF on their surface is both necessary and sufficient to initiate blood coagulation in vivo. FVII bound to TF is exquisitely sensitive to activation and while many enzymes are capable of converting FVII to FVIIa the most important one in vivo is likely FXa. However, how is this triggered if FXa is the product of TF-FVIIa? In blood 4% of total circulating FVII is in the form of FVIIa that has little functional activity in the absence of its cofactor TF [26]. It is this pool of FVIIa that most likely serves to prime the system to respond upon exposure to TF. Activation of FVII to the protease FVIIa and formation of a high affinity complex of TF-FVIIa results in activation of FIX and FX by the TF–FVIIa complex. A schematic of TF-initiated thrombin generation, including the important feedback reactions of thrombin, is shown in Figure 2.

The TF–FVIIa complex is rapidly inactivated by tissue factor pathway inhibitor (TFPI) a Kunitz-type inhibitor released from thrombin-activated platelets or found on the surface of endothelial cells. TFPI inhibits TF-FVIIa by the formation of a quaternary inhibited complex of TF-FVIIa-FXa-TFPI in a FXa dependent manner (Figure 2) (Recently reviewed in [27,28]). The duration of the functional TF-FVIIa complex is therefore dependent on the relative concentrations of TF and TFPI and these may vary at a particular site of vascular injury. This may help explain the differential bleeding phenotypes seen in different coagulation deficiencies. The sites most commonly affected by spontaneous bleeding in haemophilia A or B patients, skeletal muscle and joints, have undetectable to low TF expression.

Mutations in the gene encoding TF would be 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 [29-31] and therefore loss in early pregnancy most probably accounts for the lack of TF deficient individuals seen in clinical practice.

Tissue factor expression

TF is constitutively expressed on extravascular cells of many tissues such as skin, organ surfaces, vascular adventitia and epithelial–mesenchymal surfaces [32,33]. The cellular distribution of TF at biological boundary layers led to the proposal that it functions as a ‘haemostatic envelope’, ensuring that following disruption of vascular integrity blood is immediately exposed to cells expressing TF, leading to the initiation of blood coagulation.

Cells within the vasculature including endothelial cells (EC) do not normally express TF. However, induced expression of TF in cells within the vasculature is implicated in the pathogenesis of thrombosis in atherosclerosis [34], disseminated intravascular coagulation [35], malignancy [36] and hyperacute rejection of xenografts [37]. In vitro TF expression can be induced in ECs and monocytes by a variety of agonists. TF expression is primarily controlled at the transcriptional level although post-transcriptional stabilization of TF mRNA also contributes to its rapid induction [38,39]. The proximal promoter has been extensively studied and cis- and trans-acting factors required for appropriate gene regulation in vitro have been characterised (reviewed in [40]). 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. Although the response of both monocytes and ECs in vitro to a variety of different agonists has been well documented and shown to be very similar, evidence for EC expression of TF in vivo is scant and remains controversial. In an experimental model of Gram negative sepsis in baboons EC expression of TF was reported only in the splenic vasculature [41]. More recent data using this model demonstrated immune-reactive TF in the aorta, however this was restricted to granular structures on the surface of ECs and it could not be determined whether the TF was produced by ECs or transferred to the EC surface [42]. Two studies in mice and rabbits have reported TF immunoreactivity in ECs following treatment with lipopolysaccharide (LPS) but the data is open to other interpretation [43,44]. In contrast, a number of other studies have failed to detect TF expression in ECs in LPS treated mice and rabbits [40,45-47]. Genetic approaches to generate cell lineage-specific TF gene deletion transgenic mice has shown that TF expression in ECs in vivo does not contribute to the initiation of coagulation in a mouse model of endotoxemia or carotid artery thrombosis. In contrast, in vivo induced expression of TF on monocytes/macrophages is clearly associated with disease conditions. 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.

