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Disulfide reduction abolishes tissue factor cofactor function

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Abstract

Background—Tissue factor (TF), an *in vivo* initiator of blood coagulation, is a transmembrane protein and has two disulfides in the extracellular domain. The integrity of one cysteine pair, Cys186–Cys209, has been hypothesized to be essential for an allosteric “decryption” phenomenon, presumably regulating TF procoagulant function, which has been the subject of a lengthy debate. The conclusions of published studies on this subject are based on indirect evidences obtained by the use of reagents with potentially oxidizing/reducing properties.

Methods—The status of disulfides in recombinant TF_{1–263} and natural placental TF in their non-reduced native and reduced forms was determined by mass-spectrometry. Functional assays were performed to assess TF cofactor function.

Results—In native proteins, all four cysteines of the extracellular domain of TF are oxidized. Reduced TF retains factor VIIa binding capacity but completely loses the cofactor function.

Conclusion—The reduction of TF disulfides (with or without alkylation) eliminates TF regulation of factor VIIa catalytic function in both membrane dependent FX activation and membrane independent synthetic substrate hydrolysis.

General significance—Results of this study advance our knowledge on TF structure/function relationships.

Keywords

tissue factor; disulfides; mass-spectrometry; extrinsic factor Xase; fluorogenic assay

1. INTRODUCTION

Tissue factor (TF) contains five cysteines (Cys), four of them (Cys49, Cys57, Cys186, Cys209) reside in the extracellular domain and one (Cys245) in the cytoplasmic domain. Two disulfide bridges between Cys49–Cys57 and Cys186–Cys209 have been reported [1]. Over twenty years ago, Bach *et al.* suggested that preservation of these disulfides is necessary for the proper folding and activity of TF [2]. Based on mutagenesis studies, a non-

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functional role has been assigned to the NH₂-terminal disulfide between Cys49–Cys57 [3]. The formation of the Cys186–Cys209 bridge has been hypothesized to account for the “decryption” of TF during which reduced TF is oxidized and emerges from its cryptic form to the fully active decrypted form [4].

The C-terminal cysteine bridge Cys186–Cys209 of the extracellular domain of TF has been hypothesized to be an allosteric disulfide, which controls protein function by triggering conformational changes upon its reduction or oxidation [4]. Unlike a catalytic disulfide bond, which enzymatically mediates thiol-disulfide interchanges in substrate proteins, the hypothesized allosteric bond changes the intra- or inter-molecular protein structure [5]. The subsequent change in TF conformation is hypothesized to affect the intermolecular interactions between TF, an enzymatic component of the extrinsic factor (F)Xase, FVIIa, and the natural substrate FX, leading to altered dynamics of FX activation and consequential thrombus formation [6].

While there is common agreement about the leading role of TF in the initiation of blood coagulation *in vivo*, there are significant controversies related to the expression and regulation of TF activity on the cell surface. It has been suggested that the majority of TF molecules located on the cell surface have low activity or are “encrypted” and that “decryption” is essential for the expression of TF function [7]. Several mechanisms, often contradictory, have been suggested in an attempt to explain “encryption/decryption” of TF function [8–18]. More recently, the role of the Cys186–Cys209 bond in the “encryption/decryption” phenomenon was suggested [4]. The presumed formation of this bond using an oxidizing agent (HgCl₂) increased TF-related FVIIa activity, although the subject of the disulfide bridge formation between two unpaired cysteines by this treatment remains controversial. In studies published previously, it was concluded that HgCl₂ can modify only a single thiol group [19, 20]. Moreover, an increase in TF activity on cell surfaces similar to that caused by HgCl₂ can be achieved by treating TF-bearing cells with other metal compounds, such as silver nitrate and phenylmercuric acetate [21]. Additionally, several studies showed that such increase in TF function is related to the elevated exposure of phosphatidylserine (PS) [21, 22] on cell surface upon treatment with HgCl₂. More recent data from Hogg’s laboratory, however, suggest that HgCl₂ can possibly trigger the Cys186–Cys209 bond formation in TF [23].

Protein disulfide isomerase (PDI) has been suggested as an important player in the oxidation and reduction of Cys186–Cys209 disulfide bond and consequently in the enhancement and reduction of TF activity, respectively [24,25]. Recently, Furlan-Freguia et al. [26] and Liang et al. [23] reported their observations in support of the oxidation of Cys186 and Cys209 and its contribution to TF function. Furlan-Freguia et al. described a pathway through which TF procoagulant activity is generated via a PDI mechanism. Liang et al. studied the redox potential and spacing of the two cysteines, suggesting that TF activators enhance TF function through oxidation of Cys186 and Cys209. In contrast to these publications, lack of influence of PDI on TF function has also been reported [27,28]. Moreover, Bach and Monroe reported that the TF Cys186–Cys209 bridge is inaccessible to PDI manipulation when the cofactor is bound to the enzyme FVIIa. As a consequence of these conflicting studies, a review on PDI and TF activity concludes that the topic itself remains “cryptic” [29].

