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Role of thiol pathways in TF procoagulant regulation

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Summary

The generation of procoagulant Tissue Factor (TF) is crucial for thrombosis. TF contains a surface exposed allosteric disulfide bond that stabilizes the carboxyl-terminal domain involved in ligand interactions with coagulation factors VIIa and X. TF procoagulant activation typically occurs following cellular perturbations that also cause the appearance of procoagulant phosphatidylserine in the outer leaflet of cell membranes. However, thiol modifying agents, without suppressing phosphatidylserine exposure, can prevent TF activation, implicating thiol-disulfide exchange reactions in the regulation of TF procoagulant activity of primary cells. Protein disulfide isomerase (PDI), a regulator of extracellular thiol exchange, is associated with cell surface TF and required for TF-dependent thrombosis *in vivo*. PDI regulates the thiol-dependent biogenesis of procoagulant microparticles that are released from myeloid cells and smooth muscle cells following activation of the purinergic P2X7 receptor. Genetic deletion of P2X7 signaling attenuates $FeCl_3$-induced carotid artery thrombosis in mice, indicating that TF prothrombotic activity is regulated by specific cell signaling pathways *in vivo*.

Biochemistry and cell biology of the allosteric Cys^{186}-Cys^{209} disulfide of TF

Although we have a detailed view of the biochemistry of macromolecular substrate recognition by the TF-VIIa complex in purified systems, mechanisms that regulate the functional activity of TF on cells remain a major topic of ongoing research. TF-VIIa complex formation involves multiple interactions of TF with the VIIa protease, EGF- and Gla-domains to provide an extended surface for the ordered assembly of the substrate X protease domain with VIIa and of the X light chain with TF (1). Protein-protein interactions with TF-VIIa are sufficient for enhanced activation of substrate X (2), but reconstitution of TF into a procoagulant lipid environment markedly increases the activation of macromolecular substrate (3). Thus, measured TF procoagulant activities are inherently dependent on both, the membrane environment and a TF conformation capable of properly engaging in enzyme and substrate interactions.

In primary cells, distinct cell surface pools of TF are revealed by differences in the affinity for its ligand VIIa (4,5), in binding of substrate-dependent inhibitors (6), in antibody reactivity (7), or in relative procoagulant versus signaling activity (7,8). Procoagulant TF has high affinity for VIIa (K_D <1 nM), whereas higher concentrations of VIIa (5-20 nM) saturate pools of TF with no apparent procoagulant activity, but functions in TF-VIIa

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mediated cleavage of protease activated receptor (PAR) 2. The TF carboxyl-terminal fibronectin type III module is the contact site for the VIIa Gla-domain contributing to high affinity binding to TF and one of the exosites for docking of macromolecular substrate. We hypothesized that these interactive surfaces are stabilized by the solvent-exposed Cys^{186}-Cys^{209} disulfide bond that is located between two adjacent β-strands and has the geometry of an allosteric disulfide bond (9). Indeed, mutagenesis of Cys^{186} and Cys^{209} to Ser reduced the affinity for VIIa and severely impaired X activation. Remarkably a mutant with a broken allosteric disulfide retained the TF-VIIa signaling activity and supported PAR2 cleavage (7,10).

While these mutagenesis data demonstrate a critical role of the allosteric Cys^{186}-Cys^{209} disulfide for TF procoagulant activity, several lines of evidence further support the conclusion that thiol exchange reactions are important for TF procoagulant activation in primary cells. The redox potential of the the Cys^{186}-Cys^{209} disulfide bond has been determined at - 278 mV in accord with functional disulfides in other cell surface receptors (11). Procoagulant activation of TF by different stimuli is prevented by thiol modifying agents (7,9,11,12). TF procoagulant activity increases following cellular knock-down of protein disulfide isomerase (PDI), which regulates extracellular thiol exchange (13), and reducing PDI levels attenuated oxidant-stimulated TF procoagulant activity (7,14). However, constitutively active TF expressed by cancer cells (15) or in certain overexpression systems (16) shows little regulation by thiol exchange. Thus, experimental systems should be carefully chosen to study physiologically relevant TF activation.

P2X7 receptor signaling promotes thiol- and PDI-dependent activation of prothrombotic TF

Hematopoietic cells often express predominantly non-coagulant TF on the cell surface and these cellular models have provided important insight into mechanisms of TF activation. Chemical crosslinking and modification (6) showed that agonist-induced TF activation involved conformational changes in TF, consistent with data in the same cell model indicating that thiol blockade was more efficient than phosphatidylserine inhibition to attenuate the upregulation of TF procoagulant activity (9). In studying TF signaling in murine macrophages, we found that TF is also largely non-procoagulant on the cell surface. The P2X7 receptor is known for its role in the release of the pro-inflammatory cytokine IL-1β, but we found that ATP stimulation also rapidly activated TF procoagulant activity on interferon γ and LPS primed macrophages (12). ATP stimulation resulted in a marked increase in solvent accessible cell surface free thiols, indicating that thiol exchange reactions were induced by the cellular activation.

