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Review

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Comparison of natural and recombinant tissue factor proteins: new insights

Abstract: Tissue factor (TF), an initiator of blood coagulation *in vivo*, is expressed in a variety of cells. Sufficient natural TF has been isolated to clone and express recombinant proteins ranging from full-length TF to its extracellular domain. Because of the limited availability of natural TF, recombinant proteins have been used as surrogates. Despite the differences in their post-translational modifications, it has been accepted that membrane-anchored recombinant TFs are quite similar to the natural TF. Recent studies, however, have shown that post-translational modifications play an important role in TF-triggered thrombin generation.

Keywords: amidolytic activity; extrinsic factor Xase; factor VIIa; post-translational modifications; thrombin generation; tissue factor.

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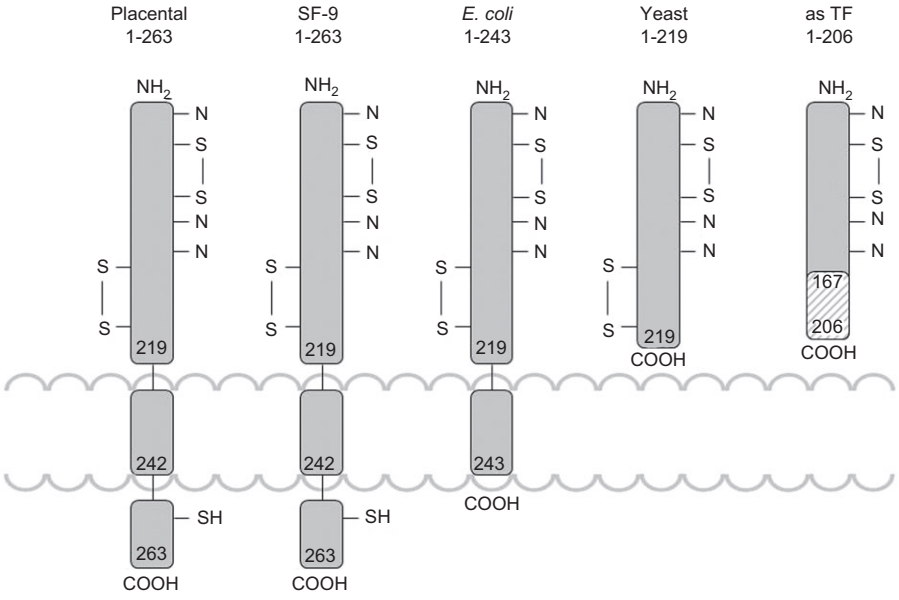
Introduction

Tissue factor (TF) is a non-enzymatic component of the factor VIIa-TF complex, which is essential for the initiation of the blood coagulation process (Lawson et al., 1992; Mackman, 2004). Many cells express TF, especially when they are stimulated by various agents (Drake et al., 1989; Fleck et al., 1990). Monocytes and macrophages are known to express TF upon stimulation by inflammation-related agents (Nijziel et al., 2001; Broussas et al., 2002; Levi et al., 2006). In addition to normal tissues and cells, TF is also known to be expressed by tumor cells, where it is related to the metastatic potential of those cells (Edwards et al., 1981; Lopez-Pedrera et al., 2006). Furthermore, a role for TF in the progression of cardiovascular disease has been suggested (Mumford and McVey, 2004; Jude et al., 2005). On the basis of the data accumulated in our laboratory, we concluded that there is no detectable (<20 fM) active TF circulating in healthy individuals (Butenas and Mann, 2004; Butenas et al., 2005), although a study by Key and co-workers (1998) reported that normal individuals have on average 0.3 pM TF in their blood. Upon mechanical or chemical damage of the vascular wall, subendothelial TF is expressed/exposed to the blood flow and binds factor VIIa, which circulates as an inefficient enzyme (Lawson et al., 1992). When factor VIIa binds the exposed TF, the catalytic efficiency of the complex enzyme formed (factor VIIa-TF; extrinsic factor Xase) increases by several orders of magnitude and initiates blood coagulation by activating the zymogens factor IX and factor X to their respective serine proteases, factor IXa and factor Xa (Komiyama et al., 1990).