In the current study, we analyzed the status of oxidized, reduced and reducedcarboxyamidomethylated cysteines in human placental TF (pTF) and recombinant TF (rTF_{1–263}) proteins and evaluated their effect on membrane independent fluorogenic substrate hydrolysis and membrane dependent FXa generation. Mass spectrometry was used

to assess the status of the cysteines. Our data allowed a conclusion that reduction of TF cysteines eliminates TF cofactor function in the TF/FVIIa complex.

2. EXPERIMENTAL PROCEDURES

2.1 Proteins

rTF₁₋₂₆₃ was a gift from Dr. Jenny and sheep anti-human TF polyclonal antibody (Ab) was purchased (Haematologic Technologies Inc, Essex Junction, VT). Anti-TF-5 monoclonal antibody (mAb) and anti-FVII-1 mAb were produced and purified in house. rFVIIa was a gift from Dr. Hedner (Novo Nordisk, Denmark). Human FX was isolated from fresh frozen plasma using an anti-FX mAb-coupled Sepharose [30]. Streptavidin-horse radish peroxidase (HRP), HRP-goat anti-mouse Ig and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Tetramethylbenzidine (TMB) peroxidase substrate was purchased from KPL, Inc. (Gaithersburg, MD). Protein disulfide isomerase (PDI) was purchased from BioVision, Inc. (Milpitas, CA). Trypsin was from Promega Corporation (Madison, WI).

2.2 Materials

1,2-Dioleoyl-sn-Glycero-3-Phospho-L-serine (PS) and 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (PC) were from Avanti Polar Lipids Inc. (Alabaster, AL). PCPS vesicles (75% PC and 25% PS) were made as described previously [31]. Spectrozyme FXa was from American Diagnostica Inc. (Stamford, CT). Fluorogenic substrate D-FPR-ANSNH-C₄H₉·2HCl (FPRnbs) was synthesized in-house [32]. ProteaseMaxTM Surfactant was from Promega Corporation. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Triton X-100 were from ThermoScientific (West Palm Beach, FL). Iodoacetamide (IAA), β-mercaptoethanol (BME), DL-dithiothreitol (DTT), NaSCN Tris-HCl, pre-activated Sepharose® CL-4B resin, benzamidine hydrochloride, guanidinium HCl, reduced and oxidized glutathione (GSH and GSSG, respectively) were from Sigma (St. Louis, MO). Calcium chloride (CaCl₂), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and Polyethylene glycol 8000 (PEG) were from Fisher Scientific (Pittsburgh, PA).

2.3 Isolation of pTF

Placental TF was isolated from fresh frozen human placentas as described, with slight modifications [2,33]. The placentas were homogenized in 1.5 L ice-cold acetone using PolyTron 2000 tissue homogenizer (Cole-Parmer, Vernon Hills, IL). Homogenate was stirred for 30 minutes in a cool dry-ice methanol bath and centrifuged at 10,000g for 30 minutes. The supernatant was discarded and pellet extracted 3 times with 1.5 L acetone. The pellet was homogenized in 1.5 L of TBS-0.2% NaSCN-10 mM Benzamidine-HCl pH 7.4, stirred at room temperature for 1 hour and centrifuged at 10,000g for 30 minutes. Pellet homogenization, extraction, and centrifugation were repeated with 1.5 L TBS-0.1% Triton X-100 - 0.2% NaSCN-10 mM Benzamidine-HCl pH 7.4 followed by 4L TBS-2% Triton X-100-0.2% NaSCN-10 mM Benzamidine-HCl pH 7.4. The supernatant of the TBS-2% Triton X-100 extract was incubated overnight with anti-TF-5 mAb [33] or sheep anti-TF polyclonal Ab coupled to pre-activated CL-4B resin. Resin was collected, washed, and eluted with HBS-10 mM CHAPS-3M NaSCN. Eluate was dialyzed into HBS-10 mM CHAPS.