Another controversial and complex area of interest is that of ‘blood borne’ extracellular TF and its role in propagation of thrombosis. Immuno-reactive TF is detectable in plasma either as a soluble protein or on circulating microparticles (MPs; also known as microvesicles or extracellular vesicles). MPs are defined as heterogeneous, submicron (0.1-1 μ m) vesicles released from cells in response to various stimuli. They have an intact phospholipid bilayer membrane and carry membrane proteins specific to their cellular origin. However, transfer from one MP to another or to other cell types via specific receptor-mediated interactions or fusion may mean surface antigens do not accurately reflect their cellular origins. Under normal conditions cells maintain a phospholipid asymmetry with negatively charged PS confined primarily to the inner leaflet of the plasma membrane. The collapse of this asymmetry appears critical to the formation of MPs and therefore the majority are PS positive, as assessed by binding of fluorescently labelled annexin V or lactadherin. The role of MPs in many pathological conditions, including thrombosis, has been extensively studied (Recently

reviewed in [48]). The contribution of MPs to a procoagulant phenotype may arise through the provision of a negatively charged phospholipid surface to promote the assembly of the procoagulant complexes or because the MPs are also TF positive. MPs positive for TF are low to undetectable in healthy individuals but increase significantly in various pathological conditions. The analysis of circulating MPs and their procoagulant phenotype is however fraught with difficulties as pre-analytical parameters can impact the outcome. These include: the type of anticoagulant, phlebotomy, transport, centrifugation and freezing of blood samples. The International Society of Thrombosis and Haemostasis (ISTH) issued guidance on standardization of protocols to reduce variability in sample preparation [49]. Similarly, there are several analytical variables that can affect the detection and quantification of MPs by flow cytometry, which is the most common method of MP analysis in biological samples. These include: the type of flow cytometer and its ability to measure submicron particles, gating strategies and the choice of assay to detect TF. The ISTH has also established a standardized method for quantification of MPs by flow cytometry that uses size calibrated fluorescent beads which may help to address some of these issues [50]. MP TF levels can be measured either antigenically using flow cytometry or ELISA or functionally by measuring FXa generation. In the case of antigen measurement careful choice of appropriate antibodies is essential to ensure specific detection of total TF. For example, the inhibitory anti-human TF monoclonal antibody HTF-1 competes with FVII/FVIIa for binding to TF and may not adequately detect TF-FVII complex thereby underestimating TF levels [51]. Plasma TF concentrations have been quantified in multiple studies using the Imubind TF ELISA (American Diagnostica) which uses monoclonal capture and polyclonal detection antibodies, however comparison of results obtained with other monoclonal assays suggest the Imubind assay detects other cross-reactive proteins and may not accurately reflect plasma TF levels [52]. Activity assays offer the advantage of specificity and sensitivity as well as detecting functional TF-FVIIa activity toward its macromolecular weight substrate FX [53]. However, it should be noted that antigen and activity based assays may not correlate with each other, which may be a result of TF encryption (discussed below) rather than a technical problem with the assays.

Tissue factor encryption

In virtually every cell type TF exists in two forms: a latent or encrypted form that binds FVII/FVIIa but does not activate FX and an active or de-encrypted form that activates FX to FXa and is therefore procoagulant. Both forms of TF are expressed on the surface of cells and encrypted TF can be converted to a procoagulant active form in a number of experimental ways that include cell lysis, calcium ionophore treatment or LPS, thrombin or cytokine treatment. The mechanism(s) that regulate the activity of TF are controversial. The two schools of thought will be briefly described in this review but the reader is referred to the following articles for an in depth discussion of the conflicting views [54,55].

Membrane phospholipid asymmetry is actively maintained by the action of a number of molecular mechanisms involving: flippases (transport PS to the inner leaflet), floppases (transport PS to the outer leaflet) and scramblases (transport PS bidirectionally). Inhibition or activation of any of these enzymes may lead to a collapse of the PS asymmetry. The phospholipid content of membranes has a significant impact on the procoagulant function of TF. Relipidation of purified TF in vesicles containing PS greatly accelerates TF procoagulant activity. One school of thought is that TF procoagulant activity is regulated by the local phospholipid microenvironment. An alternative school

of thought suggests TF is encrypted in an inactive form by reduction of the disulfide bond closest to the membrane (C275-C295) and activated by oxidation to form the disulfide bond. It is proposed that this thiol-disulfide exchange is regulated by protein disulphide isomerase (PDI), an abundant oxido-reductase predominantly expressed in the lumen of the endoplasmic reticulum but secreted and associated with the surface of various cell types, including platelets, monocytes and macrophages. The role of the disulfide bond in TF function remains controversial and has polarised opinion.