TF is a 263-amino-acid lipoprotein containing three domains (Figure 1): (1) an extracellular domain representing the amino-terminal part of the molecule (residues 1–219); (2) a trans-membrane domain, which anchors TF to the membrane (residues 220–242); and (3) a cytoplasmic carboxy-terminal domain (residues 243–263) (Spicer et al., 1987). The extracellular domain of TF participates in interactions with factor VIIa at multiple sites forming a complex enzyme. Factor VIIa binding to TF increases the amidolytic activity of this enzyme by approximately two orders of magnitude for small molecular weight synthetic substrates (Butenas et al., 1993). This activity is primarily dependent on the structure of the substrate and is not influenced by the binding of TF to the membrane (Lawson et al., 1992). In contrast, to express maximum proteolytic activity toward natural substrates factor IX, factor X, and factor VII, the factor VIIa-TF complex must be bound to the appropriate membrane (Fiore et al., 1994). There are several studies describing factor VIIa and TF interaction to form a complex enzyme and factor X binding to the formed factor VIIa-TF complex. However, an interaction of TF with lipids of the membrane and with factor VIIa and factor X on the membrane surface were lacking. Lately, attempts have been made to fill this gap. Interactions of soluble TF with the synthetic membrane and with factor VIIa on the membrane were investigated using molecular dynamics simulations in full atomic detail (Boettcher et al., 2010; Ohkubo et al., 2010). These studies could



Mol wt	Placental	SF-9	*E. coli*	Yeast	as TF
AA	29 592	29 592	27 285	24 672	23 671
SDS-PAGE	45 000	39 000	32 000	37 000	-
MALDI-TOF	36 179	32 705	27 795	29 208	-

Figure 1 Structure of various TF species.
The 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 Butenas et al. (2007).

lead to a better understanding of factor VIIa-TF-factor X (and IX) interactions on cell membranes. Thus, two of the three domains of TF (extracellular and trans-membrane) play distinct roles in the blood coagulation process. The major role of the cytoplasmic domain is related to signal transduction (Schaffner et al., 2012). As a consequence, it has been generally accepted that TF lacking the cytoplasmic domain is functionally identical to the full-length protein in the initiation of blood coagulation. However, recombinant TF lacking both the cytoplasmic and transmembrane domains cannot bind to the membrane, and therefore while forming a complex with factor VIIa, does not activate factor VII and has a decreased efficiency toward factor IX and factor X (Fiore et al., 1994).

A variety of human recombinant TF species have been produced, from those containing only the extracellular domain to the full-length protein (Paborsky et al., 1989; Fiore et al., 1994). These recombinant proteins have been extensively used in research and clinical laboratories worldwide because of the limited availability of natural TF. Alternatively, many laboratories have been using natural 'thromboplastin' reagents as a source of TF in their experiments (van den Besselaar et al., 2010). These reagents are made by homogenizing natural tissues (most often non-human) that contain a relative abundance of TF such as the brain, placenta, and lung, or by reconstituting recombinant TF. Experimental results acquired using recombinant TF proteins and thromboplastins *in vitro* are frequently used for understanding the coagulation processes occurring *in vivo*. The non-availability of isolated natural TF does not allow the confirmation (or rejection) of results obtained with recombinant proteins or those present in homogenates of natural tissues. Additionally, the use of artificial membranes could significantly alter the functional activity of TF in comparison with that observed on native membranes.

Overall, the non-availability of natural TF led to a scarcity of data addressing the influence of some structural components of TF on its function. As a consequence, there are plenty of controversies in published studies related to the structure/function of this protein. Two of the controversies are related to the influences of post-translational modifications and the environment provided by the living cell on TF function. In this review, TF controversies are discussed in light of recent experimental data.

Post-translational modifications and activity of TF proteins

Gel electrophoresis and Western blotting of various species of purified natural and recombinant TF proteins showed

Table 1 Molecular masses (Da) of TF proteins.

TF species	Source	Calculated[a]	MALDI-TOF	Difference	SDS-PAGE
Recombinant TF_{1-218}	Yeast	24 672	29 208	4536	37 000
Recombinant TF_{1-243}	*E. coli*	27 285	27 795	510	32 000
Recombinant TF_{1-263}	SF-9 cells	29 592	32 705	3113	39 000
Natural human	Placenta	29 592	36 179	6587	45 000
Monocytic	Cell line THP-1	29 592	–	–	47 000

[a]Based on amino acid composition.

different mobility for each protein, including those with the same amino acid composition (Krudysz-Amblo et al., 2010). The relative mobility of TF proteins reflected their molecular weights, although values observed by this method were significantly higher than those determined by mass spectrometry (Table 1). The data presented in the table display the only fundamentally sound molecular weights for purified TF proteins based on mass spectrometry. The comparison of mass spectrometry data and those of gel electrophoresis shows that the real molecular weight of the full-length recombinant TF 1-263 is 32 705 Da, whereas gel electrophoresis suggests approximately 39 000 Da. Similar observations were made for other TF species. Purified placental and monocyte TF proteins had a lower mobility than the corresponding recombinant counterpart (full-length TF 1-263), suggesting a higher molecular weight for natural proteins. This observation was verified by mass spectrometry, which showed that placental TF has a molecular weight of 36 179 Da. These differences in molecular weights of proteins are caused by a different level of post-translational modifications, which are determined by the protein expression system (see Table 1). According to mass spectrometry data, the level of post-translational modifications of various forms of TF ranges from 377 Da for recombinant TF 1-243 to 3604 for recombinant TF 1-263 and to 6605 Da for the natural placental TF protein (Krudysz-Amblo et al., 2010).