2.4 Sample preparation

To determine the optimal condition for complete reduction of TF a total of 7 reduction conditions were set up using rTF₁₋₂₆₃ (Table 1). First, we reduced TF with 8 mM DTT at pH 8.0 for 4 hours at either 25°C or 57°C. Both samples were then carboxyamidomethylated

with 20 mM IAA for 30 minutes at room temperature. Two other samples were reduced at 37 °C with 8 mM and 20 mM DTT. Both samples, after reduction, were dialyzed in HBS-10 mM CHAPS pH 6.0 overnight and then treated with 20 mM and 50 mM IAA, respectively. We also prepared 3 samples, which were denatured with 6 M guanidium-HCl in HBS-10 mM CHAPS pH 8.0 for 60 minutes prior to reduction. The first sample was then treated with 8 mM DTT, the second with 20 mM DTT and the third with 20 mM BME. All were dialyzed into HBS-10 mM CHAPS pH 6.0 followed by carboxyamidomethylation with 20, 50, and 50 mM IAA, respectively. All samples were analyzed by liquid chromatography/mass spectrometry (LC-MS/MS). The efficiency of the reduction was established on the basis of the Cys49–Cys57 bond. For the sample with 99% of this bond reduced, a complete reduction of the Cys186–Cys209 bond was verified. For all further experiments a total of 10 TF samples, 5 for each TF protein, were prepared including native protein, non-reduced/non-alkylated (NR/NA), reduced/non-alkylated (R/NA), reduced/alkylated (R/A) and non-reduced/alkylated (NR/A). All samples except the native protein were first denatured with 6 M guanidinium-HCl in HBS-10 mM CHAPS pH 8.0 for 60 minutes. The NR/NA sample was not treated with either DTT or IAA and was dialyzed into HBS-10 mM CHAPS pH 7.4 following denaturation. The R/NA sample was prepared by adding 20 mM DTT followed by incubation for 4 hours at 37°C. The reduction was followed by immediate dialysis into HBS-10 mM CHAPS pH 6.0 to conserve the reduced state of the thiols. Following activity assays, the R/NA sample was carboxyamidomethylated with 50 mM IAA for MS analysis. The R/A sample was prepared by treating TF with 20 mM DTT for 4 hours at 37°C followed by carboxyamidomethylation with 50 mM IAA for 30 minutes in the dark and dialysis in HBS-10 mM CHAPS pH 7.4. The NR/A samples were carboxyamidomethylated with 50 mM IAA without prior reduction. All samples were analyzed by LC-MS/MS.

2.5 Relipidation of TF

The relipidation of NR/NA, R/NA and R/A TF samples was performed with slight modification of the previously described procedure [33]. Five nM TF was incubated with 10 μ M PCPS, 5 mM CaCl_2 in HBS pH 7.4 for 30 min at 37°C [31]. The R/NA TF sample, dialyzed into HBS-10 mM CHAPS pH 6.0 after reduction, was relipidated at pH 6.0 to prevent oxidation of reduced cysteines. The concentration of relipidated TF on the surface of PCPS vesicles was quantitated by an immunoassay as previously described [33].

2.6 Quantitation of NR and R TF

Concentrations of both the non-relipidated and relipidated TF proteins were quantitated by an immunoassay utilizing a sheep anti-human TF polyclonal Ab using a previously described method [33]. This antibody recognizes both the reduced and oxidized forms of TF identically. For the quantitation of oxidized TF in mixtures with reduced form of protein anti-TF-5 mAb was used, which recognizes only oxidized form of TF. Polyclonal or monoclonal Ab was immobilized on polystyrene immunoassay plates at a concentration of 5 μ g/mL. Various dilutions (0–5 nM) of TF proteins in HBS-0.1% BSA-0.2% TritonX-100 (TritonX-100 was not present for relipidated sample analyses) were added to the plate and incubated for 2 hours. Binding was probed with biotinylated sheep anti-human TF and horseradish peroxidase (HRP)-conjugated streptavidin. Additionally, concentrations of TF in all samples were confirmed by absorbance.

2.7 Binding of NR/NA and R/NA TF to rFVIIa

To determine whether the reduced form of TF binds to rFVIIa, a direct binding assay and a competition assay was performed. In the direct binding assay, NR and R TF proteins were coated on the microtiter plate at 5 μ g/mL and various dilutions (0–500 nM) of rFVIIa were added for 2 hour incubation. The binding was probed with anti-FVII-1 mAb and HRP-goat anti-mouse Ig. In the competition assay, various concentrations (0–2500 nM) of both the NR

and R forms of TF were incubated with rFVIIa (150 nM final concentration) in HBS-0.2% TritonX-100 pH 7.4 for 10 minutes and added to a plate coated with NR TF. Binding of the rFVIIa to the immobilized TF was probed with anti-FVII-1 mAb and HRP-goat anti-mouse Ig. For the non-specific binding control, albumin was added instead of FVII.

