Review Article

Tissue Factor Structure and Function

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Tissue factor (TF) is an integral membrane protein that is essential to life. It is a component of the factor VIIa-TF complex enzyme and plays a primary role in both normal hemostasis and thrombosis. With a vascular injury, TF becomes exposed to blood and binds plasma factor VIIa, and the resulting complex initiates a series of enzymatic reactions leading to clot formation and vascular sealing. Many cells, both healthy, and tumor cells, produce detectable amounts of TF, especially when they are stimulated by various agents. Despite the relative simplicity and small size of TF, there are numerous contradictory reports about the synthesis and presentation of TF on blood cells and circulation in normal blood either on microparticles or as a soluble protein. Another subject of controversy is related to the structure/function of TF. It has been almost commonly accepted that cell-surface-associated TF has low (if any) activity, that is, is "encrypted" and requires specific conditions/reagents to become active, that is, "decrypted." However there is a lack of agreement related to the mechanism and processes leading to alterations in TF function. In this paper TF structure, presentation, and function, and controversies concerning these features are discussed.

1. Introduction

TF is an integral transmembrane protein expressed by various cells, is a component of the factor VIIa-TF complex enzyme and is essential for normal hemostasis [1, 2]. Under normal circumstances cells in contact with blood do not express physiologically active TF [3]. When mechanical or chemical damage of the vascular wall occurs, subendothelial TF is expressed/exposed to blood flow and binds plasma factor VIIa, which circulates as an enzyme at a concentration of approximately 0.1 nM (1% of plasma factor VII) [4] and escapes the inhibition by serine proteases inhibitors because of its poor enzymatic qualities [1, 5]. The factor VIIa-TF complex initiates blood coagulation by activating the zymogens factor IX and factor X to their respective serine proteases, factor IXa and factor Xa. Factor IXa and factor Xa form complex enzymes with their nonenzymatic cofactors (factor VIIIa and factor Va, resp.) on the surface of membranes containing acidic phospholipids, robustly producing thrombin, the final enzymatic product of the process. Thrombin accelerates its own generation via several feedback reactions, cleaves fibrinogen, and activates factor XIII, which leads to the formation of a crosslinked insoluble fibrin clot [6, 7] (Figure 1).

Current literature supports the notion that blood coagulation reactions are driven by the enzymatic complexes consisting of the vitamin K-dependent serine protease and a non-enzymatic cofactor assembled on a membrane surface in a calcium-dependent manner [13, 14]. The significance of such complexes could be illustrated by the alterations in catalytic efficiency displayed in proteolysis of their natural substrates. For example, the proteolytic efficiency of factor VIIa in the absence of TF is negligible [15, 16]. The addition of the cofactor, TF, leads to the assembly of a potent enzymatic complex, which proteolyses factor X at an approximately $2 \cdot 10^7$-fold higher rate than factor VIIa alone. Similar differences in the efficiencies of enzymatic complexes versus those of enzymes alone were observed for the factor IXa-factor VIIIa complex and the factor Xa-factor Va complex [15, 17]. The significance of the membrane surface (cell or artificial) in the assembly of these complexes should not be overlooked, since in the absence of membrane the formation of the complex is either abolished or impaired [17–20]. Similarly, calcium is also an essential component for efficient complex formation [21].