While for the efficient proteolysis of natural substrates (factors IX, X, and VII), the complex factor VIIa-TF enzyme has to be located on a membrane containing acidic phospholipids (Mann et al., 1990; Fiore et al., 1994), the enzymatic activity of the protein-protein complex with small molecular weight synthetic substrates is phospholipid independent (Lawson et al., 1992). The apparent affinities of the TF proteins for factor VIIa and catalytic efficiencies of formed complex enzymes were evaluated by functional titrations of factor VIIa in a fluorogenic assay in the absence of phospholipids (Table 2) (Krudysz-Amblo et al., 2010). Recombinant full-length TF had a higher affinity for factor VIIa than its placental counterpart (dissociation constants of 0.31 and 0.92 nM, respectively). Recombinant truncated TF 1-243 also showed a tighter binding to factor VIIa (0.41 nM) than the natural placental protein. The dissociation constant for the complex enzyme formed by factor VIIa and the soluble form of TF 1-218 was higher by approximately one order of magnitude (7.5 nM) than those observed for transmembrane domain-containing proteins. A more pronounced difference between human placental TF and recombinant TF proteins was observed when the maximum rate of substrate hydrolysis at saturating concentrations of factor VIIa was analyzed. The rate was the highest for the natural placental TF (173 pM s^{-1}) and significantly lower for recombinant TF proteins. All forms of TF formed a complex with factor VIIa with a 1:1 stoichiometry. These factor VIIa titration data suggested that natural TF is a more efficient cofactor for factor VIIa than its recombinant counterparts.

For the expression of activity of the factor VIIa-TF complex toward its natural substrates, the complex enzyme has to be located on the membrane surface (Mann et al., 1990; Fiore et al., 1994). Comparison of relipidated TF proteins in the extrinsic factor Xase (factor X activation by the factor VIIa-TF complex) show that the most efficient activator of factor X is the complex formed by placental TF and factor VIIa (Table 3, Figure 2). However, the highest activity was observed for the extrinsic factor Xase formed by factor VIIa and monocyte TF presented on the cell surface or in cell lysate.

The observed Michaelis constants (K_M) are somewhat higher for all TF proteins tested than those published

Table 2 Binding of TF to factor (F)VIIa in a fluorogenic assay.

TF species	$K_{d(app)}$ (nM)	V_{max} (pM s^{-1})	Stoichiometry (TF:FVIIa)
rTF_{1-243}	0.41±0.40	59.7±1.2	1.1:1.0
$rTF_{1-243*D'}$	0.47±0.03	61.2±1.0	0.9:1.0
rTF_{1-263G}	0.31±0.02	75.8±1.6	0.9:1.0
rTF_{1-263D}	0.35±0.02	78.3±1.4	1.0:1.0
pTF_{G}	0.92±0.15	173.1±8.4	0.9:1.0
pTF_{D}	0.69±0.09	172.8±10.0	1.0:1.0
rTF_{1-218G}	7.5±0.62	45±0.9	–

G, glycosylated; D, deglycosylated.

Table 3 TF proteins in the extrinsic factor Xase.

TF species	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	Relative activity[a]
Recombinant TF_{1-218}	N/A	N/A	N/A	0
Recombinant TF_{1-243}[b]	1.2	1.8	1.5	1.0
Recombinant TF_{1-263}[b]	0.54	2.8	5.2	3.5
Human placental[b]	0.32	8.7	26.8	17.9
Monocytic *in situ*	0.70	59.7	85.3	56.9
Monocytic in lysates	0.40	44.9	112.2	74.8

[a]Ratio of k_{cat}/K_m.
[b]Relipidated in PCPS.