Importantly, P2X7 activation promoted the release of TF^{+} procoagulant microparticles (MP). Blockade of extracellular free thiols or PDI with an inhibitory monoclonal antibody prevented the release of procoagulant MP, but these inhibitors were without appreciable effects on the exposure of procoauglant PS. Thus, PDI is part of a thiol exchange pathway leading to MP generation in the context of P2X7-dependent TF activation. It is notable that the same inhibitory antibody to PDI (RL90) or bacitracin, which inhibits PDI's chaperone activity by binding to the hydrophobic pocket, attenuated thrombus formation in laser-induced injury (17,18) or the $FeCl_3$ model in mice (12). Although PDI has multiple platelet redox targets that may contribute to thrombus formation, platelets are not absolutely required for PDI-dependent fibrin formation *in vivo* (17,19). Inhibition studies also implicated PDI in preventing PS exposure on endothelial cells (14), which predicted that a PDI antibody should promote a prothrombotic state, but this outcome appeared to be at odds with *in vivo* observations in thrombosis models.

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When tested side by side with the anti-thrombotic antibody RL90, the effects of this particular anti-PDI antibody (Clone 34) (14) produced effects on primary macrophages that were entirely different from RL90. Unlike the inhibitory effects of RL90, Clone 34 promoted the release of TF^{+} MP in the absence of ATP stimulation and mimicked other cellular events that follow activation of the P2X7 receptor, including opening of a plasma membrane pore for larger solutes and the increased solvent accessibility of free thiol groups on the cell surface. Consistent with an activating effect of Clone 34 that bypasses P2X7 to produce TF activation, administration of Clone 34 *in vivo* reversed the thrombosis-protected phenotype of P2X7 signaling-deficient mice in the $FeCl_3$ carotid artery occlusion model. Thus, PDI plays a regulatory role in controlling TF cell surface activity, the generation of procoagulant MP, and TF-dependent thrombosis.

It is of note that cellular expression of TF with a mutated allosteric disulfide results in faster electrophoretic mobility relative to wild-type, consistent with altered carbohydrate structures of TF (7). TF from recombinant and natural sources also differs in carbohydrate composition that influences the activity towards macromolecular substrate in purified systems (20). Taken together these data may suggest that the allosteric disulfide not simply acts a redox switch, but rather engages in dynamic interactions required for TF to acquire the proper posttranslational modifications required for full procoagulant activity. However, these independent studies strongly implicate thiol-disulfide exchange in cellular TF procoagulant activation that occurs in the context of alterations in cytoskeletal and membrane structures and PS exposure.

Concluding remarks

While the precise structure of a broken allosteric Cys^{186}-Cys^{209} disulfide remains to be defined and may vary between physiologically relevant cell types, other studies have entirely dismissed the possibility that carbohydrate modifications or the allosteric disulfide bond play roles in the cellular regulation or encryption/decryption of TF's procoaglant activity (15,16,21-23). Bach originally proposed the term decryption to describe the procoagulant activation of completely inactive TF on myeloid cells and showed that this process involves both, structural changes in TF and the exposure of procoagulant PS (6). Recently, decryption has been used more deliberately to describe any change in TF procoagulant activity, even in cell models that have constitutively active TF (15,16,21-23). It is readily apparent that it is difficult to distinguish in these models between activation of TF due to structural changes versus increased availability of PS that amplifies the procoagulant reactions driven by pre-existing active pools of TF.

Importantly, some of these models may lack components that alter the cellular behavior of TF with a modified allosteric disulfide. The biochemical evidence in the absence of phospholipid clearly shows that disulfide bond mutated TF has diminished affinity for VIIa and is markedly impaired in macromolecular substrate activation (24). However, the reduced Cys^{186} and Cys^{209} residues stay within 3–6 Å of each other (11), excluding a major disruption of TF's tertiary structure upon reduction. Therefore, it is not surprising that supra-physiological concentrations of enzyme VIIa and substrate X on a supporting phospholipid surface can promote an induced fit that entirely normalizes the functional defect of TF with a broken disulfide on certain cells (10,16). Importantly, in other cell models cell surface expressed, disulfide-mutated TF remains inactive (7,25), even when analyzed at 1000-fold the physiological VIIa concentration (~ 100 pM) (26). Thus, TF with a broken disulfide likely engages in cell type-specific protein interactions that modulate TF procoagulant activity. Future studies are required in cellular models that mimic at least some of the aspects of TF decryption in primary cells and it remains a challenge to develop molecular probes for the conformational heterogeneity of TF.

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