Tissue factor signalling

As discussed above the TF-FVIIa complex catalyses the activation of FIX and FX leading to thrombin generation and subsequently formation of a fibrin clot. Over the last twenty years it has become clear that activated coagulation proteases play an important role in cellular signalling independent of fibrin generation. This occurs through activation of protease-activated receptors (PARs), a subfamily of seven-transmembrane G-protein-coupled receptors. There are four members of the PAR family (PAR-1, -2, -3 and -4) encoded by the their respective genes (*F2R*, *F2LR1*, *F2RL2* and *F2RL3*). Their unique mechanism of activation involves specific enzymatic cleavage of the PAR extracellular domain, generating a neo-amino terminus that functions as a 'tethered ligand' by binding intramolecularly and generating an intracellular signal through G-proteins leading to PI hydrolysis, Ca^{2+} transients and activation of intracellular kinases. Vitamin K-dependent coagulation factors with the exception of FIXa are able to selectively activate cell surface PARs. Thrombin can directly activate PAR-1, -3 and -4 but not PAR-2, however PAR-2 can be trans-activated by thrombin activated PAR-1 when the N-terminal PAR-1 tethered ligand is able to interact with the binding pocket of an adjacent PAR-2 receptor. Although thrombin can activate both PAR-1 and PAR-4 markedly higher concentrations of thrombin are required to activate PAR-4. FXa can directly activate both PAR-1 and -2 as well as through the ternary TF-FVIIa-FXa complex, whereas the TF-FVIIa complex can only activate PAR-2. The PARs activation has been shown to elicit intracellular signalling that results in a plethora of cellular responses including proliferation, migration, cell shape change, secretion of cytokines/chemoattractants, degranulation as well as the initiation of various transcriptional responses. It has become clear that coagulation protease activation of PARs plays an important role in normal haemostasis, vascular development, inflammation in a variety of disease models, cancer biology as well as fibrosis seen in a number of pathological situations across all major organ systems [56].

Coagulation protease signalling through PARs is complex. PARs are expressed on a variety of different cell types in a cell-type specific manner; however the same cell type isolated from different sources may display different expression patterns of PARs. For example, primary fibroblasts of different origin (dermal vs lung) show distinct PAR expression profiles and respond differently to FXa stimulation in vitro [57]. Similarly, ECs from different origins have been shown to respond differently to PAR agonist peptides suggesting differential PAR expression [58]. Furthermore, although following tissue injury cells may be exposed to blood containing FVII/FVIIa, FX and prothrombin, in the absence of tissue damage the source of coagulation factors may be local and may not reflect the makeup or concentrations found in plasma and therefore protease specific responses may occur [59-62]. Initially, because TF-FVIIa and FXa ultimately lead to the generation of thrombin and PAR-1 signalling the significance of the other protease complexes in activating PARs and initiating cellular responses was largely overlooked. However, the recognition that PAR-2 signalling plays a key role in

a number of pathological responses has reignited interest in FXa as an important mediator of inflammatory responses [63].