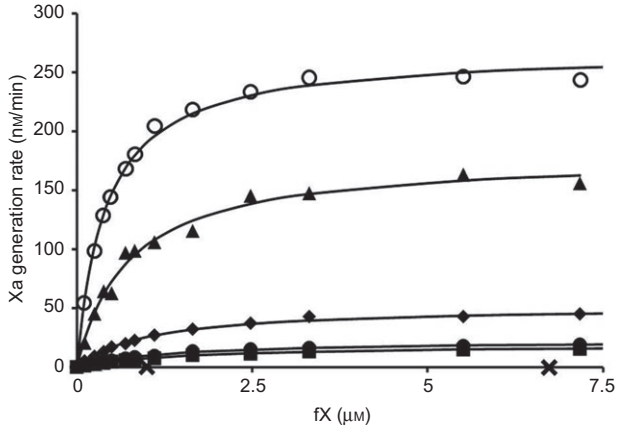


Figure 2 TF proteins in the extrinsic factor Xase.
Purified and relipidated recombinant TF 1-243 (●), recombinant TF 1-263 (■), placental TF (♦), recombinant TF 1-218 in solution (×), monocyte TF *in situ* (▲), and monocyte TF in monocyte lysate (○) were incubated with factor VIIa. Varying concentrations of factor X were added and the rates of factor Xa generation were evaluated.

previously (Komiyama et al., 1990). This apparent decrease of the substrate (factor X) affinity for the complex enzyme is most likely caused by the dilution of enzyme and substrate on the phospholipid vesicles. As the surface area increases, the local concentration of enzyme and substrate decreases and, as a consequence, an increase in the apparent K_M is observed. Similar phospholipid effects have been reported for prothrombin activation by the prothrombinase complex (Nesheim et al., 1984) and for factor VII activation by factor Xa (Butenas and Mann, 1996).

Glycosylation

It was predictable that the prevailing post-translational modification of TF is 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 (Figure 1) and one (Asn^{261}) in the cytoplasmic domain (Paborsky et al., 1989; Paborsky and Harris, 1990). The latter site is not present in truncated TF proteins. Although the sites of glycosylation of the extracellular domain and a partial identification of carbohydrates attached to those sites was accomplished, a more detailed analysis of carbohydrate moieties and their role on TF function, affinity for factor VIIa, and influence on the factor VIIa-TF complex enzyme activity toward natural and synthetic substrates were missing. A possible lack of interest to TF glycosylation could be explained by the influence of several studies published approximately two decades ago. In those studies, the carbohydrates were reported to be insignificant for the activity of TF (Waxman et al., 1992; Stone et al., 1995; Rickles et al., 1996) owing to a similar activity of the non-glycosylated recombinant protein from *Escherichia coli* and the glycosylated one from kidney 293 cells (Paborsky and Harris, 1990). In contrast to those publications, data from other laboratories (Pitlick, 1975; Shands, 1985; Bona et al., 1987) suggested that carbohydrates could play a role in the activity of TF. Pitlick observed that concanavalin A inhibits the coagulant activity of TF by binding reversibly to the carbohydrate moiety of the protein. Shands and Bona and coworkers both observed the loss of function and inability of TF to be incorporated into membranes after treatment with tunicamycin, an inhibitor of protein glycosylation (Heifetz et al., 1979). However, the direct evidence for the effect of glycosylation on the activity of TF came from our laboratory (Krudysz-Amblo et al., 2010).

We started characterization of modifications from identifying the extent of glycosylation and structure of carbohydrates at each potential glycosylation site of all three TF proteins. Both full-length TF proteins analyzed (placental and recombinant) had all three potential glycosylation sites in the extracellular domain (Asn^{11}, Asn^{124}, and Asn^{137}) modified with carbohydrates. No carbohydrates were detected on recombinant TF 1-243 produced in *E. coli* (Krudysz-Amblo et al., 2010). All three TF proteins, both native and deglycosylated, were tested for their effect on factor VIIa activity in a membrane-independent fluorogenic assay and in a membrane-dependent extrinsic factor Xase assay. For both recombinant TF 1-263 and placental TF, deglycosylation had little effect on the affinity for factor VIIa and on the activity of the formed complex in the membrane-independent fluorogenic assay. Nevertheless, the activity of the placental TF remained >2-fold higher than that of the recombinant protein. In the extrinsic factor Xase, deglycosylation marginally decreased the catalytic constant for factor X activation from 2.8 s^{-1}