2.8 Membrane independent amidolytic activity of the TF/rFVIIa complex

Amidolytic activity of the TF/rFVIIa complex was measured in a fluorogenic assay [34]. TF (0.5 nM) was incubated in HBS-5 mM CaCl₂ pH 7.4 with rFVIIa (10 nM or varying between 0–10 nM) for 10 min at 37°C. Fluorogenic substrate FPRnbs was added (50 μM final concentration) and the rate of substrate hydrolysis as the change in fluorescence intensity (FIU) over time (5 min) was measured at the excitation wavelength of 350 nm and emission wavelength of 470 nm using a 450 nm cut-off filter in Jobin Yvan-Spex FluoroMax-2 (Instruments S.A. Inc., Edison, NJ). The parameters of Michaelis-Menten kinetics were calculated using GraphPad Software (La Jolla, CA) program.

2.9 Membrane dependent FXa generation

Relipidated TF preparation (0.1 nM TF) was incubated with rFVIIa (5 nM) for 10 min at 37°C and FX was added (4 μM or varying between 0–8 μM). At 30 second intervals (0–5 min) the reaction mixture was quenched in HBS-0.1% PEG-20 mM EDTA and Spectrozyme FXa was added at a final concentration of 0.2 mM. Substrate hydrolysis was monitored and the FXa generation rate was calculated from a standard curve prepared by serial dilutions of FXa [33]. Parameters of Michaelis-Menten kinetics were calculated using the GraphPad software program.

2.10 Treatment of reduced TF with PDI

Five μM of R/NA rTF1–263 in HBS-10 mM CHAPS-0.1% PEG-5 mM CaCl₂ pH 7.4 was treated with 500 nM PDI, 0.2 mM GSH and 0.2 mM GSSG for 30, 60, 90, 120 minutes and 15 hours at 37°C. All reaction mixtures were diluted 200-fold into HBS-0.1% PEG-5 mM CaCl₂ containing 5 nM rFVIIa. Fluorogenic substrate FPRnbs (50 μM) was added and the amidolytic activity of the TF/rFVIIa complex was evaluated as described.

2.11 LC-MS/MS analysis of TF proteolytic peptides

Five μg of each TF sample, NR/NA, R/NA (the sample was alkylated prior to MS analysis for the accurate determination of the reduced species), and R/A were resolved by electrophoresis (4–12% acrylamide SDS-PAGE gradient gel) and stained with Coomassie Blue. Protein bands were digested with trypsin in ProteaseMax™ Surfactant according to manufacturer's instructions. Peptides were subjected to LC-MS/MS analysis using a ThermoFinnigan Surveyor HPCL and LCQ Deca XPplus MS. Peptides were separated on an in-house made 100 μm ID × 110 mm length nano-spray column packed with Phenomenex's Jupiter Proteo 4 μm phase (Torrence, CA). The MS analysis was run as previously described [33], except for this analysis the full MS scan range was from 500–1100 m/z. Peptides were identified using the Sequest algorithm [35,36]. This database search identified TF peptides (Table 2) with and without carboxyamidomethylation of cysteine residues (carboxyamidomethylation increases the mass of each cysteine by 57 Da). All Sequest-identified peptide data were manually inspected to confirm correct b and y ion [33] designations and to rule out possible false positive identifications.

3. RESULTS

3.1 Sample preparation

The extent of reduction, as calculated by MS fractional abundance (represented as % of reduced species in a given sample), of TF proteins at 25°C and 57°C with 8 mM DTT and 20 mM IAA was 32% and 36% of reduced species, respectively (Table 1). The percent of reduced species increased to 66% in a sample reduced at 37°C followed by dialysis at pH 6.0. Increasing DTT to 20 mM (37°C followed by dialysis in pH 6.0) and IAA to 50 mM did not increase the fractional abundance of the reduced species (50%). However, denaturation with guanidinium-HCl enabled a more efficient reduction of TF using both the 8 mM DTT/20 mM IAA (88%) and 20 mM DTT/50 mM IAA (99%). The use of 20 mM BME as a reducing agent produced the lowest fractional abundance of reduced species even after denaturation (18%). These data suggest that a complete TF reduction can not be achieved without prior denaturation of the protein.

3.2 Quantitation of NR and R forms of TF

The NR and R forms of both TF proteins were recognized by our in-house immunoassay using polyclonal Ab almost identically. The titration curves (0–5 nM) of the NR and R TF proteins were similar and both forms of TF proteins (individually or as mixtures) were quantitated by this method using the rTF1–263 as a standard (not shown). The detectability limit of the assay is 40 pM.

3.3 Binding of NR and R TF to rFVIIa

Figure 1A shows the direct binding of rFVIIa to both the NR and R TF immobilized on a microtiter plate. The solution phase rFVIIa binds to both forms of TF similarly. Figure 1B shows an increase in rFVIIa binding to the solution phase NR and R rTF1–263 proteins. Both solution phase forms of TF displace rFVIIa from the immobilized TF in a concentration dependent manner.