The observation that tissue extracts, especially brain extracts, play an important role in activation of blood coagulation was reported already in the middle of the
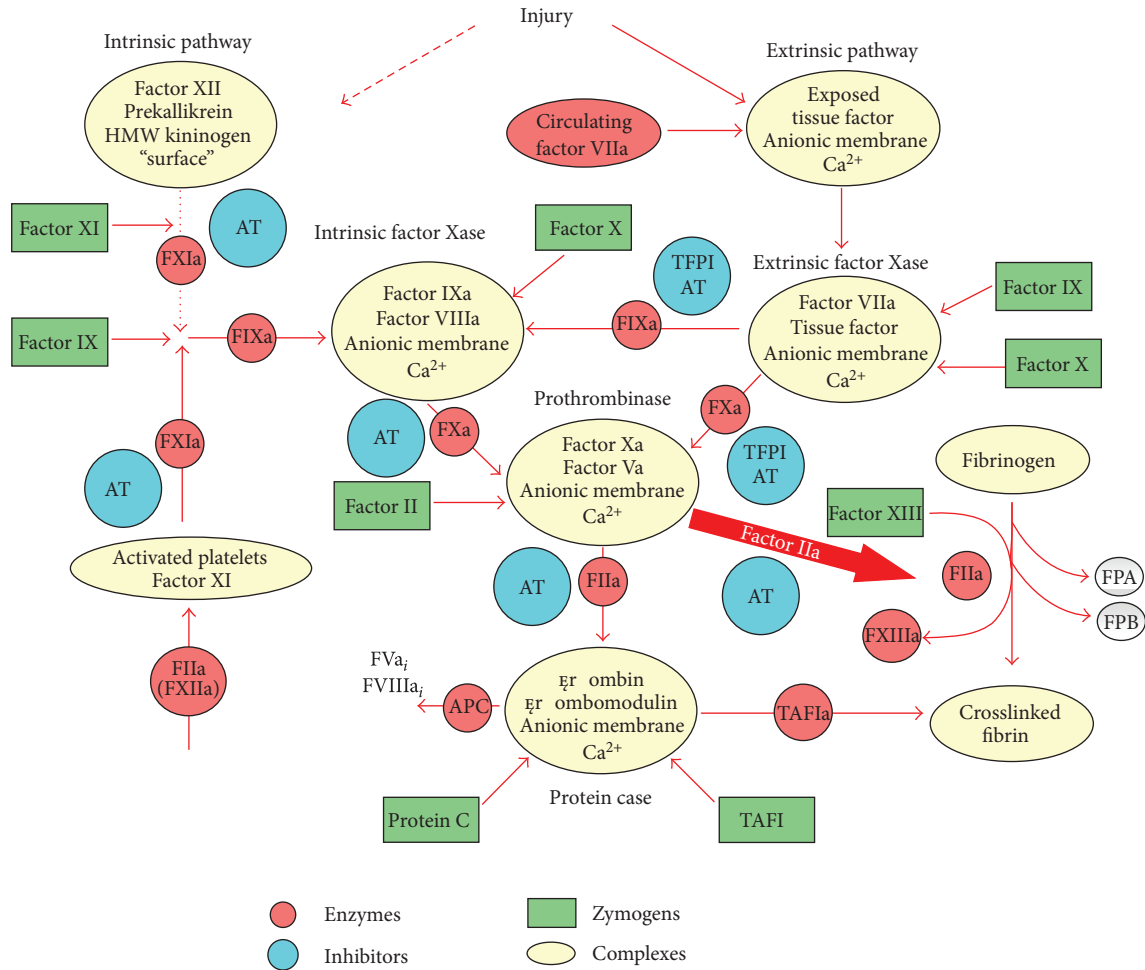
FIGURE 1: Overview of hemostasis. There are two pathways to initiate coagulation: the primary extrinsic pathway (shown on the right) and the intrinsic (also called the contact pathway) (shown on the left). The components of these multistep processes are illustrated as follows: enzymes (pink circles), inhibitors (blue circles), zymogens (green boxes), or complexes (cream ovals). The intrinsic pathway has no known bleeding etiology associated with it; thus, this pathway is considered accessory to hemostasis. Upon an injury to the vessel wall, TF, a membrane-bound cofactor, is exposed to circulating factor VIIa, forming the extrinsic factor Xase, a complex enzyme. Factor IX and factor X are converted to the serine proteases factor IXa and factor Xa, which are the enzymatic components of the intrinsic factor Xase and the prothrombinase complexes, respectively. The combined action of all three complexes lead to an explosive burst of thrombin (FIIa). Once thrombin is generated, it cleaves fibrinogen (releasing fibrinopeptides A and B (FPA and FPB, resp.)) and activates factor XIII to form a cross-linked fibrin clot. Thrombin-thrombomodulin also activates thrombin-activatable fibrinolysis inhibitor (TAFI) that slows down fibrin degradation by plasmin. Thrombin has also been shown to activate factor XI. In addition to its multiple procoagulant roles, thrombin also acts as an anticoagulant when combined with the cofactor thrombomodulin in the protein Case complex. The product of the protein Case reaction, activated protein C (APC), inactivates the cofactors factors Va and VIIIa. The cleaved species, factors Va_i (FVa_i) and $VIIIa_i$ ($FVIIIa_i$), no longer support the respective procoagulant activities of the prothrombinase and the intrinsic factor Xase complexes. TF-triggered procoagulant response is also down-regulated by the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin (AT). TFPI serves to attenuate the activity of the extrinsic factor Xase, the trigger of coagulation. AT directly inhibits thrombin, factor IXa, and factor Xa. High molecular weight (HMW) kininogen is one of components of the intrinsic pathway. (An original version of this figure was published in Wintrobe's Clinical Hematology) [8].

nineteenth century [22]. In the late eighteen hundreds, it was identified that the substance responsible for this effect is a phospholipin-protein complex [23]. The role for TF as the blood coagulation trigger (then named thromboplastin) was assigned by Loeb and Morawitz in the early twentieth century and it was found to be expressed in most animal tissues and to be released upon tissue injury [24, 25]. Over the next decades, the new findings in the field of hemostasis led to

the elucidation of the coagulation cascade and its key players. The coagulation trigger, now commonly known as TF, was isolated by Nemerson's group in 1981 [26], which enabled the cloning of the protein and gene sequencing [27] and expression of recombinant forms of the protein. Since then, important roles of TF in hemostasis, thrombosis, cancer, inflammation, angiogenesis and embryogenesis have been established [28]. However, in spite of dramatically expanding

knowledge related to TF, multiple controversies regarding the structure/function relationship of this protein remain subjects of scientific publications.

2. Structure

TF is a 263/261 amino acid transmembrane protein containing three domains (Figure 2): (1) an extracellular domain (residues 1–219) representing the NH_2-terminal part of the molecule composed of two fibronectin type III domains. It is involved in complex formation with factor VIIa and increases, in a membrane dependent fashion, the activity of the protease toward its natural substrates factor IX, factor X, and factor VII by several orders of magnitude [29, 30]; (2) a transmembrane domain (residues 220–242), which anchors TF to the membrane; and (3) a cytoplasmic COOH-terminal domain (residues 243–263) [27], which is involved in signal transduction [31–33].

TF binds factor VIIa with relatively high affinity, although reported dissociation constants for the factor VIIa-TF interaction vary over a wide range (from 1 pM to 20 nM) [34, 35]. TF binding to factor VIIa increases the amidolytic activity of this enzyme by approximately two orders of magnitude for small molecular weight synthetic substrates [36]. This activity is primarily dependent upon the structure of the substrate and is not influenced by the binding of TF to the membrane [1]. In contrast, to express maximum proteolytic activity toward natural substrates factor IX, factor X, and factor VII, the factor VIIa-TF complex must be formed on the surface of an appropriate membrane [29, 30]. Thus, two of the three domains of TF (extracellular and transmembrane) play distinct roles in the blood coagulation process. It has been generally accepted that TF lacking the cytoplasmic domain is functionally identical to the full-length protein in the initiation of thrombin generation. On the other hand, TF proteins lacking both the cytoplasmic and transmembrane domains cannot bind to the membrane, and therefore, while forming a complex with factor VIIa, are not efficient (if active at all) in proteolyzing natural substrates factors VII, IX, and X [29, 30].