Alternatively Spliced Tissue factor

This review has concentrated on the expression and function of full-length TF, however another isoform of TF generated by alternative splicing of the TF transcript has been described [64]. The alternatively spliced TF mRNA transcript lacks exon 5 which results in a translation product (AS-TF) of 206 amino acids that shares the first 166 amino acids with full-length TF but lacks 3 of the β -strands (E, F & G) that comprise the C-terminal immunoglobulin-like domain which includes the disulfide bridge C275-C295 (Figure 3). In addition AS-TF also lacks the transmembrane and cytoplasmic domains and is therefore a soluble protein with a unique 40 amino acid C-terminus (Figure 3). The unique neo-C-terminus does not appear to be highly conserved as protein database searches fail to identify TF sequences with sequence identity with the exception of some primate sequences. The mouse AS-TF mRNA has been cloned [65] and the sequence of the C-terminal region shares limited amino acid identity (18/40) and is an additional 53 amino acids longer. The expression pattern of AS-TF essentially mirrors that of full-length TF but it is expressed at significantly lower levels. Perhaps not surprisingly given its structure AS-TF has no procoagulant activity. However, AS-TF has been associated with increased cancer cell migration, invasion, metastasis and proliferation. The effect of AS-TF appears to be mediated through direct interaction with integrins ($\alpha v\beta 3$ and $\alpha 6\beta 1$), leading to activation of cell signalling and subsequent modulation of cancer cell biology [66-68]. In contrast to full-length TF the biological actions of AS-TF appear to be independent of PAR signalling.

Conclusion

Tissue factor is a cellular receptor that plays a pivotal role in regulating the initiation of blood coagulation and wound repair processes. Despite many decades of research that have increased our understanding of the structure and function of this molecule there still remains much to learn about its role in the pathophysiology of disease.

Key points

- TF is the cellular receptor and cofactor for blood coagulation factor VII/VIIa
- Exposure of flowing blood to cells expressing TF is sufficient and necessary to initiate blood coagulation
- TF initiated thrombin generation leads to cellular signalling through activation of PARs
- TF initiated cellular signalling plays a pivotal role in pathophysiology of a number of diseases

Acknowledgements

Financial support and sponsorship

This work was supported by the School of Biosciences & Medicine, University of Surrey, UK.

Conflicts of interest

The author has no conflicts of interest.

References

(1) Davidson CJ, Tuddenham EG, McVey JH. 450 Million Years of Hemostasis. *J.Thromb.Haemost.* 2003;1:1487-1494.

(2) Davidson CJ, Hirt RP, Lal K et al. Molecular evolution of the vertebrate blood coagulation network. *Thromb.Haemost.* 2003;89:420-428.

(3) Larkin MA, Blackshields G, Brown NP et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23:2947-2948.

(4) Goodeve AC, Reitsma PH, McVey JH, Working Group on Nomenclature of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. Nomenclature of genetic variants in hemostasis. *J.Thromb.Haemost.* 2011;9:852-855.

- This publication details the recommendations of the ISTH that the HGVS nomenclature should be adopted to describe genes/proteins of the coagulation network. This changes the amino acid numbering of a number of proteins in the network.

(5) Dickinson CD, Kelly CR, Ruf W. Identification of surface residues mediating tissue factor binding and catalytic function of the serine protease factor VIIa. *Proc.Natl.Acad.Sci.U.S.A.* 1996;93:14379-14384.

(6) Dickinson CD, Shobe J, Ruf W. Influence of cofactor binding and active site occupancy on the conformation of the macromolecular substrate exosite of factor VIIa. *J.Mol.Biol.* 1998;277:959-971.

(7) Ruf W, Miles DJ, Rehemtulla A, Edgington TS. Tissue factor residues 157-167 are required for efficient proteolytic activation of factor X and factor VII. *J.Biol.Chem.* 1992;267:22206-22210.

(8) Kelley RF, Yang J, Eigenbrot C et al. Similar molecular interactions of factor VII and factor VIIa with the tissue factor region that allosterically regulates enzyme activity. *Biochemistry* 2004;43:1223-1229.

(9) Kirchhofer D, Eigenbrot C, Lipari MT et al. The tissue factor region that interacts with factor Xa in the activation of factor VII. *Biochemistry* 2001;40:675-682.

(10) Neuenschwander PF, Morrissey JH. Roles of the membrane-interactive regions of factor VIIa and tissue factor. The factor VIIa Gla domain is dispensable for binding to tissue factor but important for activation of factor X. *J.Biol.Chem.* 1994;269:8007-8013.

(11) Eigenbrot C, Kirchhofer D, Dennis MS et al. The factor VII zymogen structure reveals reregistration of beta strands during activation. *Structure* 2001;9:627-636.