3.4 Membrane independent amidolytic activity of the TF/rFVIIa complex

The amidolytic activity of pTF and rTF1–263 in a complex with rFVIIa and in the absence of phospholipids was evaluated in the fluorogenic assay (Figure 2; n=3). The activity of NR/NA preparations of pTF was approximately 2.5-fold higher than those of NR/NA rTF1–263 preparations (2323±192 and 875±7.6 FIU/sec, respectively), consistent with our previous observation [33]. The affinity of freshly purified NR/NA pTF for rFVIIa was similar to that previously reported for pTF purified from Thromborel S (1.8±0.2 and 0.9±0.2 nM, respectively) [33]. Treatment with guanidinium HCl (NR/NA) and carboxyamidomethylation of TF without prior reduction (NR/A) did not alter the cofactor function. Reduction of rTF1–263 or pTF with (R/A) or without (R/NA) IAA treatment abolished the ability of TF to enhance the hydrolysis of the substrate by rFVIIa. In the mixture of NR and R TF, only the former was able to enhance amidolytic activity of FVIIa, and specific activity of NR TF in the mixture was almost identical to that of purified NR TF.

3.5 Membrane dependent FXa generation

Generation of FXa by rFVIIa in complex with relipidated pTF and rTF1–263 in the presence of phospholipids was evaluated (Figure 3). Consistent with the fluorogenic assay and previously published data [33], FXa generation rates are greater using NR/NA pTF preparations than those of NR/NA rTF1–263. The kinetics of FXa generation by pTF from fresh placenta was similar to that measured for pTF purified from Thromborel S [33] (k_{cat} of 7.4±0.1 and 8.7±0.2 s⁻¹, respectively). Treatment with the IAA of the native TF did not significantly change the function of TF. The reduction of rTF1–263 and pTF with and without

carboxyamidomethylation abolished the ability of TF to enhance rFVIIa activity in the extrinsic FXase. The activity of rFVIIa in complex with R/NA and R/A TF was similar to that of rFVIIa alone. Only NR TF enhanced FVIIa in FX proteolysis when the mixture of NR and R TF was used in the reaction. The specific activity of NR TF in the mixture was almost identical to that of pure NR TF.

3.6 Treatment of reduced TF with PDI

The treatment of R/NA TF with PDI for up to 15 hours did not restore TF cofactor function, i.e. rFVIIa activity remained unaltered (data not shown). The MS analysis showed that 40% of Cys49–Cys57 became oxidized upon PDI treatment and 60% remained as unpaired cysteines. No oxidized form for Cys186 and Cys209 was observed.

3.7 LC-MS/MS analysis

Trypsin digestion of native TF should generate 31 peptides including three peptides of interest for the present study, 49-CFYTTDTECDLTDEIVK-65, 182-GENYCFSVQAVIPSR-196, and 202-STDSPVECMGQEK-214 containing all four cysteines of the extracellular domain, i.e. Cys49, Cys57, Cys186 and Cys209 [33] (Table 2). After tryptic digestion, correctly cleaved peptides would result in one peptide containing both Cys49 and Cys57, whereas Cys186 and Cys209 would be located on two different peptides. We searched for the correctly cleaved and miscleaved peptides using Sequest program and manual inspection. After activity assays, all samples were carboxyamidomethylated for MS analysis.

According to the amino acid sequence, the molecular weight of the singly charged peptide containing reduced thiols of Cys49 and Cys57 is expected to be 1996 Da. If this peptide exists in an oxidized form, the mass would be expected to be 1994 Da. Figure 4 Panel a shows that in the NR samples only a peptide of 1994 Da corresponding to the oxidized form of Cys49 and Cys57 is observed at a retention time of 31.2 minutes as the $M+2H^+$ ion (997 Da). Panel b shows that upon reduction and carboxyamidomethylation, both Cys49 and Cys57 are alkylated and a corresponding peptide of 2110 Da appears at a retention time of 30.6 minutes as the $M+2H^+$ ion (1055.5 Da). The presence of b and y ions in the MS/MS scan matching the fragmentation pattern of the oxidized and alkylated peptides, confirm the identity of both. We also noted the lack of most of the b and y ions located between the two cysteines in the oxidized species (data not shown). According to previous reports [37, 38], the absence of these ions between the two cysteines is indicative of an intrapeptide disulfide bridge. Figure 4 Panels c and d show the presence of two peptides containing carboxyamidomethylated Cys209 (1466 Da, $M+2H^+ = 734$) and Cys186 (3116 Da, $M+3H^+ = 1039.5$), respectively. These two peptides are detectable only in the R/A samples at retention times of 24.7 minutes for Cys209 and 32.6 minutes for Cys186 and not found in NR/A samples. The peptide containing Cys186 is observed as a miscleaved peptide. The MS/MS fragmentation pattern confirms their identity (data not shown). The absence of peptides containing oxidized Cys186 or Cys209 (3060 Da and 1409 Da respectively) suggests that they are quantitatively involved in a disulfide bridge.