The gene of human TF is located on chromosome 1 p21-22 spanning approximately 12.4 kilobases. The amino acid sequence of TF has been determined from the cloned 2.3 kilobase cDNA, containing 263 amino acid residues after the cleavage of a 32 amino acid residue leader sequence [27, 37–40]. The coding sequence is made up of six exons. Exon one corresponds to the propeptide and the translation initiation sites. Exons two to five contain sites for the translation of the extracellular domain of the molecule and exon six constitutes transmembrane and cytoplasmic domains [41]. The analysis of the TF sequence revealed a distant homology to the cytokine receptor superfamily [42]. The primary sequence, as well as structural homology, places TF with a group of receptors such as the human growth hormone receptor, prolactin, erythropoietin receptor, the interferon-γ receptor, CD2, and CD4 [43–48]. The comparison of the three dimensional structure of the extracellular regions of these proteins and that of TF shows a domain formed by two immunoglobulin like modules. The amino (extracellular) terminus of TF (residues 1–219) is composed of two domains joined at an angle of 125 degrees [48]. The cleft formed at the interface between the two immunoglobulin-like modules was predicted to serve as the ligand binding site (Figure 3).

TF is a member of the class two cytokine receptor superfamily and fibronectin type III family. The cytokine receptor superfamily comprises a diverse group of proteins with highly homologous binding domains. The binding domains contain an approximately 200 amino acid segment with conserved regions of beta-strands. The structural analysis shows a homology in sequence and structure topology with characteristic antiparallel beta-sandwich fold with a Greek key motif. These motifs are found in the extracellular domains of a subgroup of receptor family proteins such as interferon-α/β and γ receptors and TF. The motif is also linked to the immunoglobulin superfamily with the analogous antiparallel β-sandwich topology [42, 49].

Fibronectins are glycoproteins involved in numerous cellular processes such as blood coagulation, tissue repair, cell differentiation and embryogenesis, and so forth. The wide spectrum of activities of these molecules in signaling and binding explains their interaction with various ligands including collagen, DNA, heparin, actins, fibrin, and cytokine receptors on cell surfaces [42, 50]. Fibronectin is composed of three types of homologous repeating modules, with the type III module being the most abundant. This region in fibronectins is composed of approximately 100 amino acid residues. The extracellular domain of TF contains two type III modules. Each module is composed of two overlapping beta sheets with the top sheet containing three antiparallel beta strands and the bottom sheet containing four beta strands [42, 49–52]. The strands are connected by β-loops between strand βA and βB, βC and βD, βE and βF, all of which are conserved in conformations in the two modules. There are three short alpha helix segments connecting the β-strands. A unique feature to TF is a 17 residue β hairpin between strand $\beta 10$ and strand $\beta 11$, which is not a common element of the fibronectin superfamily. The N-terminal domain also contains a loop, an insertion of 12 residues between $\beta_6 F$ and $\beta_7 G$, which is not seen in the C-terminal domain and is unique to TF [48] (Figure 4).

Sequences of proteins with fibronectin III modules are diverse, however superposition of structures shows a conserved backbone conformation and core packing [52]. Nevertheless, some residues are specific for TF. They include a conserved Trp14 residue of strand $\beta_1 C$ which points toward the hydrophobic core in the interface of the two domains, not conserved within the cytokine receptor superfamily. Its side chain is accommodated by Ser97 of strand $\beta_7 G$ Pro98 found between $\beta_7 G$ and $\beta_8 A$ of the C-terminal domain. Asn137 of the β turn between strand β_{11} and $\beta_{12} C$ is found in human TF but is substituted by a glycine in rabbit, rat, and mouse protein, suggesting a specific role for this residue in humans.