for glycosylated to 2.2 s^{-1} for deglycosylated recombinant TF 1-263 (Table 4, Figure 3). A more pronounced effect of deglycosylation was observed for placental TF. Deglycosylation decreased the catalytic constant for factor X activation from 8.7 to 2.3 s^{-1}, having less pronounced effect on the K_M (Krudysz-Amblo et al., 2010). After deglycosylation, the catalytic efficiency of factor Xa generation was almost identical for the recombinant TF 1-263 and placental TF, and was similar to that of TF 1-243. The native (glycosylated) form of placental TF was 3.1-fold more active than the native form of the recombinant protein 1-263 and 4.8-fold more active than TF 1-243. Owing to the threshold nature of the TF-triggered thrombin generation, this <5-fold difference in the catalytic efficiency leads to dramatic changes in thrombin generation in complex systems representing the blood coagulation process (Figure 4). These data indicate that, in contrast to previously published statements suggesting that post-translational modifications have no effect on TF activity (Paborsky and Harris, 1990; Waxman et al., 1992), TF glycosylation has a pronounced effect on factor X proteolysis by the factor VIIa-TF complex and, as a consequence, on thrombin generation.

Table 4 Effect of glycosylation on activity of the TF-factor (F)VIIa complex in the extrinsic factor Xase.

TF species	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($μM^{-1}$ s^{-1})	Relative activity[a]	Stoichiometry (TF:FVIIa)
rTF_{1-243}	1.19±0.22	1.8	1.5	1.0	1.0:1.0
$rTF_{1-243'D'}$	1.31±0.26	1.9	1.4	0.9	1.0:1.0
rTF_{1-263G}	0.54±0.05	2.8	5.2	3.5	1.0:1.0
rTF_{1-263D}	0.57±0.17	2.2	3.8	2.5	0.9:1.0
pTF_G	0.32±0.04	8.7	26.8	17.9	0.9:1.0
pTF_D	0.57±0.07	2.3	4.0	2.7	0.9:1.0

G, glycosylated; D, deglycosylated.
[a]Ratio of k_{cat}/K_m.

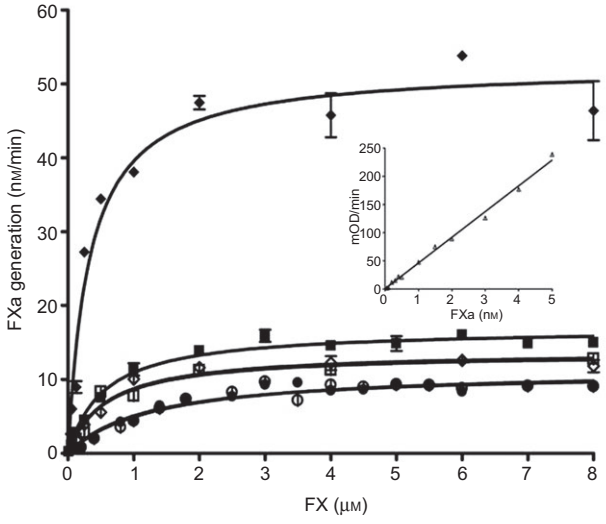


Figure 3 Effect of TF deglycosylation on its function in the extrinsic factor Xase.
Varying concentrations of factor X were incubated with the complex of factor VIIa and glycosylated (●) and deglycosylated (○) recombinant TF 1-243, glycosylated (■) and deglycosylated (□) recombinant TF 1-263, and glycosylated (♦) and deglycosylated (◇) placental TF. Factor Xa generation rates were evaluated from the factor Xa calibration curve (inset). This figure was originally published in Krudysz-Amblo et al. (2010).

The native and deglycosylated forms of recombinant 1-263 and placental TF were used for the characterization of carbohydrates attached to the corresponding asparagines. The analyses revealed that out of four potential sites for N-linked glycosylation (Asn^{11}, Asn^{124}, Asn^{137}, and Asn^{261}), two (Asn^{124} and Asn^{137}) were found to undergo complete glycosylation in both the recombinant and placental proteins (Krudysz-Amblo et al., 2011a). An incomplete glycosylation occurs at Asn^{11} of recombinant TF 1-263 with a relative abundance of carbohydrates at 20%, whereas the extent of 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 the recombinant and natural protein as well as between each site within each protein. Recombinant TF 1-263 predominantly contains high mannose (Mann) sugars at Asn^{11} with a varying number of Mann residues ($Mann_1$-$Mann_4$); however, a fucosylated core structure ($GlcNAc_1Mann_3$) is also found as a minor modification.

