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Synergies of phosphatidylserine and protein disulfide isomerase in tissue factor activation

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Summary

Tissue factor (TF), the cellular receptor and cofactor for factor VII/VIIa, initiates haemostasis and thrombosis. Initial tissue distribution studies suggested that TF was sequestered from the circulation and only present at perivascular sites. However, there is now clear evidence that TF also exists as a blood-borne form with critical contributions not only to arterial thrombosis following plaque rupture and to venous thrombosis following endothelial perturbation, but also to various other clotting abnormalities associated with trauma, infection, or cancer. Because thrombin generation, fibrin deposition, and platelet aggregation in the contexts of haemostasis, thrombosis, and pathogen defence frequently occur without TF *de novo* synthesis, considerable efforts are still directed to understanding the molecular events underlying the conversion of predominantly non-coagulant or cryptic TF on the surface of haematopoietic cells to a highly procoagulant molecule following cellular injury or stimulation. This article will review some of the still controversial mechanisms implicated in cellular TF activation or decryption with particular focus on the coordinated effects of outer leaflet phosphatidylserine exposure and thiol-disulfide exchange pathways involving protein disulfide isomerase (PDI). In this regard, our recent findings of ATP-triggered stimulation of the purinergic P2X7 receptor on myeloid and smooth muscle cells resulting in potent TF activation and shedding of procoagulant microparticles as well as of rapid monocyte TF decryption following antithymocyte globulin-dependent membrane complement fixation have delineated specific PDI-dependent pathways of cellular TF activation and thus illustrated additional and novel links in the coupling of inflammation and coagulation.

Keywords

Tissue factor; phosphatidylserine; protein disulfide isomerase; thiol-disulfide exchange; thrombosis

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Introduction

Tissue factor (TF) is a 47-kDa glycoprotein composed of an extracellular (residues, 1–219), a transmembrane (220–244), and an intracellular domain (245–263). Following binding of activated factor VII (FVIIa), membrane-expressed TF serves as the cellular cofactor for efficient activation of FX and FIX. Despite accumulating evidence for important contributions of the contact pathway to arterial thrombosis [1], TF-initiated coagulation remains an integral component of haemostasis and thrombosis in physiology and pathology [2].

Characterization of the tissue distribution suggested that TF was only present in separation from the circulating blood in perivascular locations where it forms a haemostatic envelope to ensure rapid haemostasis upon vascular injury [3]. Likewise, pathological TF expression by macrophages and smooth muscle cells sequestered in atherosclerotic plaques initiates intravascular thrombosis upon plaque rupture [4]. However, there is now increasing evidence that TF exists as a blood-borne form, being either released on microparticles (MPs) or expressed by vascular cells [5]. Intravascular TF may contribute to coagulation activation in various disease states, including trauma, infection, and cancer [6–8].

The vascular cell type that has been conclusively shown to synthesize TF *in vivo* is the blood monocyte, although intravascular TF may also be expressed by neutrophils, eosinophils, endothelial cells, and platelets. Monocytes and tissue macrophages are considered key players in systemic clotting abnormalities such as disseminated intravascular coagulation (DIC) [9], but animal models also suggest that non-haematopoietic sources of TF contribute to coagulation activation in sepsis [10]. In addition, macrophages and dendritic cells can release TF on procoagulant MPs which can be taken up e.g. by endothelial cells [11–13]. Thus, one needs to consider that certain cell types in the vasculature become TF positive due to MP transfer from monocytic cells [14,15]. Importantly, recent evidence from independent *in vivo* studies suggests that not only arterial, but also venous thrombosis is triggered by intravascular TF in the context of heterotypic multicellular interactions at sites of endothelial perturbation [16–19].

The rapid kinetics of thrombus formation after vessel wall perturbation indicate that TF is not *de novo* synthesized through the well characterized mechanism of inflammatory immediate early gene induction, but rather is exposed or activated in the context of cell injury. Likewise, following pathogen invasion, immediate thrombin generation and fibrin deposition are needed to form a physical barrier and to effectively control further bacterial spreading. Based on the observation that TF is frequently cell surface expressed, but non-coagulant, considerable efforts are still devoted to understand how cells control the activation of TF from a predominantly non-coagulant or cryptic state on intact cells to a procoagulant molecule following stimulation. This article will review some of the still controversial molecular mechanisms implicated in cellular TF activation or decryption with particular focus on the coordinated effects of outer leaflet phosphatidylserine (PS) exposure and thiol-disulfide exchange pathways involving protein disulfide isomerase (PDI).