Based on the distant sequence similarities, topology and a receptor like function, TF is also characterized as a member of the cytokine/hematopoietic growth factor receptor family. Proteins of this family are cell-surface molecules with a single transmembrane domain and a cytoplasmic domain
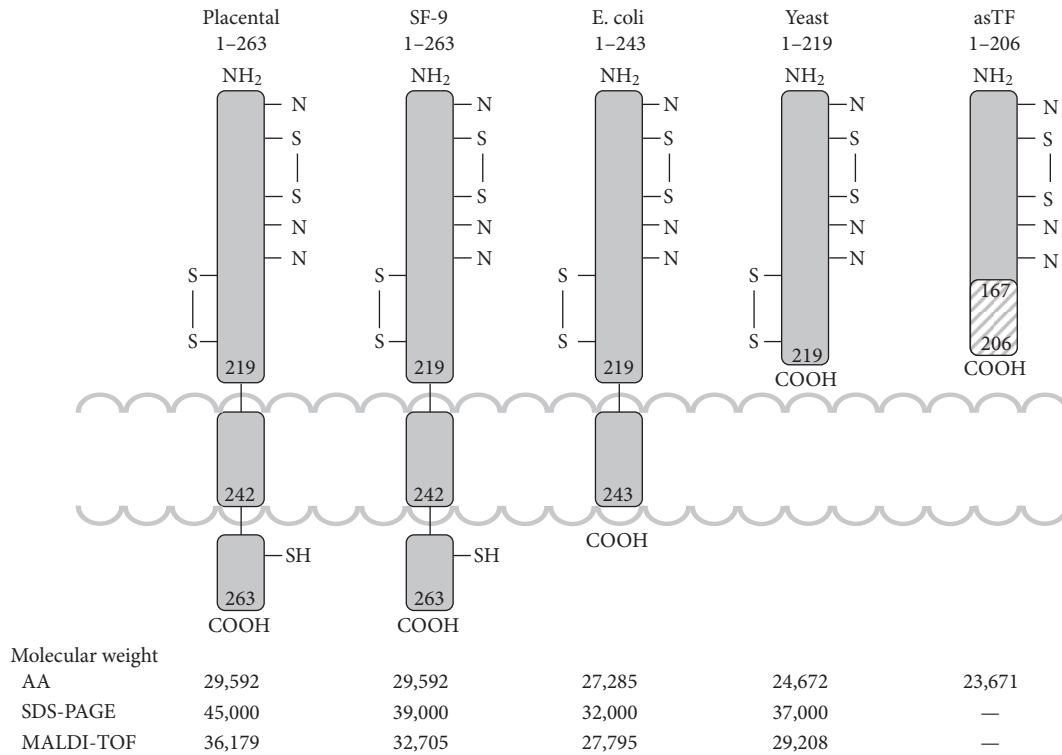
FIGURE 2: The structure of various TF species. Indicated molecular weights were determined from the amino acid composition (AA), gel electrophoresis (SDS), and mass-spectrometry (MALDI-TOF). (This figure was originally published in Surgery) [9].
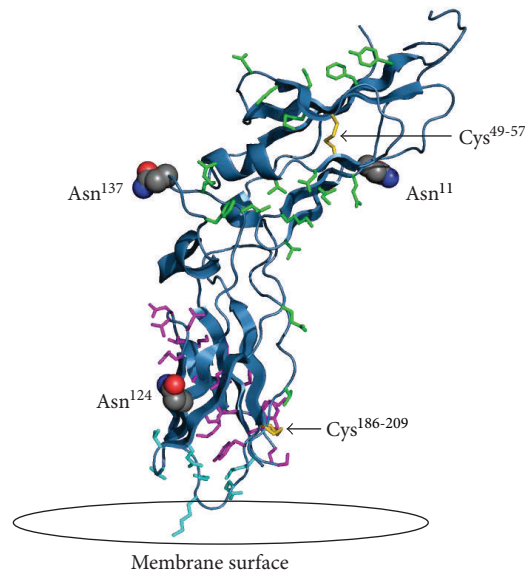

Membrane surface

FIGURE 3: The extracellular domain of TF on a modeled lipid membrane. The figure shows in red the three sites of glycosylation (Asn[11], Asn[124] and Asn[137]). Highlighted in green are residues important for TF interaction with factor VIIa (Thr[17], Lys[20], Ile[22], Glu[24], Gln[37], Asp[44], Lys[46], Lys[48], Asp[58], Thr[60], Phe[76], Tyr[78], Gln[110], Leu[133], Arg[135], Phe[140], and Val[207]). Highlighted in magenta are residues important for the interaction with factor X (Thr[154]-Glu[174] and Tyr[185]). Highlighted in aqua are the residues important for TF interaction with the membrane (Gln[118], Val[119], Thr[121], Lys[159], Asp[180], Lys[181], and Glu[183]). Also shown in yellow are the two disulfide bridges of TF at positions Cys[49]-Cys[57] and Cys[186]- Cys[209]. (This figure was originally published in Biochim Biophys Acta) [10].
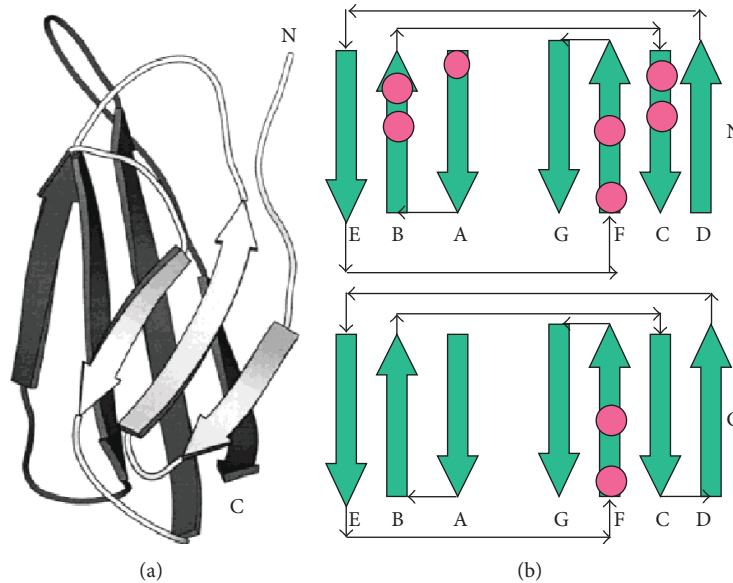
FIGURE 4: (a) Schematic presentation of fibronectin III type toplogy consisting of seven antiparallel beta-sheets. (b) Presentation of fibronectin III type N-and C-domains showing connectivity of the antiparallel beta sheets and their arrangement into a sandwich Greek key motif. The N- and C-domains constitute the extracellular domain of fibronectin III type proteins including TF.

with structural diversity. The unique characteristic of this family is a distinct disulfide bond in the extracellular domain further dividing the group into class 1 receptors and class 2 receptors with the latter including tissue factor [53].

3. Presentation

TF is constitutively expressed by cells associated with the vessel wall including vascular smooth muscle cells, adventitial fibroblasts and pericytes [54, 55]. At physiologic conditions, relatively high levels of TF are found specifically in the astrocytes of brain tissue, epithelial cells of the lung and cardiomyocytes of the heart and endothelial cells of placenta [56–59]. Many healthy cells produce detectable amounts of TF when they are stimulated by various agents [57–64]. TF has also been known to be expressed by tumor and tumor-like cells, where it is related to the metastatic potential of those cells [65–69]. Furthermore, TF has been identified in atherosclerotic plaques, which has suggested a role for TF in the progression of cardiovascular disease [70, 71]. However the concentration of TF in tissues and cells is low, which makes it difficult to detect, quantify and purify enough natural TF for the characterization and use in research and clinical laboratories.

