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Role of tissue factor disulfides and lipid rafts in signaling

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Abstract

Tissue factor (TF) is a cellular receptor for coagulation factor VIIa (FVIIa), and the assembly of TF:FVIIa complexes on cell surfaces not only triggers the coagulation cascade but also transduces cell signaling via activation of protease-activated receptors. This brief review summarizes recent controversial data on the importance of protein disulfide isomerase-mediated disulfide bond switching in extracellular domain of TF in regulating its coagulant and cell-signaling activities. The review also discusses the importance of TF localization in lipid rafts and caveolae in maintaining its coagulant and signaling functions.

Keywords

Tissue factor; Factor VIIa; Protein disulfide isomerase; Lipid rafts; Coagulation; Cell signaling

Introduction

Tissue factor (TF) is a cellular receptor for clotting factor VIIa (FVIIa). Formation of TF:FVIIa complexes on cell surfaces not only triggers the coagulation cascade but also transduces cell signaling via activation of protease-activated receptors (PARs) [1,2]. It is generally believed that only a small fraction of the TF found on cell surfaces is active in coagulation whereas the vast majority is non-functional (encrypted) in coagulation [3–5]. It is unclear how coagulation-active TF differs from the encrypted form, or what mechanisms are involved in decryption of TF. In contrast to the restriction of coagulant activity to a limited number of TF sites on cell surfaces, most of the TF on cell surfaces appears to be capable of transducing TF:FVIIa-induced cell signaling, indicating that encrypted TF retains the cell-signaling function. It is puzzling how TF:FVIIa, a relatively poor activator of PAR2, can induce a robust cell-signaling response in many cell types. In this article we critically review recent studies that examine the role of disulfide bonds in the extracellular domain of TF in regulating its coagulant and cell-signaling functions. The review also includes a discussion on the role of lipid rafts in TF:FVIIa signaling.

Modulation of TF function by disulfide bonding/protein disulfide isomerase

Tissue factor, a 47 kD glycoprotein, has structural homology with the class-II cytokine receptor family of proteins. The extracellular domain of TF has two disulfide bonded loops (Cys49–Cys57 and Cys186–Cys209) that are remarkably conserved in all class-II cytokine receptor family members [6], suggesting that they could be critical for an essential conserved global folding pattern or for forming a recognition/binding structure for the ligand. Site-directed

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mutagenesis studies revealed that the Cys49–Cys57 bond is not essential for the proper folding of TF or for its procoagulant function, but the Cys186–Cys209 disulfide bond is required to maintain proper conformation, FVIIa binding and procoagulant activity [7]. Review of the crystral structure of TF revealed that the Cys186–Cys209 disulfide bond is exposed to solvent and has the features of an allosteric bond, i.e., links adjacent strands in the same β sheet, has a high mean potential energy and a narrow energy distribution [8]. Emerging evidence suggests that the allosteric disulfide bond could play a role in regulating protein function by triggering a conformational change when it breaks and/or forms [8].

Recent studies imply that the Cys186–Cys209 disulfide bond is reduced in the encrypted form of TF on cell surfaces and that activation involves the formation of the disulfide [9]. Both nonprotein-oxidizing agents, such as $HgCl_2$, and oxidoreductase enzymes, such as protein disulfide isomerase (PDI), were shown to be capable of inducing the formation of the Cys186–Cys209 disulfide bond [9,10]. However, $HgCl_2$ treatment of intact cells has been shown also to increase the concentration of anionic phospholipids on the outer cell surface by us [11] and others [12]. Furthermore, the exposure of anionic phospholipids on the outer cell surface is the most potent mechanism to induce the conversion of encrypted TF into the coagulant-active form [4,5,13]. Consistent with the hypothesis that increased anionic phospholipids are primarily responsible for increased TF activity at the cell surface, at least in cells treated with the oxidizing agent $HgCl_2$, is the observation that annexin V, a phospholipid binding protein, markedly reduced the increase in TF activity associated with $HgCl_2$ treatment [11]. Another important observation that questions the role of PDI as a physiologically relevant oxidizing agent for the TF transformation is the fact that it is largely an intracellular protein associated with endoplasmic reticulum, and its presence in extracellular locations is debatable [14]. In recent studies from our laboratory, we found no evidence for the presence of PDI on the cell surface in multiple cell types, including MDA-231 tumor cells, fibroblasts and HUVEC [11]. Furthermore, PDI silencing had no effect on TF procoagulant activity [11]. These data further undermine the validity of the proposed hypothesis that PDI-mediated disulfide exchange plays a role in regulating TF coagulant activity at the cell surface. However, recent preliminary studies from other investigators seem to provide further evidence that PDI does play a role in TF-dependent thrombin generation and fibrin formation *in vivo* [15,16]. It is possible that in these *in-vivo* model systems endothelial cell injury and/or platelet activation may lead to PDI release and that this PDI could selectively activate TF in microparticles. However, in our opinion, one should exercise caution in interpreting these data since the injury also results in exposure of anionic phospholipids at the injury site and that PDI may influence thrombin generation and fibrin formation by its known actions on platelet activation [17] and integrin ligation [18]. It should also be noted that bacitracin, one of the inhibitors used to block PDI in these studies, is not selective in its activity to PDI alone. Finally, there is no evidence that TF *in vivo* exists in the reduced form, and it is pertinent to note that purified unreduced human TF had no free sulfhydryl groups, which indicates four cystines in the extracellular domain of TF are in disulfide bonds [19]. Further studies are essential to resolve some of above conflicting data and to establish whether disulfide bond formation and PDI have any role in regulating TF activity.