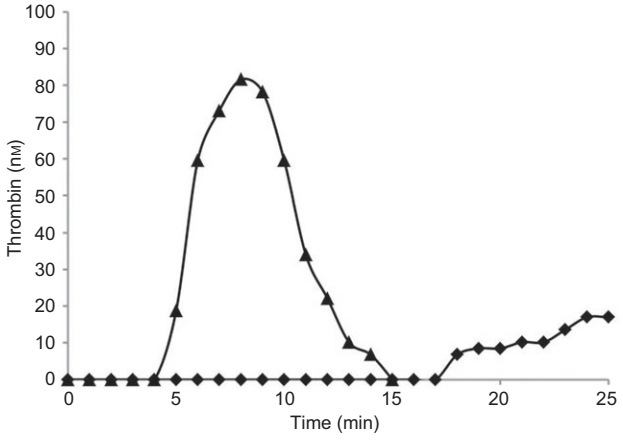


Figure 4 Thrombin generation in the synthetic coagulation proteome.
Placental (▲) or recombinant TF 1-243 (♦) at equimolar concentrations were added to the mixture of platelets; factors II, V, VII, VIIa, VIII, IX, X, and XI; antithrombin-III; and TF pathway inhibitor (all at mean physiologic concentrations) and thrombin generation over time was evaluated.

Asn^{124} in recombinant protein contains high mannose ($Mann_2$-$Mann_7$) and hybrid ($Mann_1HexNAc_1$-$Mann_2HexNAc_2$) carbohydrates. High-mannose carbohydrates are also found on Asn^{137} ($Mann_2$-$Mann_6$) accompanied by complex sugars composed of a fucosylated core and terminal HexNAcs ($HexNAc_1$-$HexNAc_2$). A unique characteristic of placental TF is the presence of sialic acid on all three sites, Asn^{11}, Asn^{124}, and Asn^{137}. All three sites contain either hybrid or complex carbohydrates. In contrast to recombinant protein, high-mannose sugars are absent in placental TF. The number of Mann, HexNAc, and sialic acid residues varies, contributing to a high heterogeneity at each site ($Fuc_1Mann_2HexNAc_3$-$Fuc_1Mann_4HexNAc_5SA_2$). An interesting observation related to carbohydrates at Asn^{124} is that fucosylation of this site in both recombinant and placental TF is not favored, and fucosyl residues are absent at this site in both proteins. The overall abundance and mass of carbohydrates on placental TF is greater than that found on recombinant protein, which might be attributed to the presence of sialic acid on the former. The N-terminal analysis showed that both recombinant and placental proteins exist in two forms, a full-length form composed of 263 amino acid residues and a truncated form composed of 261 residues, where the first two amino acids on the N-terminus of the protein are missing. This observation was consistent with previously published data (Spicer et al., 1987; Paborsky et al., 1989). The fractional abundance of each form in the two proteins is different. Recombinant TF 1-263 is predominantly (77%) expressed in the truncated form, whereas only 31% of placental protein is truncated (Krudysz-Amblo et al., 2011a).

These findings make a contribution in elucidating the structural aspects of TF that are important in its interaction with factor VIIa and factor X (Ruf et al., 1994). Factor X binding to the factor VIIa-TF complex occurs close to the membrane surface and in close proximity to Asn^{124} (Martin et al., 1995). How glycosylation affects the interaction of TF with its molecular substrates is still unclear. It is possible that the difference in the extent and the nature of glycosylation of some or all of the three glycosylation sites contributes to the difference in the activity of the extrinsic factor Xase formed by either recombinant or placental TF.

Phosphorylation and acetylation

Protein phosphorylation plays a critical role in the regulation of many protein functions. Protein phosphorylation is carried out by protein kinases, which transfer phosphate to the hydroxyl groups of the side chains of three amino acids – serine, threonine, and tyrosine (Cohen, 1982). Hydroxyl groups of serine represent the major site of phosphorylation in proteins (90–95% of total phosphorylation sites) and 5–10% of total phosphorylation occurs at the threonine side chain, whereas tyrosine phosphate represents <1% of the total protein phosphorylation in eukaryotic cells.

Zioncheck et al. (1992) determined that TF contains two phosphorylation sites, both of them in the cytoplasmic domain. They suggested that phosphorylation is regulated by the protein kinase C (PKC)-dependent mechanism because it was induced by the PKC activator phorbol 12-myristate 13-acetate, whereas staurosporine, a potent PKC inhibitor, abolished the phosphorylation of TF. From the alignment of cDNA sequences of several 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 (1997) suggested that the cytoplasmic domain of TF can be phosphorylated *in vitro* at multiple sites, particularly at Ser^{253} and Ser^{258} (Mody and Carson, 1997). The mutational data presented by Dorfleutner and Ruf (2003) suggested that initial phosphorylation at Ser^{253} enhances the subsequent phosphorylation at Ser^{258} (Dorfleutner and Ruf, 2003).