During the last ten years, numerous conflicting studies related to the presence, concentration, and functional activity of TF circulating in blood as a soluble protein and on/in various blood cells and platelets have been published. Several groups of investigators reported the presence of TF antigen circulating in blood at the concentrations as high as 5–10 nM [72] and those of active protein reaching (sub)nanomolar concentrations [73]. In contrast, data published by several other groups indicate that if there is TF-related activity either

in blood or plasma from healthy humans, the concentration of active protein does not exceed 20 fM [11, 74, 75]. Moreover, based upon the experience accumulated in several laboratories, blood or plasma activated with (sub)picomolar concentrations of functional TF clots within several minutes [76–80] (Figure 5).

It has been reported that the blood-borne TF is located on blood cells, platelets, and microparticles or that it circulates as a soluble protein. There is a common agreement that stimulation of circulating monocytes with lipopolysaccharides induces TF expression *in vitro* and *in vivo* [81–83]. It has also been shown that the expression of monocyte and monocyte-derived macrophage TF could be induced by oxidized low density lipoprotein [84, 85]. As a consequence, an increased TF-related procoagulant activity was observed. Similarly, the stimulation of monocytes through inflammatory pathways also leads to TF expression at low picomolar concentrations [86]. This expression of TF by monocytes could be enhanced by the presence of platelet-monocyte aggregates [87].

The existence of TF on platelets has been controversial and is still unresolved. The sources of hypothetical TF in platelets have been described to include denovo synthesis and storage in α-granules as well as absorption of monocyte-shed TF-containing microparticles [88, 89]. Studies by Zill-mann et al. suggested that platelets isolated from stimulated blood contain functional TF [90] and Müller et al. claimed the presence of TF in α-granules of resting platelets [91]. Panes and coworkers believed that platelets synthesize TF in response to activation [92] and other studies suggested the presence of TF mRNA in platelets [93–96]. These suggestions have been challenged by our data based on the observation that no detectable TF activity or antigen are detected either on resting and ionophore-treated washed platelets or lipopolysaccharide-treated blood platelets [11,
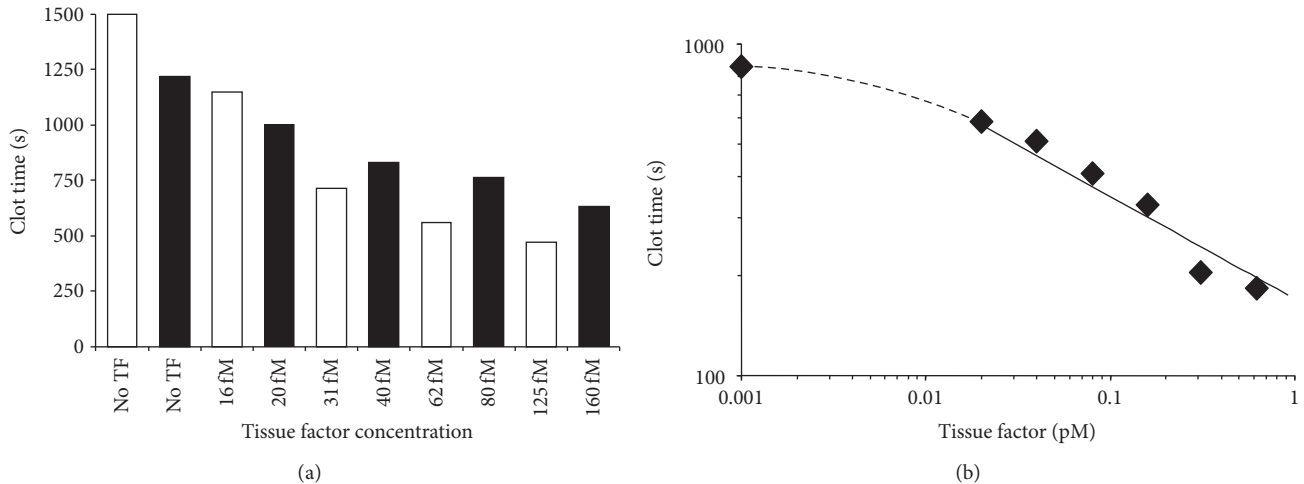
(a)

(b)

FIGURE 5: TF titrations in contact pathway inhibited whole blood (a) and plasma (b) from healthy individuals. Black and white bars represent two healthy donors. (This figure was originally published in Blood) [11].

97]. Similarly, Osterud and coworkers failed to detect any TF activity on collagen stimulated platelets [98, 99]. Bouchard and coworkers also did not observe TF expression by human platelets stimulated with PAR-1 and PAR-4 agonist peptides [97]. In contrast to Camera et al. transient TF expression was not detected upon platelet stimulation for a short (15 min) time [100, 101].

Similar to the subject of platelet TF, there is little agreement related to the presence of TF on granulocytes. Maugeri et al. suggested that granulocytes produce TF upon stimulation [102] while other authors have reported the expression of TF in neutrophils [103] and eosinophils [104]. However, data from Osterud's laboratory show no evidence of TF expression in any granulocytic cells [105–107].