Figure 5 demonstrates that freshly purified NR human pTF does not contain any reduced cysteines in the extracellular domain. The NR pTF was carboxyamidomethylated and subjected to MS analysis. Figure 5 Panel a shows that Cys49 and Cys57 are observed in the oxidized form only at a retention time of 34.6 minutes. Panel b demonstrates the absence of the alkylated form of the peptide containing Cys49 and Cys57 (expected retention time of approximately 33 minutes) which suggests that they are involved in an intrapeptide disulfide bridge. Panels c and d demonstrate the absence of alkylated Cys209 and Cys186 (expected retention time of 27 and 35 minutes respectively) in the NR/A pTF sample suggesting that

these two cysteines are involved in an interpeptide disulfide bridge. The fragmentation patterns of all peaks in the figure were inspected for identification. The same results were obtained for the analysis of the rTF₁₋₂₆₃ (data not shown).

4. DISCUSSION

The data of this study indicate that: 1) cysteines of the extracellular domain of TF play a role in TF cofactor function; 2) in freshly purified human pTF, cysteines of the extracellular domain exist in the oxidized (disulfide) form, as no alkylation is detectable without prior chemical reduction of the protein. However, it does not rule out the possibility that in a native environment these cysteines could exist in a completely or partially reduced form with oxidation occurring during pTF purification; 3) treatment of TF with guanidinium HCl (without reduction) does not alter TF function; 4) reduction of TF cysteines abolishes the catalytic function of the TF/FVIIa complex in both membrane-independent and membrane-dependent reactions; 5) reduced TF retains its capability to bind FVIIa; 6) treatment of the reduced TF with PDI does not restore the Cys186–Cys209 disulfide bond and, as a consequence, does not restore TF function.

The debate over the role of the disulfides of TF, specifically over that formed by Cys186 and Cys209, started more than twenty years ago. Bach *et al.* suggested that the disulfides are necessary for the proper folding of partially pure bovine brain TF and consequently for its function [2]. Mutagenesis studies performed with TF expressed in Chinese hamster ovary cells demonstrated that mutation of Cys186 and Cys209 to serine decreased (albeit did not abolish) the ability of the TF/FVIIa complex to generate FXa, whereas mutation of Cys49 and Cys57 did not affect such activity [3].

More recently, the concept of allosteric disulfide bonds has been widely propagated [4,5]. Chen and co-workers assigned to the disulfide between Cys186 and Cys209 of TF, expressed in human myeloid leukemia HL-60 and baby hamster kidney cells, the role of an allosteric bond, which can be non-enzymatically formed or reduced contributing to its pro-coagulant and cryptic states, respectively [4]. Ahamed *et al.* supported this notion and suggested that the switch between procoagulant and cryptic TF, expressed in human HaCaT keratinocytes and umbilical vein endothelial cells, is regulated by the disulfide between Cys186 and Cys209 and that PDI mediates this switch [24]. This observation was supported by the data acquired in a mouse model vessel injury of Reinhardt and coworkers, who stated that PDI is essential for the activation of TF following injury [25]. Additionally, Liang *et al.* studied the redox properties of Cys186 and Cys209 in a recombinant soluble TF and suggested that the oxidation of the cysteines plays a role in its activation [23]. However, a report by Pendurthi and coworkers challenged this concept by showing that the increase in activity of cell surface TF is not related to the formation of a disulfide but is caused by the exposure of anionic phospholipids upon stimulation of cells [27]. These authors did not observe any detectable PDI or any association of TF with the isomerase. Furthermore, Bach and Monroe analyzed the crystal structure of TF and FVIIa and concluded that the half cysteines proximal to the membrane are buried within the TF/FVIIa complex and could not contribute to the activation of FX, nor could be available for interaction with PDI [39]. However, this conclusion is questionable due to the reversible nature of the TF and FVIIa interaction. Upon the dissociation of the TF/FVIIa complex, TF could become available for an interaction with PDI. The most recent study reports that in mouse myeloid cells, ATP-triggered signaling through P2X7 receptor utilizes PDI to generate procoagulant TF, linking inflammation and thrombosis [26].