The mass spectrometry analyses showed that there are distinct differences in phosphorylation of recombinant and natural (placental) TF. Recombinant TF 1-263 is phosphorylated only on Ser^{258} with 7% containing phosphate (Krudysz-Amblo et al., 2011b). In contrast, phosphorylation on the cytosolic domain of placental TF takes place only at Ser^{253}, with 36% phosphorylation. Additional phosphorylation sites at Thr$^{170/172}$ were identified in the extracellular domain of placental TF with a fractional abundance of 22% and at one of the residues between Ser^{42} and Lys^{65}. No phosphorylation at these sites was observed in recombinant TF 1-263. An acetylation at Lys^{181} was also identified in placental TF but not in the recombinant protein. Certain heterogeneity in acetylation was observed in placental TF purified from different placentas. The extent of these modifications is under investigation.

The role of phosphorylation and acetylation in the extracellular domain of placental TF remains to be determined. However, an observation that deglycosylated placental TF has a lower affinity for factor VIIa and higher activity with a low molecular weight synthetic substrate than deglycosylated full-length recombinant TF 1-263 suggests that these modifications could have an effect on TF cofactor function (Krudysz-Amblo et al., 2010).

TF environment and activity

All reactions leading to TF-initiated thrombin formation and consequential blood coagulation are presumed to occur on membrane surfaces provided by platelets, microparticles, monocytes, endothelial cells, and other cells (Stern et al., 1985; Tracy et al., 1985; Tans et al., 1991; Bouchard and Tracy, 2003). Artificial membranes composed of several phospholipids have been widely used in experiments presumably representing the processes occurring during blood coagulation *in vivo* (Mann et al., 1990). The composition of these membranes has been based on the knowledge of natural membranes, primarily those of platelets. An essential, 'active', component of such membranes is an acidic phospholipid, phosphatidylserine, which is exposed on the membrane surface upon platelet activation (Bevers et al., 1982) and serves for the binding of proteins involved in processes leading to thrombin generation. Artificial membranes are expected to provide a surface similar to that present on cells. However, the surface of a living cell is a much more complex entity than just a phospholipid membrane. It also contains receptors, which serve as docking sites for the assembly of complex enzymes of coagulation (McGee et al., 1989; McGee and Li, 1991; Bouchard and Tracy, 2003). Thus, it is not surprising that mechanisms of the reactions occurring on the cell surfaces are qualitatively and quantitatively different from those occurring on artificial membranes.

Monocytes can play a distinctive role in blood coagulation. They can provide surface for the assembly of complex enzymes involved in the TF pathway to thrombin (McGee et al., 1989; McGee and Li, 1991; Bouchard and Tracy, 2003). However, the most important role of monocytes in blood coagulation is the synthesis and expression of TF upon stimulation by bacterial lipopolysaccharides (LPS) (Broussas et al., 2002; Bouchard and Tracy, 2003). A variety of agonists and interactions can upregulate the expression of TF (Carlsen and Prydz, 1988; Cermak et al., 1993; Celi et al., 1994). More recently, it has been suggested that the toll-like receptors TLR4 and TLR6 contribute to the expression of TF on monocytes and monocytic cells (Xu et al., 2011; Zhou et al., 2011; Owens et al., 2012) induced by oxidized low-density lipoproteins and anti-β_2-glycoprotein I/β_2-glycoprotein I. It has been also shown that platelet-derived growth factor CC (Gebhard et al., 2010) and the platelet factor 4/heparin-antibody complex (Kasthuri et al., 2012) can induce monocyte TF expression. It has been suggested that the majority of the cell surface TF is latent or 'encrypted' (Bach, 2006), has little (if any) activity, and that cell lysis (Le et al., 1992) or treatment (Wolberg et al., 1999) can lead to the 'decryption'. However, our data using quantitated concentrations of TF on LPS-stimulated monocytes and in their lysates (Parhami-Seren et al., 2004) indicate that TF activity in both cases is similar, with the latter being only 1.3-fold more efficient than the former (Table 3, Figure 2). The difference in TF activity on monocyte surface and their lysates is, most likely, related to the release of TF from the intracellular stores upon the treatment of stimulated cells (Egorina et al., 2005, 2006). Monocyte-expressed TF binds factor VIIa and initiates proteolytic activation of factor X (Edwards and Rickles, 1984) and factor IX (Worfolk and Tracy, 1995). Propagation of the coagulant response on the surface of stimulated monocytes is accomplished subsequently through the assembly of the intrinsic factor Xase (McGee and Li, 1991) and prothrombinase (Tracy et al., 1983). Data related to the efficiency of the intrinsic Xase and prothrombinase suggest that rates of proteolyses of substrates by these complex enzymes do not exceed rates observed on the synthetic phospholipid membranes (Tracy et al., 1983; McGee and Rothberger, 1986; McGee et al., 1989; McGee and Li, 1991).