(12) Banner DW, D'Arcy A, Chene C et al. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* 1996;380:41-46.

(13) Bajaj SP, Schmidt AE, Agah S et al. High resolution structures of p-aminobenzamidine- and benzamidine-VIIa/soluble tissue factor: unpredicted conformation of the 192-193 peptide bond and mapping of Ca²⁺, Mg²⁺, Na⁺, and Zn²⁺ sites in factor VIIa. *J.Biol.Chem.* 2006;281:24873-24888.

(14) Paborsky LR, Caras IW, Fisher KL, Gorman CM. Lipid association, but not the transmembrane domain, is required for tissue factor activity. Substitution of the transmembrane domain with a phosphatidylinositol anchor. *J.Biol.Chem.* 1991;266:21911-21916.

- (15) Parry GC, Mackman N. Mouse embryogenesis requires the tissue factor extracellular domain but not the cytoplasmic domain. *J.Clin.Invest.* 2000;105:1547-1554.
- (16) Krishnaswamy S. Exosite-driven substrate specificity and function in coagulation. *J.Thromb.Haemost.* 2005;3:54-67.
- (17) Thiec F, Cherel G, Christophe OD. Role of the Gla and first epidermal growth factor-like domains of factor X in the prothrombinase and tissue factor-factor VIIa complexes. *J.Biol.Chem.* 2003;278:10393-10399.
- (18) Ndonwi M, Broze GJ,Jr, Agah S et al. Substitution of the Gla domain in factor X with that of protein C impairs its interaction with factor VIIa/tissue factor: lack of comparable effect by similar substitution in factor IX. *J.Biol.Chem.* 2007;282:15632-15644.
- (19) Ke K, Yuan J, Morrissey JH. Tissue factor residues that putatively interact with membrane phospholipids. *PLoS One* 2014;9:e88675.
- (20) Huang Q, Neuenschwander PF, Rezaie AR, Morrissey JH. Substrate recognition by tissue factor-factor VIIa. Evidence for interaction of residues Lys165 and Lys166 of tissue factor with the 4-carboxyglutamate-rich domain of factor X. *J.Biol.Chem.* 1996;271:21752-21757.
- (21) Ruf W, Miles DJ, Rehemtulla A, Edgington TS. Cofactor residues lysine 165 and 166 are critical for protein substrate recognition by the tissue factor-factor VIIa protease complex. *J.Biol.Chem.* 1992;267:6375-6381.
- (22) Gajsiewicz JM, Nuzzio KM, Rienstra CM, Morrissey JH. Tissue Factor Residues That Modulate Magnesium-Dependent Rate Enhancements of the Tissue Factor/Factor VIIa Complex. *Biochemistry* 2015;54:4665-4671.
- (23) Sekiya F, Yoshida M, Yamashita T, Morita T. Magnesium(II) is a crucial constituent of the blood coagulation cascade. Potentiation of coagulant activities of factor IX by Mg²⁺ ions. *J.Biol.Chem.* 1996;271:8541-8544.
- (24) Lawson JH, Butenas S, Mann KG. The evaluation of complex-dependent alterations in human factor VIIa. *J.Biol.Chem.* 1992;267:4834-4843.
- (25) McCallum CD, Hapak RC, Neuenschwander PF et al. The location of the active site of blood coagulation factor VIIa above the membrane surface and its reorientation upon association with tissue factor. A fluorescence energy transfer study. *J.Biol.Chem.* 1996;271:28168-28175.
- (26) Ghaddar HM, Folsom AR, Aleksic N et al. Correlation of factor VIIa values with factor VII gene polymorphism, fasting and postprandial triglyceride levels, and subclinical carotid atherosclerosis. *Circulation* 1998;98:2815-2821.
- (27) Wood JP, Ellery PE, Maroney SA, Mast AE. Biology of tissue factor pathway inhibitor. *Blood* 2014;123:2934-2943.
- (28) Mast AE. Tissue Factor Pathway Inhibitor: Multiple Anticoagulant Activities for a Single Protein. *Arterioscler.Thromb.Vasc.Biol.* 2016;36:9-14.