Microparticles, small anucleoid cell membrane fragments, are released by various cells upon their stimulation or during cell apoptosis or death [108]. Microparticles are quite heterogenous with respect to size (usually from 100 to 1000 nm) and membrane lipid and protein composition, all of them dependent upon the microparticles' cellular origin and their generation pathway [109]. It is predictable that microparticles shed by the TF-bearing cells would contain TF on their surface. However, due to the controversy related to the presence of TF on some cell types and platelets, the presence of TF on microparticles shed by those cells remains a subject of the discussion. For example, while the role of lipopolysaccharide-stimulated monocytes as a source of microparticles containing active TF has been established [98, 110–112], that of platelets and granulocytes remains questionable [110, 113–115]. Despite the controversies related to sources of TF-bearing microparticles, there has been a growing body of evidence that this form of TF is associated with pathologic conditions, such as pulmonary embolism [116], venous thromboembolism [117], and disseminated intravascular coagulation [118], particularly in patients with various types of malignancy [118–122]. On the bright side, microparticle TF has a potential to promote hemostasis in hemophilia situation [123].

The presence, source, and function of a soluble form of alternatively spliced TF in blood have also been subjects of controversy [124–127]. It has been suggested that this form of TF is procoagulant [128] and stimulates clot growth [124]. However subsequent studies showed that alternatively spliced TF has no procoagulant activity [125–127] but that it could protect cells from apoptosis [129] and promote tumor growth and angiogenesis [126, 130–132]. Alternatively, Khan et al. suggested that soluble TF can bind to peripheral monocytes and platelets and efficiently activate factor VII [133]. The potential origin of this discrepancy could be assigned to the physiologically irrelevant conditions [3, 124] used and the lack of validated commercial assays for the detection of alternatively spliced TF activity at its physiologic concentrations [134–136]. TF antigen in blood may also be detected as a degradation product and not necessarily as an alternatively spliced form.

The majority of studies reporting high concentrations of TF in plasma and the presence of TF in platelets and blood cells use commercial assays. For example, in a study by Bis et al. which used a commercial TF assay, nanomolar concentrations of TF in plasma from patients with acute coronary syndrome a were reported [137]. Using validated assays for the quantitation of TF antigen [135] and activity [138] developed in our laboratory, we found that the TF antigen concentrations in plasmas from patients with a similar diagnosis are at low picomolar levels, with an average functional concentration less than 0.4 pM [138]. Until there is agreement in the scientific community concerning the validity of the assays used by various laboratories, incongruent reports will continue to accumulate in the literature.

4. Posttranslational Modifications

While contributions of various regions of the primary structure of TF on its activity are relatively well established, the data related to the influence of posttranslational modifications on the function of TF are scarce, if available at all,

primarily due to the shortage of natural TF protein. To compensate for this shortage, a variety of human recombinant TF species have been produced in different expression systems [27, 30, 139]. These recombinant proteins have been extensively used worldwide, and the experimental results acquired using recombinant proteins in vitroare frequently extrapolated to coagulation processes occurring in vivo. It has been commonly accepted that recombinant TF proteins are functionally identical to natural TF [140]. Unfortunately, the nonavailability of isolated natural TF does not allow the confirmation (or rejection) of results obtained with recombinant proteins or with those present in homogenates of natural tissues. Additionally, it leads to a scarcity of data addressing the influence of some structural components of natural TF on its activity. As a consequence, there is plenty of controversy in published studies related to the structure/activity of natural TF. A major obstacle arises as to the protein's genuine folding, activity, and function caused by different posttranslational modifications as per specific expression system. Limited work has been done on the contribution of each modification to the activity of TF, although recently more attention has been directed to the subject of structure-function relationship of natural TF [12].

According to the mass-spectrometry data, the level of posttranslational modifications of various forms of TF varies from 377 Da for recombinant TF 1–243 to 6,605 Da for the natural placental TF protein [12, 136]. Recombinant TF 1–263 is less modified (3,604 Da) than placental (Figure 2).

It was predictable that the prevailing posttranslational modification of TF would be related to carbohydrates. The amino acid sequence data indicate that full-length TF has three potential glycosylation sites at Asn^{11}, Asn^{124}, and Asn^{137} in the extracellular domain of the protein and one (Asn^{261}) in the cytoplasmic domain [27, 141]. The Asn^{261} site is not present in the truncated TF 1–243. Already in 1944, Chargaff et al. observed the presence of carbohydrtaes in TF [142], with the suggested sugar content constituting between 7–13% of total protein mass. A thorough analysis of the carbohydrate content of TF protein by Bjorklid [143] showed a carbohydrate content of 6%, mostly composed of fucose, mannose, galactose and N-acetylneuranimidase. The linkage of carbohydrates to the TF backbone was presumed to be via asparagine. Paborsky and Harris determined three potential sites for TF glycosylation in the extracellular domain, all of which were within the recognized sequence for N-linked glycosylation, that is, N-X-T/S [141]. Based on a similar activity of carbohydrate-free recombinant TF from *E. coli* and glycosylated protein expressed by kidney cells, it was concluded that carbohydrates play no role in TF function. Rickles, Waxmann, Stone, and their coworkers [140, 144, 145] also suggested that glycosylation is not required for the function of TF. In contrast, Pitlick, Shands, Bona, and data from our laboratory demonstrated that carbohydrates play a considerable role in TF activity [12, 146–148]. Pitlick observed that concanavalin A inhibits the coagulant activity of TF by binding reversibly to the carbohydrate moiety of the protein [146]. Shands and Bona both observed the loss of function and inability of TF to be incorporated into membranes after treatment with tunicamycin [147, 148].