Interestingly, the Cys186–Cys209 disulfide bond, which is critical for coagulation, is not required for TF:FVIIa-induced PAR2-mediated cell signaling as a TF mutant (TF C209A) with an unpaired Cys186 retains TF:FVIIa signaling [10]. Further, it had been shown that TF coagulant activity is suppressed during association of extracellular PDI with TF and that disulfide/thiol exchange pathways are required for TF:PAR2 complex formation and TF:FVIIa signaling [10]. Based on these data, it had been concluded that TF:FVIIa-mediated coagulation and cell signaling involves two distinct cellular pools of TF, that is, TF with an intact Cys186–Cys209 disulfide bond that is active in coagulation and TF with a reduced Cys186–Cys209 disulfide bond that is encrypted, but functions in TF:FVIIa-mediated cell signaling; and the

disulfide exchange mediated by PDI acts as a regulatory mechanism that switches TF between coagulation and signaling. Although this hypothesis is novel and interesting, compelling evidence supporting the hypothesis has not been presented at this time.

The notion that encrypted TF is functional in TF:FVIIa signaling seems valid and consistent with our earlier observations that FVIIa binds to encrypted TF and that the resultant TF:FVIIa complexes are catalytically active [3]. It also fits with our observation of a good correlation between the dose-dependent kinetics of FVIIa binding to TF and TF:FVIIa signaling [20]. However, it is unclear whether it is the redox status of the Cys186–Cys209 bond that actually determines whether TF is functionally active in either coagulation or cell signaling, nor is there clear evidence that shows that coagulant-active TF is not also functionally capable of inducing TF:FVIIa-mediated cell signaling.

The hypothesis also does not fully explain other observations made using TF mutants. For example, in contrast to the mutation of Cys209 to alanine, Cys186 to alanine, the other residue involved in the disulfide bond which would also result in unpaired cysteine, resulted in the complete loss of TF:FVIIa signaling [10]. If the breakage of the Cys186–Cys209 disulfide bond were the critical factor then both the mutants should have behaved similarly, i.e., retain the signaling function. Earlier studies showed that preclusion of the Cys186–Cys209 bond by pairwise substitution of serine residue for cysteine resulted in a marked impairment in its ability to interact with FVII (Kd ≳100 nM) [7]. In contrast to this, FVIIa binds to encrypted TF with relatively high affinity (Kd < 5 nM) [3] and TF:FVIIa cell signaling reaches saturation at 10 to 50 nM of FVIIa [20]. Finally, TF lacking Cys186–Cys209 disulfide bonds is not completely inactive in its procoagulant activity, and the decrease in the activity could be partially compensated by the addition of large amount of FVIIa [7].

Association of TF with PDI, increased TF coagulant activity following partial inhibition of PDI by siRNA, and inhibition of TF:FVIIa signaling by bacitracin have all been cited as evidence that PDI regulates TF function by targeting the disulfide bond in the extracellular domain of TF [10] and switching it between coagulant (oxidized/disulfide form) and signaling (reduced non-disulfide form) functions. However, as discussed above, the presence of PDI at the outer cell surface is questionable; the stimulation of cells with FVIIa, thrombin or other agonists failed to relocalize PDI to the cell surface [11]; and PDI-neutralizing antibodies or gene silencing of PDI had no effect on either TF coagulant or signaling function [11].

At present the reasons for the marked differences between our study [11] and the earlier study [10] on the potential role for PDI in regulation TF:FVIIa signaling function remain unclear, but it may pertinent to note that these two studies used different cell model systems (HaCaT keratinocytes vs. MDA-231 breast tumor cells) and experimental strategies varied considerably. Future studies may resolve the discrepancies; nonetheless, the differences highlight that PDI regulation of TF function at cell surfaces, even if it does occur, may not be the universal and general regulatory mechanism implied in the earlier study [10].