Our study shows that in a purified system both in the presence or absence of a synthetic membrane, reduction of TF disulfides impairs the activity of the TF/FVIIa complex by

abolishing TF cofactor function, despite the ability of reduced TF to bind FVIIa at least as efficiently as an oxidized protein. These data are somewhat contradictory with early mutational studies by Rehemtulla *et al* [3], where a decrease but not abolishment of FVIIa binding and TF cofactor function was observed upon mutations of Cys186 and Cys209. This apparent discrepancy related to the affinity of reduced TF for FVIIa is, most likely, caused by the different methodologies used in our study and that study. In the Rehemtulla *et al*. [3] study, the affinity was estimated by the activity-based assay, whereas in our study direct binding of TF to FVIIa was measured by an immunoassay. With respect to the functional impact of the Cys186–Cys209 disulfide bond on TF, more recent mutational studies with human and murine TF showed that in the absence of an intact allosteric disulfide coagulant TF activity is abolished [40, 41], i.e. they are in an agreement with our observation. In contrast to these observations, Kothari *et al*. observed that at saturating FVIIa concentrations the Cys186–Cys209 disulfide bond is not essential for the TF function [42].

We also observed that to achieve a complete reduction of TF disulfides, a denaturation of the protein is essential. This observation could explain (at least in part) the discrepancies in published studies related to the role of TF disulfides on TF activity. It is quite possible that under the reduction conditions used in some of those studies, only a fraction of disulfides were disrupted and the observed activity was assigned to the reduced TF protein, whereas in reality it came from the oxidized form of TF. It is essential therefore to use reliable quality control methods, such as MS, to confirm complete reduction/oxidation of cysteines being studied. Another possible source of discrepancies could be related to the “side effects” of reagents used in the studies analyzing TF activity on the cell surface. These reagents may change the overall environment of the cell, primarily that of the membrane, and lead to certain alterations (e.g. increased PS exposure) which would affect TF function.

4.1. Conclusions

The data of this study indicate that in freshly purified placental TF all four extracellular cysteines are oxidized and that the reduction of the Cys186–Cys209 disulfide bond abolishes TF function, despite an efficient binding of this form of TF to FVIIa. Although it has been shown in a previous publication that disruption of the Cys49–Cys57 disulfide bond has no effect on TF function [3], it cannot be ruled out that this disruption could indirectly effect properties of the Cys186–Cys209 bond. Additional studies are required to understand why formation of the complex between FVIIa and reduced TF does not alter proteolytic activity of FVIIa. Determining the status of cysteines of the cell surface TF and to establishing the effect of their reduction/oxidation on TF function will also be a subject of the future studies.

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The abbreviations used are

TF	tissue factor
FVII	factor VII
FVIIa	factor VIIa
Cys	cysteine
FX	factor X
FXase	extrinsic factor Xase
PS	phosphatidylserine
PC	phosphotidylcholine
PCPS	synthetic vesicles of (1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine) and (1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-L-serine)
PDI	protein disulfide isomerase
LPS	lipopolysaccharide
pTF	placental tissue factor
rTF₁₋₂₆₃	recombinant tissue factor 1–263
mAb	monoclonal antibody
HRP	horse radish peroxidase
BSA	bovine serum albumin
TMB	tetramethylbenzidine
FPRnbs	D-FPR-ANSNH-C ₄ H ₉ · 2HCl
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
IAA	iodoacetamide
DTT	dithiothreitol
GSH	reduced glutathione
GSSG	oxidized glutathione
EDTA	ethylenediaminetetraacetic acid disodium salt dihydrate
PEG	polyethylene glycol
NR/NA	non-reduced/non-alkylated
R/NA	reduced/non-alkylated
R/A	reduced/alkylated
NR/A	non-reduced/alkylated
MS	mass spectrometry
LC	liquid chromatography
FIU	fluorescence intensity units
Ig	immunoglobulin

Highlights

1. The reduction of tissue factor (TF) disulfides abolishes TF cofactor function.
2. Reduced TF binds to factor VIIa but does not increase factor VIIa activity.
3. Treatment of reduced TF with PDI does not restore TF function.

Figure 1. Binding of NR/NA and R/NA TF to rFVIIa determined by a direct binding and a competition assay

(A) NR and R TF proteins were coated on the microtiter (5 μ g/ml) plate and various dilutions of rFVIIa (0–500 nM) were added for incubation. The binding was probed with anti-FVII-1 mAb and HRP-goat anti-mouse Ig then developed with chromogenic TMB substrate. (B) Various concentrations (0–2500 nM; 0–200 nM in inset) of the NR and R TF were incubated with rFVIIa (150 nM) in solution and added to a plate coated with NR TF (5 μ g/ml). Binding of the rFVIIa to the immobilized TF was probed with anti-FVII mAb and HRP-goat anti-mouse Ig then developed with chromogenic TMB substrate.

Figure 2. Membrane-independent amidolytic activity of the TF/FVIIa complex

The native, NR/NA, R/NA and R/A TF preparations (0.5 nM) were incubated with rFVIIa (10 nM) for 10 min at 37°C in HBS-0.1% PEG-5 mM CaCl₂. In a control experiment rFVIIa was incubated without TF. FPRnbs fluorogenic substrate (50 μM) was added and substrate hydrolysis was measured for 5 min and recorded in fluorescence intensity units (FIU). Each value of the NR/NA samples is a mean of three independent measurements ± one standard deviation. All other samples were a mean of two independent measurements ± one standard deviation.