A direct evidence for the effect of glycosylation on TF function came from our laboratory when we compared carbohydrate-free recombinant TF 1–243 expressed in *E.coli,* glycosylated recombinant TF 1–263 from Sf9 insect cells and natural TF from human placenta. Deglycosylated forms of the latter two proteins were included into analysis as well. The extent of glycosylation and structure of carbohydrates at each potential glycosylation site of all three TF proteins were somewhat different. No carbohydrates were detected on recombinant TF 1–243 produced in *E. coli* [12]. All three TF proteins analyzed were tested for their effect on FVIIa activity. For recombinant TF 1–263, deglycosylation had little effect on the affinity for FVIIa and it only marginally decreased the activity of the formed factor VIIa-TF complex. A more pronounced effect of deglycosylation was observed for placental TF. Deglycosylation significantly decreased the catalytic efficiency of the factor VIIa-TF complex towards the natural substrate factor X [12]. After deglycosylation, the catalytic efficiency of factor Xa generation became comparable for the placental, recombinant 1–263 and native recombinant TF 1–243 (Figure 6). Analysis of the relative carbohydrate abundance revealed that out of the four potential sites for N-linked glycosylation, two (Asn^{124} and Asn^{137}) were found to undergo complete glycosylation in both recombinant 1–263 and placental TF proteins [10]. An incomplete glycosylation occurs at Asn^{11} of recombinant TF 1–263 with a relative abundance of carbohydrates of 20%, whereas the glycosylation on Asn^{11} of placental TF reaches 76%. No carbohydrates were found at Asn^{261} in either protein. The composition of carbohydrates varied between these two proteins and between each site within each protein. At all three glycosylation sites, recombinant TF 1–263 predominantly contains high mannose sugars, whereas natural placental protein contains either hybrid or complex carbohydrates with high mannose sugars absent. A unique characteristic of placental TF is the presence of sialic acid on all three glycosylation sites. Thus in contrast to previously published statements that posttranslational modifications have no effect on TF activity [140, 141], these data indicate that glycosylation and the structure of carbohydrates have a pronounced effect on TF function.

Phosphorylation is another important posttranslational modification because it plays a critical role in the regulation of many protein functions. Upon protein phosphorylation, the phosphate is transferred to the hydroxyl groups of the side chains of three amino acids—serine, threonine, and tyrosine [149], with the hydroxyl groups of serine representing the major site of protein phosphorylation (90–95% of total phosphorylation sites). In 1992, Zioncheck et al. determined that TF contains two phosphorylation sites, both of them located in the cytoplasmic domain [150]. From the alignment of cDNA sequences of several TF species (including human) it was concluded that phosphorylation sites contain a conserved amino acid sequence X-Ser*/Thr*-Pro-X with the asterisk indicating the phosphorylation residue. In a later publication, Mody and Carson suggested that the cytoplasmic domain of TF can be phosphorylated in vitro at multiple sites, particularly at Ser^{253} and Ser^{258} [151]. The mutational data presented by Dorfleutner and Ruf suggested that initial
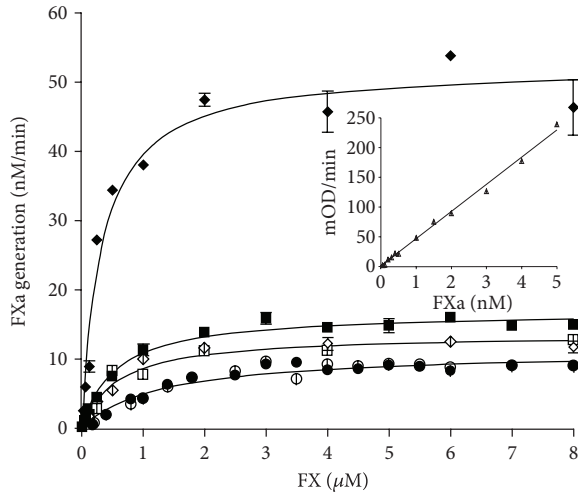
FIGURE 6: Proteolytic activity of the extrinsic factor Xase formed by factor VIIa and relipidated placental TF (♦ glycosylated and ◇ deglycosylated), full-length recombinant TF 1–263 (■ glycosylated and □ deglycosylated) and truncated recombinant TF 1–243 (● native and ○ "deglycosylated"). Factor VIIa (5 nM) was incubated with 0.1 nM relipidated TF and increasing concentrations of factor X were added. Factor Xa generation was monitored in a chromogenic assay. (This figure was originally published in J Biol Chem) [12].

phosphorylation at Ser[253] enhances the subsequent phosphorylation at Ser[258] [152]. In several publications the influence of phosphorylation on cell TF activity has been suggested [153], primarily by altering TF expression [154] and cell signaling, migration and angiogenesis [31, 155, 156]. Rydén and coworkers showed that TF phosphorylation related protease-activated receptor-2 signaling plays an important role in breast cancer recurrence [157].

Another common posttranslational modification of eukaryotic proteins is acylation with a fatty acid palmitate, which occurrs at a cysteine residue via a thioester bond formation [158]. S-palmitoylation is almost an exclusive feature of membrane proteins, although there is no well-defined sequence for this modification other than the presence of a free cysteine. S-palmitoylation occurs in the vicinity of cell membranes and directs proteins to the membrane lipid rafts, presumably due to a high affinity of proteins modified with fatty acids for these subdomains of cell membranes [159]. It has been shown in a study by Bach and coworkers that TF has one S-palmitoylation site in the intracellular domain of the protein at Cys[245] [160]. The Cys[245] is located at the amino terminus of the intracellular domain and close to the membrane surface. The extent of palmitoylation at this site, however, is not clear because it has been shown in several publications that in purified TF proteins, Cys[245] can also participate in an inter-molecular disulfide bond formation [160–163]. It has been suggested that S-palmitoylated TF should target cell membrane's lipid rafts, which are enriched in sphingolipids and cholesterol [164]. Increasing experimental data suggest a role for these rafts in modification of tissue factor expression [165, 166] and activity [167–170], although the latter subject remains

controversial [171]. Another mechanism for TF activity regulation by S-palmitoylation is based upon its effect on the phosphorylation of the intracellular domain of the protein. Dorfleutner and Ruf have shown that S-palmitoylation at Cys[245] inhibits phosphorylation at Ser[258] in the intracellular domain of TF [152]. It has been suggested by the authors that palmitoylation favors the association of TF with caveolin-containing lipid rafts. Thus, S-palmitoylation can indirectly alter the procoagulant activity of TF by influencing phosphorylation of the intracellular domain.