The concept of cryptic TF

In virtually every cell type, TF procoagulant activity (PCA) is significantly increased upon lysis with select detergents or physical disruption. In particular, TF PCA has been shown to be 30–50fold increased in lysed as compared to intact myeloblasts from patients with acute myelogenous leukaemia (AML) and decompensated DIC [20]. This observation has led to the hypothesis that TF released from intracellular storage pools following spontaneous or chemotherapy-induced apoptosis/necrosis is responsible for systemic coagulation activation in AML. However, it has become clear that TF is primarily cell surface expressed on activated monocytes, myeloblasts, and other cancer cells, because preincubation of intact cells with an inhibitory TF antibody completely abolishes TF PCA after removal of unbound antibody, even after cell disruption [21,22].

Indirect evidence that membrane alterations are critically important for cellular TF decryption has been provided by transmission electron microscopy of the bone marrow from a patient with AML-associated DIC [23]. Extensive fibrin deposition was almost exclusively seen around fragmented myeloblasts. It was therefore concluded that membrane damage was closely related to the development of DIC in this patient. In a subsequent study using the AML cell line, HL60, Bach and Moldow [24] demonstrated that upon induction of TF synthesis by phorbol myristate acetate, short-term treatment with calcium ionophore was significantly more effective in decrypting TF PCA than physical cell disruption, indicating that an altered, but structurally intact cell membrane provides a superior catalytic surface for TF activation or TF-dependent coagulation than membrane dispersion by cell lysis.

Cryptic TF binds FVIIa, but with lower affinity compared to coagulant active TF. Early studies on stimulated, highly procoagulant monocytes reported K_d values of <0.1 nM [25] and on adherent tumour cells found a subpopulation of TF that cooperatively bound FVIIa with high affinity [26]. Subsequent studies with tumour cell lines or fibroblasts confirmed that the majority of TF bound FVII/FVIIa with K_d values of well over 1 nM [27–32]. Side by side titration of TF activity and saturation of specific binding sites for FVII/VIIa demonstrated that the low affinity pool of TF did not contribute to FX activation [33]. In addition, while binding of FVII/FVIIa to coagulant active TF reaches equilibrium within a few minutes, saturation of FVII/FVIIa binding to cryptic TF requires hours. However, a distinct feature of the cryptic TF-FVIIa complex is its ability to promote cleavage of small peptidyl substrate or the TF-FVIIa signalling receptor PAR2 [34], despite being highly inefficient in the catalysis of macromolecular substrates [27–33].

Contributions of PS membrane exposure to TF decryption

Calcium ionophore, which induces a rapid influx of extracellular calcium, is used as a standard agent to increase cellular TF PCA. The rapid elevation of cytosolic calcium disturbs the phospholipid asymmetry of the plasma membrane resulting in increased exposure of negatively charged phospholipids, i.e. PS, on the outer membrane leaflet [35–37]. Phospholipids are critically important for the PCA of recombinant or purified TF, and the presence of anionic phospholipids such as PS greatly accelerates TF PCA. Studies using purified TF reconstituted into liposomes have shown that a high PS content of

approximately 30% is required for optimal PCA of TF-FVIIa complexes [38]. However, many phospholipids not containing a choline head group (e.g. phosphatidylethanolamine) can lower the percentage of PS required for optimal TF PCA [39].

Increased PS availability contributes to the enhanced TF PCA observed after calcium ionophore treatment, physical cell disruption, or cell lysis with non-ionic detergents such as n-octyl- β -D-glucoside. PS exposure also plays an important role in more pathophysiologically relevant scenarios such as activation of monocytes by high concentrations of endotoxin [40], complement-mediated cell lysis [41], or induction of apoptosis [42].

Binding of fluorescently labelled annexin V or lactadherin or measuring prothrombinase activity are commonly used to demonstrate PS membrane externalization in the context of cellular TF activation. However, these methodologies cannot resolve the exact membrane topology of exposed PS relative to TF, although annexin V and lactadherin may have preferential affinity for PS presented in different membrane structures [43,44]. PS exposed in the close vicinity of cryptic TF is more likely to be involved in the activation process than PS randomly distributed across the cell surface. Annexin V and lactadherin bind and neutralize PS and are also used to prove the functional involvement of PS exposure during cellular TF activation. On cell membranes, basal levels of TF PCA are typically not affected by PS neutralization, but TF activation achieved by any of the above means is variably, but in most cases not completely neutralized by saturating concentrations of annexin V or lactadherin [37,45–47], pointing to additional molecular mechanisms involved in cellular TF activation.