However, in our more complex systems [blood (Rand et al., 1996), plasma (Butenas et al., 2005), and synthetic coagulation proteome (Lawson et al., 1994; Butenas et al., 1999)], a dramatic difference in the activity of natural monocyte TF located on a native membrane (living monocytic cells) and that of recombinant proteins presented on an artificial membrane composed of phosphatidylcholine and phosphatidylserine (PCPS) was observed. Monocytic TF *in situ* displayed functional activity up to approximately 200-fold greater than the recombinant protein (Figure 5). This elevated activity can be assigned primarily

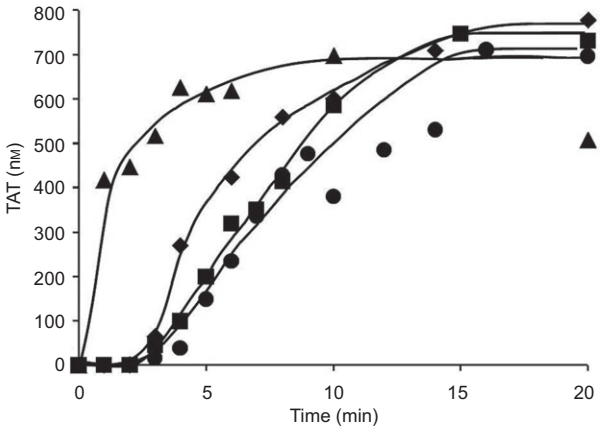


Figure 5 Thrombin generation in whole blood.
Thrombin generation in contact pathway-inhibited whole blood was triggered with recombinant TF 1-263 (■), recombinant TF 1-243 (●), placental TF (♦) (all relipidated and used at 5 pM), or 1.2 pM monocyte TF *in situ* (▲).

to TF because a monoclonal anti-TF antibody inhibited 97% of this activity. Additionally, this elevated activity of monocyte TF *in situ* was not related to platelets because it has been observed in their absence (citrated plasma and synthetic coagulation proteome) as well as in their presence (whole blood and synthetic coagulation proteome) (Butenas et al., 2004).

During the last several years, increasing experimental data suggest a role for cell membrane lipid rafts in the modification of TF expression (Fortin et al., 2005; Mandal et al., 2006; Davizon et al., 2010; Henriquez et al., 2011) and activity (Sevinsky et al., 1996; Dietzen et al., 2004; Mandal et al., 2005). It has been shown that one of the components of the lipid rafts, cholesterol, might play an important role in TF function (Dietzen et al., 2004; Mandal et al., 2005) and TF-bearing microparticle shedding (Del Conde et al., 2005), although the data related to its effect are contradictory. Additionally, an observed effect of lipid rafts on TF function is quite limited and does not explain an increase in that function by more than two orders of magnitude. It is possible that a unique lipid or a composition of lipids not related to the lipid rafts could be responsible for a high monocyte TF activity when this protein is presented in a native environment. Alternatively, a TF receptor similar to those for protein C (Galligan et al., 2001), factors X and Xa (McGee and Rothberger, 1986; Altieri et al., 1988; Worfolk et al., 1995), and the prothrombinase complex (Tracy et al., 1985) could be the cause of this phenomenon. Further studies are required to elucidate the nature of high monocyte TF activity.

Conclusions

It has been widely accepted for the last two decades that recombinant TF proteins are indistinguishable from the natural TF with respect to their factor VIIa cofactor function in reactions leading to thrombin generation and blood coagulation. As a consequence, it has been believed by the majority of researchers in the field of coagulation that post-translational modifications of TF have no effect on the procoagulant activity of this protein, although no convincing (if any) data were provided to confirm such statements. However, more recent data, primarily based on mass spectrometric analyses, challenged these statements and demonstrated that natural TF proteins have (significantly) higher specific activity than their recombinant counterparts and that this difference in activity is defined by differences in post-translational modifications and by the environment/presentation of TF.

There are several aspects of TF structure/function not addressed in this review, particularly the regulation of TF function by the Cys^{186}-Cys^{209} disulfide bond. However, the controversy related to this bond has been discussed in three reviews from our laboratory published in 2012 (Butenas, 2012; Butenas and Krudysz-Amblo, 2012; Butenas et al., 2012).

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