- This and reference 27 provide excellent recent reviews of the biology of tissue factor pathway inhibitor (TFPI).

(29) Carmeliet P, Mackman N, Moons L et al. Role of tissue factor in embryonic blood vessel development. *Nature* 1996;383:73-75.

(30) Toomey JR, Kratzer KE, Lasky NM et al. Targeted disruption of the murine tissue factor gene results in embryonic lethality. *Blood* 1996;88:1583-1587.

(31) Bugge TH, Xiao Q, Kombrinck KW et al. Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. *Proc.Natl.Acad.Sci.U.S.A.* 1996;93:6258-6263.

(32) Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am.J.Pathol.* 1989;134:1087-1097.

(33) Luther T, Flossel C, Mackman N et al. Tissue factor expression during human and mouse development. *Am.J.Pathol.* 1996;149:101-113.

(34) Tatsumi K, Mackman N. Tissue Factor and Atherothrombosis. *J.Atheroscler.Thromb.* 2015;22:543-549.

(35) Levi M, van der Poll T, ten Cate H. Tissue factor in infection and severe inflammation. *Semin.Thromb.Hemost.* 2006;32:33-39.

(36) Ruf W. Tissue factor and cancer. *Thromb.Res.* 2012;130 Suppl 1:S84-7.

(37) Lin CC, Cooper DK, Dorling A. Coagulation dysregulation as a barrier to xenotransplantation in the primate. *Transpl.Immunol.* 2009;21:75-80.

(38) Crossman DC, Carr DP, Tuddenham EG et al. The regulation of tissue factor mRNA in human endothelial cells in response to endotoxin or phorbol ester. *J.Biol.Chem.* 1990;265:9782-9787.

(39) Ahern SM, Miyata T, Sadler JE. Regulation of human tissue factor expression by mRNA turnover. *J.Biol.Chem.* 1993;268:2154-2159.

(40) Mackman N, Sawdey MS, Keeton MR, Loskutoff DJ. Murine tissue factor gene expression in vivo. Tissue and cell specificity and regulation by lipopolysaccharide. *Am.J.Pathol.* 1993;143:76-84.

(41) Drake TA, Cheng J, Chang A, Taylor FB,Jr. Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal *Escherichia coli* sepsis. *Am.J.Pathol.* 1993;142:1458-1470.

(42) Lupu C, Westmuckett AD, Peer G et al. Tissue factor-dependent coagulation is preferentially up-regulated within arterial branching areas in a baboon model of *Escherichia coli* sepsis. *Am.J.Pathol.* 2005;167:1161-1172.

(43) Song D, Ye X, Xu H, Liu SF. Activation of endothelial intrinsic NF- κ B pathway impairs protein C anticoagulation mechanism and promotes coagulation in endotoxemic mice. *Blood* 2009;114:2521-2529.

(44) Semeraro N, Triggiani R, Montemurro P et al. Enhanced endothelial tissue factor but normal thrombomodulin in endotoxin-treated rabbits. *Thromb.Res.* 1993;71:479-486.

(45) Pawlinski R, Pedersen B, Kehrl B et al. Regulation of tissue factor and inflammatory mediators by Egr-1 in a mouse endotoxemia model. *Blood* 2003;101:3940-3947.

(46) Erlich J, Fearn C, Mathison J et al. Lipopolysaccharide induction of tissue factor expression in rabbits. *Infect.Immun.* 1999;67:2540-2546.

(47) Hara S, Asada Y, Hatakeyama K et al. Expression of tissue factor and tissue factor pathway inhibitor in rats lungs with lipopolysaccharide-induced disseminated intravascular coagulation. *Lab.Invest.* 1997;77:581-589.

(48) Mooberry MJ, Key NS. Microparticle analysis in disorders of hemostasis and thrombosis. *Cytometry A.* 2016;89:111-122.

(49) Lacroix R, Judicone C, Poncelet P et al. Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *J.Thromb.Haemost.* 2012;10:437-446.

(50) Lacroix R, Robert S, Poncelet P et al. Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J.Thromb.Haemost.* 2010;8:2571-2574.

