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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.

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-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 intermolecular 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

Abbreviations: 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-*sn*-glycero-3-phosphocholine) and (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine); PDI, protein disulfide isomerase; LPS, lipopolysaccharide; pTF, placental tissue factor; $rTF_{1–263}$, recombinant tissue factor 1–263; mAb, monoclonal antibody; HRP, horse radish peroxidase; BSA, bovine serum albumin; TMB, tetramethylbenzidine; FPRnbs, D-FPR-ANSNH-C_4H_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

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of the Cys186–Cys209 bond in the "encryption/decryption" phenomenon was suggested [4]. The presumed formation of this bond using an oxidizing agent ($HgCl_2$) 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 $HgCl_2$ can modify only a single thiol group [19,20]. Moreover, an increase in TF activity on cell surfaces similar to that caused by $HgCl_2$ 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 $HgCl_2$. More recent data from Hogg's laboratory, however, suggest that $HgCl_2$ 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 reduced-carboxyamidomethylated 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

$rTF_{1–263}$ 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 albumbin (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-Dioleolyl-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-C_4H_9 •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 ThermoScentific (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 ($CaCl_2$), 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 min in a cool dry-ice methanol bath and centrifuged at 10,000 *g* for 30 min. 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 h and centrifuged at 10,000 g for 30 min. 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 4 L 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-3 M 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 $rTF_{1–263}$ (Table 1). First, we reduced TF with 8 mM DTT at pH 8.0 for 4 h at either 25 °C or 57 °C. Both samples were then carboxyamidomethylated with 20 mM IAA for 30 min 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 min 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 min. 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 h 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 h at 37 °C followed by carboxyamidomethylation with 50 mM IAA for 30 min 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.