It is not completely clear how PS contributes to the enhanced PCA of TF-FVIIa complexes on cell membranes. PS exposure facilitates binding of FX to the cell membrane through its vitamin K-dependent γ -carboxyglutamic acid (Gla)-containing domain, thus lowering the FX concentration required for activation. Consistently, a decrease in the apparent K_m for FX has been demonstrated following stimulation of TF expressing cells with calcium ionophore [35]. These experiments, however, have also shown significantly accelerated FX substrate turnover with an increase in V_{max} . This finding is unlikely caused by an increased number of TF-FVIIa complexes, because PS has only minimal effects on FVIIa binding to relipidated TF [48,49]. Instead of promoting TF-FVIIa complex formation, PS most likely facilitates substrate recognition, turnover, and release by established TF-FVIIa complexes. Molecular modelling has suggested potential direct interactions of TF extracellular domain residues previously implicated in procoagulant function with negatively charged phospholipid head groups [50–52], indicating that structural effects of PS on TF and not only support of Gla-domain interactions of coagulation factors may contribute to enhanced PCA following PS exposure. Such a direct effect of PS on the quaternary structure of the TF-FVIIa complex may explain why activation mechanisms involving the tertiary structure of TF discussed below provide optimal effects on PCA in the context of simultaneous PS exposure.

Role of thiol-disulfide exchange in TF decryption

The extracellular domain of TF consists of two fibronectin type III domains. While the N-terminal domain contains a typical structural disulfide between Cys⁴⁹ and Cys⁵⁷, the Cys¹⁸⁶-Cys²⁰⁹ disulfide within the C-terminal, membrane-proximal TF domain exists in an – RHStaple configuration, which is characteristic of allosteric disulfide bonds facilitating the cystine link between two adjacent strands within a β -sheet [53,54]. Because allosteric disulfide bonds are known to control protein function in a redox-dependent manner, a role for the Cys¹⁸⁶-Cys²⁰⁹ disulfide in the regulation of TF encryption/decryption has been postulated. Mutagenesis of this bond indicated that the allosteric disulfide bond is formed (i.e. oxidized) in procoagulant and broken (i.e. reduced) in cryptic TF [34,55–59]. Specifically, cell surface-expressed cryptic TF has been shown to contain free cysteine thiols or cysteines that are either S-nitrosylated or S-glutathionylated [34,46,55]. However, TF mutated at the allosteric Cys¹⁸⁶-Cys²⁰⁹ disulfide is poorly expressed on cell surfaces and in certain cell types accumulates intracellularly [34,60], indicating that oxidation and/or reversible breaking of this bond is important for normal TF trafficking. However, when wild-type and TF disulfide mutants were expressed to similar levels, ablation of the membrane-proximal allosteric disulfide resulted in severely impaired procoagulant function at both physiological (i.e. 1–10 nM) and supraphysiological (i.e. >100 nM) concentrations of FVIIa in certain cell types [57]. Disulfide mutated TF binds FVIIa with an affinity comparable to cryptic wild-type TF, and, importantly, the mutant retaining Cys¹⁸⁶ supports TF-FVIIa signalling activities indistinguishable from wild-type TF [34].

TF can be activated after saturation with FVIIa by stimuli that decrypt TF [24]. Assuming steric hindrance of bound FVIIa, these experiments have been quoted as evidence to argue against relevance of disulfide switching in TF decryption [61]. Measurements of the distance between Cys¹⁸⁶ and Cys²⁰⁹ using molecular rulers [54] showed that reduction only minimally changed the proximity of these residues, indicating that the overall structure of TF and critical binding regions for FVIIa remain largely conformed in reduced TF. The available crystal structures of TF-FVIIa have elucidated the complex of oxidized soluble TF and show the FVIIa Gla-domain in an unusually constrained orientation that covers the allosteric disulfide bond [62]. The crystal structure with the FVIIa Gla-domain saturated with Mg²⁺, which is critical for optimal FX activation by TF-FVIIa, shows a slightly different interface of TF with the FVIIa Gla-domain that imposes stabilization on the flexible C-terminal module of TF [63].