(51) Basavaraj MG, Olsen JO, Osterud B, Hansen JB. Differential ability of tissue factor antibody clones on detection of tissue factor in blood cells and microparticles. *Thromb.Res.* 2012;130:538-546.

(52) Parhami-Seren B, Butenas S, Krudysz-Amblo J, Mann KG. Immunologic quantitation of tissue factors. *J.Thromb.Haemost.* 2006;4:1747-1755.

(53) Hisada Y, Alexander W, Kasthuri R et al. Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thromb.Res.* 2016;139:90-97.

- References 48-53 describe the problems in analysing microparticles (MPs) and in particular tissue factor positive MPs. References 49 and 50 address the technical challenges and make recommendations for the standardisation of sample preparation and analysis. References 51-53 address confounding factors in measuring either TF antigen or activity.

(54) Rao LV, Pendurthi UR. Regulation of tissue factor coagulant activity on cell surfaces. *J.Thromb.Haemost.* 2012;10:2242-2253.

(55) Langer F, Ruf W. Synergies of phosphatidylserine and protein disulfide isomerase in tissue factor activation. *Thromb.Haemost.* 2014;111:590-597.

- References 54 and 55 discuss the controversial question of TF encryption and the role of phospholipids and protein disulfide isomerase.

(56) Mercer PF, Chambers RC. Coagulation and coagulation signalling in fibrosis. *Biochim.Biophys.Acta* 2013;1832:1018-1027.

- This reference presents an overview of the emerging role of TF induced coagulation protease signalling in the development and progression of fibrosis in different organ systems.

(57) Bachli EB, Pech CM, Johnson KM et al. Factor Xa and thrombin, but not factor VIIa, elicit specific cellular responses in dermal fibroblasts. *J.Thromb.Haemost.* 2003;1:1935-1944.

(58) Fujiwara M, Jin E, Ghazizadeh M, Kawanami O. Differential expression of protease-activated receptors 1, 2, and 4 on human endothelial cells from different vascular sites. *Pathobiology* 2004;71:52-58.

(59) Scotton CJ, Krupiczko MA, Konigshoff M et al. Increased local expression of coagulation factor X contributes to the fibrotic response in human and murine lung injury. *J.Clin.Invest.* 2009;119:2550-2563.

(60) Koizume S, Jin MS, Miyagi E et al. Activation of cancer cell migration and invasion by ectopic synthesis of coagulation factor VII. *Cancer Res.* 2006;66:9453-9460.

(61) Zacharski LR, Dunwiddie C, Nutt EM et al. Cellular localization of activated factor X by Xa-specific probes. *Thromb.Haemost.* 1991;65:545-548.

(62) Yamada T, Nagai Y. Immunohistochemical studies of human tissues with antibody to factor Xa. *Histochem.J.* 1996;28:73-77.

(63) Borensztajn K, Peppelenbosch MP, Spek CA. Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. *Trends Mol.Med.* 2008;14:429-440.

(64) Bogdanov VY, Balasubramanian V, Hathcock J et al. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat.Med.* 2003;9:458-462.

(65) Bogdanov VY, Kirk RI, Miller C et al. Identification and characterization of murine alternatively spliced tissue factor. *J.Thromb.Haemost.* 2006;4:158-167.

(66) van den Berg YW, van den Hengel LG, Myers HR et al. Alternatively spliced tissue factor induces angiogenesis through integrin ligation. *Proc.Natl.Acad.Sci.U.S.A.* 2009;106:19497-19502.

(67) Kocaturk B, Van den Berg YW, Tieken C et al. Alternatively spliced tissue factor promotes breast cancer growth in a beta1 integrin-dependent manner. *Proc.Natl.Acad.Sci.U.S.A.* 2013;110:11517-11522.

- This report demonstrates a role for alternatively spliced rather than full length TF in promoting breast cancer growth .

(68) Kocaturk B, Versteeg HH. Tissue factor isoforms in cancer and coagulation: may the best isoform win. *Thromb.Res.* 2012;129 Suppl 1:S69-75.