Figure 3. Membrane-dependent extrinsic FXase activity

Five species of pTF and rTF₁₋₂₆₃ proteins (native, NR/NA, NR/A, R/NA, R/A) were evaluated for their ability to function in the extrinsic FXase reaction. Relipidated TF proteins (native, NR/NA, NR/A, R/NA, R/A), (0.1 nM) were incubated with rFVIIa (5 nM) and 100 μ M PCPS for 10 min at 37°C. In a control experiment rFVIIa was incubated without TF FX (4 μ M) was added and at selected time points (0–5 min) 10 μ l aliquots of the reaction mixture were quenched in HBS-0.1% PEG-20 mM EDTA. Spectrozyme FXa (0.2 mM) was added and the rate of substrate hydrolysis was measured. Each value of the NR/NA samples is a mean of four independent measurements $\pm$ one standard deviation. All other samples were a mean of two independent measurements $\pm$ one standard deviation.

Figure 4. Analysis of extracellular cysteines of pTF by MS

(A) In the NR/NA sample only the oxidized form of Cys49 and Cys57 is observed (retention time 31.2 min). (B) Upon reduction and carboxyamidomethylation of Cys49 and Cys57, the alkylated form is detectable (retention time 30.6 min). Unpaired Cys209 (C) and Cys186 (D) could be detected only after reduction and carboxyamidomethylation (retention time 24.7 and 32.6 min, respectively).

Figure 5. Analysis of the extracellular cysteines of NR pTF by MS

The NR preparation of pTF was treated with IAA for this analysis. (A) Only the oxidized form of Cys49 and Cys57 is observed (retention time 34.6 min). (B) No unpaired Cys49 and Cys57 are observed. No peptides corresponding to unpaired Cys209 (C) and Cys186 (D) are observed. Arrows indicate the expected retention times for the corresponding peptides containing unpaired cysteines.

Table 1

Reduction conditions of TF₁₋₂₆₃. Reduction efficiency was determined by MS/MS analysis

Guanidinium HCl		DTT			BME			IAA			
Conc.	Time	Conc.	Temp.	Time	Conc.	Temp.	Time	Conc.	Time	% Reduction	
--	N/A	8 mM	25 °C	4 hr	--	N/A	N/A	20 mM	30 min	32	
--	N/A	8 mM	37 °C	4 hr	--	N/A	N/A	20 mM	30 min	66	
--	N/A	8 mM	57 °C	4 hr	--	N/A	N/A	20 mM	30 min	36	
--	N/A	20 mM	37 °C	4 hr	--	N/A	N/A	50 mM	30 min	50	
6M	60 min	8 mM	37 °C	4 hr	--	N/A	N/A	20 mM	30 min	88	
6M	60 min	20 mM	37 °C	4 hr	--	N/A	N/A	50 mM	30 min	99	
6M	60 min	--	N/A	N/A	20 mM	37 °C	4 hr	50 mM	30 min	18	

Table 2

List of expected and observed peptides, containing Cys49, Cys57, Cys186 and Cys209

TF species	Treatment	Fragment	Expected MW (Da)	Observed MW (Da)
Human placental	NR/A	49-CFYTTDTECDLTDEIVK-65	1994	1994
	R/A	49-C#FYTTDTEC#DLTDEIVK-65	2110	2110
rTF ₁₋₂₆₃ Sf9 cells	NR/A	49-CFYTTDTECDLTDEIVK-65	1994	1994
	R/A	49-C#FYTTDTEC#DLTDEIVK-65	2110	2110
Human placental	NR/A	170-TNTNEFLIDVDKGENYCFSVQAVIPSR-196	3060	ND
	R/A	170-TNTNEFLIDVDKGENYC#FSVQAVIPSR-196	3116	3116
rTF ₁₋₂₆₃ Sf9 cells	NR/A	170-TNTNEFLIDVDKGENYCFSVQAVIPSR-196	3060	ND
	R/A	170-TNTNEFLIDVDKGENYC#FSVQAVIPSR-196	3116	3116
Human placental	NR/A	202-STDSPVECMGQEK-214	1409	ND
	R/A	202-STDSPVEC#MGQEK-214	1466	1466
rTF ₁₋₂₆₃ Sf9 cells	NR/A	202-STDSPVECMGQEK-214	1409	ND
	R/A	202-STDSPVEC#MGQEK-214	1466	1466

ND = not detected; R/A = DTT and IAA treated; # = alkylated cysteine