5. Cysteines and Disulfides

A quite popular hypothesis describing cell TF activity suggests that many cells, including those in contact with blood, contain under normal physiologic conditions inactive, that is, "encrypted" TF on their surface and that it needs "decryption" to express the procoagulant activity [172]. Several, often contradictory mechanisms have been hypothesized in attempts to explain "encryption-decryption" of TF activity. One of the proposed mechanisms is related to the disulfide bond formation in the extracellular domain of TF.

There are four cysteines (Cys[49], Cys[57], Cys[186], and Cys[209]) located in the extracellular domain of TF [160], which can potentially form disulfide bonds. The carboxyterminal cytoplasmic domain of TF contains a single Cys[245] residue, which is acylated. Two disulfide bridges between Cys[49]–Cys[57] and Cys[186]–Cys[209] have been reported [173]. The role of the latter (Cys[186]–Cys[209]) in the regulation of TF function and the mechanism by which it is formed on the cell surface has been the subject of debates for the last years [174–189]. The suggested range of importance for this bond is from essential [180, 189] to not having any effect on TF function [186, 188]. Bach and coworkers suggested in 1981 that preservation of disulfides is necessary for TF activity [160]. Based on mutagenesis studies, a nonfunctional role has been assigned to the N-terminal disulfide Cys[49]–Cys[57], whereas an important functional role has been assigned to the C-terminal disulfide Cys[186]–Cys[209], because mutation of these cysteines was shown to impair the procoagulant activity of TF [173, 190]. This C-terminal cysteine bridge has been described as an allosteric disulfide bond [174]. An allosteric bond controls protein function by triggering conformational change upon its reduction or oxidation. Unlike the catalytic disulfide bond, which enzymatically mediates thiol-disulfide interchanges in substrate proteins, the allosteric bond nonenzymatically changes the protein structure [191]. Based on an observation that TF activity increased upon the treatment of cells with mercuric chloride, an oxidizing agent [174], it has been suggested that mercuric chloride can oxidize two cysteines forming a disulfide bond. Other publications, however, show that mercuric chloride oxidizes only a single thiol group [192, 193] and that a similar effect could be achieved by treating TF-bearing cells with other metal compounds [175]. Another hypothesis related to the Cys[186]–Cys[209] disulfide bond suggests protein disulfide isomerase as a regulator of cell TF activity via its effect on the oxidation/reduction of this bond [176–180]. In contrast to these publications, several

studies from other laboratories suggested that the TF activity-enhancing effect of protein disulfide isomerase is related to the presence of acidic phospholipids either as a contaminant in the preparations of protein disulfide isomerase [181, 182] or due to their relocation to the cell surface upon treatment with protein disulfide isomerase or mercuric chloride [183–186]. Moreover, one study suggested that Cys^{186} and Cys^{209} are not available for interaction with protein disulfide isomerase when TF at physiologically relevant conditions is bound to factor VIIa [187].

There are several possible explanations for the contradictions observed in publications describing the influence of protein disulfide isomerase on TF activity: (1) variability in reagents, procedures and cell lines used in different laboratories; and (2) the lack of specificity of protein disulfide isomerase for TF. Protein disulfide isomerase has an effect on cell membrane lipid composition [184, 185] and it targets multiple proteins in the cell, catalyzing thiol-disulfide exchange [194–196]. Both of those processes could alter TF activity without changing the status of thiols/disulfides. Additionally, protein disulfide isomerase can alter thrombin generation in a TF-independent manner via coagulation factor ligation to platelets [197] or by catalyzing complex formation with participation of thrombin and antithrombin [198].

Although the status of cysteines of the extracellular domain of TF located on the surface of resting cells remains an open question, recent data from several laboratories brings new knowledge related to their effect on TF function. In contrast to Kothari et al. who suggested that the Cys^{186}–Cys^{209} disulfide bond is not essential for the cell TF procoagulant activity [188], van den Hengel and coworkers demonstrated that the absence of this bond completely abolishes function of human TF presented on the cell surface [189]. A similar conclusion has been drawn about the essential role of the Cys^{190}–Cys^{213} disulfide bond for mouse TF function [199]. Mass spectrometry-based data from our laboratory indicate that the reduction of the Cys^{186}–Cys^{209} bond completely eliminates TF co-factor function, despite that the reduced protein is still able to bind to the enzymatic component of the complex, factor VIIa.

6. Conclusions

TF is an *in vivo* initiator of blood coagulation and is essential for life. It has several types of posttranslational modifications, some of which are different for the natural and recombinant proteins. Although those modifications of TF were identified several decades ago, thorough characterization and an evaluation of their influence on TF function have been somewhat neglected. This neglection was related primarily to the scarce availability of the natural TF protein, and, as a consequence, most of the experiments were done using recombinant TF proteins, despite the known differences in structure and activity of these proteins compared to the natural TF. These differences are translated into differences in physiologically-relevant activities of TF. Thus, caution should be used in data interpretation when a recombinant protein is used as a

surrogate for the natural protein, especially in diagnostic and biological experiments. Lately, the interest in TF structure-activity relationship has been rekindled, primarily by the controversies related to the role of disulfides of the extracellular domain and that of glycosylation. Additionally, there has been an increasing number of studies accomplished using natural human TF or that present on the cell surface instead of recombinant proteins. This increased interest leads us to believe that the gap existing in the knowledge related to the structure-activity relationship of TF will be complemented with new research data.

Acknowledgment

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