Figure Legends:

Figure 1:

A: Multiple sequence alignment of predicted amino acid sequences encoding TF from various species. The accession number (gi) of each sequence is given. Sequences were aligned using Clustal W [3]. The transmembrane domain sequence is boxed. Disulfide bonds are indicated by square brackets. N-linked consensus glycosylation sites are indicated by *. The putative substrate-binding region is indicated by ★. The percent identity and similarity of each sequence with the human reference sequence is given.

B: Molecular model of the complex between active site inhibited factor VIIa and the extracellular domain of tissue factor (PDB: 1DAN). FVIIa protein chain is represented by a grey ribbon. The active site inhibitor is shown in ball and stick. Calcium ions are represented by red spheres. The TF protein chain is represented by a green ribbon with residues conserved in all species analysed shown in blue.

Figure 2:

Schematic representation of the blood coagulation network initiated by exposure of flowing blood to cells expressing TF.

Formation of a high affinity complex of TF-FVIIa results in activation of FIX and FX by the TF–FVIIa complex. In the absence of its activated cofactor FVa, FXa generates only trace amounts of thrombin from prothrombin. Although insufficient to initiate significant fibrin polymerization, thrombin formed in this initiation stage of coagulation is able to back-activate FV, FVIII and FXI by limited proteolysis and to activate platelets providing a negatively charged surface for the assembly of procoagulant complexes. In the amplification phase of coagulation, FXIa activates further FIXa which forms a complex with FVIIIa. This is the intrinsic tenase complex (FVIIIa–FIXa) that activates sufficient FXa to form a complex with FVa, producing the prothrombinase complex (FVa–FXa). This results in the explosive generation of thrombin that ultimately leads to generation of a fibrin clot. A key feature of these processes is the assembly of multimolecular complexes on phospholipid surfaces provided by activated platelets. 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. The cofactors FV and FVIII are themselves activated by limited proteolytic digestion. Positive feedback loops are represented by red dashed lines, negative feedback loops by blue dashed lines.

Figure 3:

A: Sequence alignment of predicted amino acid sequences encoding full-length TF (FL-TF) and alternatively spliced TF (AS-TF). The amino acid sequence in common is shaded black. The transmembrane domain sequence is shaded grey. Disulfide bonds are indicated by square brackets.

Molecular model the extracellular domain of tissue factor (PDB: 1DAN). The TF protein chain is represented by a green ribbon with residues conserved in all species analysed shown in blue. B: represents FL-TF and C: represents AS-TF demonstrating the loss of 3 of the β–strands that comprise the C-terminal immunoglobulin like domain.

A.

	10	20	30	40	50	60
FL-TF	SGTTNTVAAY	NLTWKSTNEK	TILEWEPKPV	NQVYTVQIST	KSGDWKSKCF	YTTDTECDLT
AS-TF	SGTTNTVAAY	NLTWKSTNEK	TILEWEPKPV	NQVYTVQIST	KSGDWKSKCF	YTTDTECDLT
	70	80	90	100	110	120
FL-TF	DEIVKDVQQT	YLARVFSYPA	GNVESTGSAG	EPLYENSPEF	TPYLETNLGO	PTIQSFEQVG
AS-TF	DEIVKDVQQT	YLARVFSYPA	GNVESTGSAG	EPLYENSPEF	TPYLETNLGO	PTIQSFEQVG
	130	140	150	160	170	180
FL-TF	TKVNVTVDEE	RTLVR RNNTF	LSLRDVEGKD	LIYTLYYWKS	SSSGKKTAKT	NTNEFLIDVD
AS-TF	TKVNVTVDEE	RTLVR RNNTF	LSLRDVEGKD	LIYTLYYWKS	SSSGKKYSTS	LELWYLVSSS
	190	200	210	220	230	240
FL-TF	KGENYCFSVQ	AVIPSRTVNR	KSTDSPVECM	GQEKGEFREI	FYIIGAVVFV	VIILVIILAI
AS-TF	LSSSWLYLYT	SVERQEWGRA	GRRTPH			
	250	260				
FL-TF	SLHKCRKAGV	GQSWKENSPL	NVS			
AS-TF						

B.

C.