Modulation of TF:FVIIa signaling by lipid rafts

Unlike thrombin and trypsin, FVIIa has to bind to its cellular receptor, TF, in order to activate PARs. Therefore, TF:FVIIa could only activate PARs that are in the close vicinity of TF. Unless TF and PAR2 are present in high density, it is unlikely that TF:FVIIa would encounter PAR2 on the cell surface, which raises the important question how TF:FVIIa could activate PAR2-mediated cell signaling. It is believed that the compartmentalization of signaling molecules in membrane microdomains, such as lipid rafts/caveolae, plays a crucial role in facilitating the signaling function of many receptors [21–25]. Cholesterol association with glycosphingolipids and other saturated long-chain lipids in biomembranes causes a phase transition in the

membrane leading to segregated specialized microdomains, lipid rafts [21,26]. Cholesterol- and sphingolipid-rich rafts in association with the structural protein caveolin-1 form caveolae, flask-shaped invaginations in the plasma membrane [24,27].

It has been shown that TF in smooth muscle cells is associated with caveolae [28] and that it is redistributed into caveolae in endothelial cells following initial formation of a transient ternary complex with TF:FVIIa:FXa and TFPI in glycosphingolipid-rich microdomains [29]. Our studies with fibroblasts [30] and tumor cells [31] have shown that a small fraction of TF is constitutively localized in caveolae and a substantial portion is localized in non-caveolar lipid rafts [30]. Recent studies from our laboratory showed that a substantial amount of PAR2 is also localized in lipid rafts/caveolae in tumor cells, and more importantly that a fraction of TF and PAR2 are co-localized in these microdomains [31]. This co-localization may be critical for TF:FVIIa to activate PAR2. Disruption of lipid rafts/caveolae by cholesterol depletion with β-methyl cyclodextrin or sequestering membrane cholesterol by filipin treatment abrogated TF:FVIIa-induced PAR2-mediated cell signaling. Similarly, disruption of caveolae by caveolin-1 silencing also attenuated TF:FVIIa-induced cell signaling, consistent with this hypothesis. Although the depletion of membrane cholesterol does impair the assembly of the TF:FVIIa complex at the cell surface [30] this alone cannot explain the complete loss of TF:FVIIa-induced cell signaling in the cholesterol-depleted cells. It is telling that filipin treatment and caveolin-1 silencing, which respectively either increased or had no effect on TF coagulant activity, also impaired TF:FVIIa signaling, supporting the contention that the integrity of these membrane domains is critical for functional TF:FVIIa signaling. Disruption of lipid rafts or caveolae might also cause uncoupling of G-proteins and other signaling proteins with PAR2, decoupling the cell signaling down-stream of the receptor activation, so it is difficult to determine whether the loss of TF:FVIIa signaling upon disruption of lipid rafts/caveolae is due to the loss of TF:FVIIa interaction with PAR2 or to PAR2 uncoupling from G-proteins from these experiments. To ascertain this we examined the cleavage of PAR2, independent of its coupling to G-proteins, and showed that disruption of lipid rafts/caveolae by either cholesterol depletion/sequestration or caveolin-1 silencing markedly reduced TF:FVIIa cleavage of PAR2. In contrast, they have no effect on trypsin cleavage of PAR2. These data demonstrate that lipid raft disruption specifically impaired TF:FVIIa cleavage of PAR2, and suggests that localization of TF and PAR2 in lipid rafts is critical for TF:FVIIa to trigger PAR2-mediated cell signaling.

Similar to our observation that TF and PAR2 co-localization in lipid rafts is essential for FVIIa signaling, the co-localization of endothelial cell protein C receptor (EPCR) and PAR-1 in lipid rafts also appears to be a key requirement for the cellular signaling activity of activated protein C [32]. Localization of PARs and cellular receptors for clotting proteins in lipid rafts that are enriched with different G-proteins and related down-stream signaling molecules therefore play a critical role in transducing robust cell signaling by clotting proteases that otherwise would not very effective. Such localization may also provide specificity to PAR-mediated cell signaling induced by a plethora of proteases.

Summary and future directions

Recent studies on TF activation and a potential role for disulfide exchange mediated by PDI in regulating TF coagulant and cell-signaling activities are interesting, but at present the evidence is somewhat murky. Increased anionic phospholipids at cell surfaces or at the injury site could explain at least some of the increased TF coagulant activity that has been assigned to PDI, and this contribution needs be taken into serious consideration in interpreting the results with PDI. A clearer and better definition of encrypted TF and its structural basis is needed. Currently the term is used to describe a range of molecules from inactive TF on cell surfaces to recombinant soluble TF. Such broad use creates potential confusion in comparing/

contrasting various studies on this subject to reach firm and valid conclusions. It is important that several questions be addressed before a final verdict is reached on the potential significance of PDI and of disulfide bond formation/breakage as the key regulatory controlling event in TF function. These questions include: Is the Cys186–Cys209 disulfide bond actually the activity-defining factor? Does non-disulfide bonded TF (either as the thiolated or as the nitroso form) exist *in vivo* and if so, in what proportion to the disulfide bonded form of TF? What is the relative contribution of anionic phospholipids vs. PDI in any given model system in activating TF? Finally, if the disulfide bond formation is the defining event in regulating TF activity, then one should ask what other enzymes, in addition to PDI, contribute to this process, and what is their localization/availability in pathophysiology of normal clotting and disease.

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