Importantly, the majority of the binding energy for formation of the TF-FVIIa complex comes from interactions that do not involve the FVIIa Gla-domain [48,64]. Only one TF residue, Val²⁰⁷, in proximity of the allosteric disulfide bond makes a measurable energetic contribution to FVIIa binding in Ala scanning mutagenesis experiments [65]. FVIIa Gla-domain deletion or structural disorder due to Ca²⁺ depletion is compatible with TF binding and the estimated loss of affinity is in the same order of magnitude as the determined affinity difference between cryptic and fully active TF for FVIIa [66]. Therefore, one cannot assume that the FVIIa Gla-domain interacts with cryptic TF as seen in the crystal structures of oxidized TF or precludes the disulfide bond of low affinity TF-FVIIa complexes. In addition, the C-terminal module of TF in the binary TF-FVIIa complex exposed a large

interactive surface [67] that may be utilized by catalysts of thiol-disulfide exchange reactions. Consistently, cellular TF decryption by antithymocyte globulin (ATG), which is dependent on complement and PDI-mediated thiol-disulfide exchange [68], is not prevented by saturation of TF binding sites with recombinant human FVIIa prior to the activation process [Figure 1].

Treatment of cells with oxidizing agents such as HgCl_2 increases TF-specific PCA [46]. In contrast, blocking free thiols with chemical agents such as N-ethylmaleimide (NEM), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), or methyl methanethiosulfonate (MMTS) inhibits cellular TF activation [34,46]. Both observations are consistent with disulfide bond formation during cellular TF decryption, but the lack of specific probes recognizing the oxidized or reduced form of TF on cell membranes makes measuring these changes on the cell surface difficult. One antibody may detect, in part, TF conformational changes on cells exposed to strong oxidants [46]. It is important to point out that both oxidizing agents and thiol blockers also increase membrane PS externalization, indicating that TF activation (by oxidizers) and its prevention (by thiol blockers) is not strictly correlated with PS exposure [47,69,70]. However, most activating stimuli also increase PS externalization, suggesting that PS exposure is an important component in facilitating thiol-disulfide exchange-dependent allosteric activation of TF.

We have recently shed new light on the complexity of TF activation on monocytic cells by studying complement-dependent TF decryption by ATG [68]. On THP1 cells, thiol alkylation by NEM increases extracellular PS and produces a concomitant increase in basal PCA. However, despite amplified PS externalization, further TF activation through the local complement fixation and activation induced by ATG is completely prevented by NEM pretreatment [68]. These data and studies with murine macrophages [13] suggest that, although required, PS exposure is not sufficient to support cellular TF activation. Instead, a complex and well-orchestrated sequence of (inter)molecular events is induced by complement activation, involving lipid-raft disruption, PS externalization, and surface-located thiol-disulfide exchange reactions. A candidate enzyme implicated herein is PDI.

Role of PDI in TF decryption

PDI is an abundant oxidoreductase with chaperone activity that is predominantly expressed in the lumen of the endoplasmic reticulum (ER) of eukaryotic cells, where it mediates the correct formation of disulfide bonds and thereby aides folding of secreted proteins. PDI breaks and reforms disulfide bonds and this disulfide isomerization involves either formation of a mixed disulfide with the protein substrate or cycles of reduction and reoxidation in the presence of redox partners such as reduced glutathione or thioredoxin (Trx) [71,72]. Additional members of the thiol-disulfide isomerase family are ERp5, ERp57, and ERp72. PDI is also secreted and associates with the surface of various cell types, including platelets, endothelial cells, keratinocytes, fibrosarcoma cells, and monocytes/macrophages [13,34, 68,73–75], where it may control the redox state of exofacial protein thiols or reactive disulfides [76]. It is unclear how PDI that contains a C-terminal KDEL retention sequence escapes the ER and remains associated with the cell surface. PDI can also

transfer nitric oxide (NO) onto sulfhydryl groups and PDI-mediated S-nitrosylation of cell surface proteins has been implicated in transport of NO across cell membranes [77].

In the human HaCaT keratinocyte cell model, free thiols of PDI can be labelled with a membrane impermeable thiol probe [34]. Co-immunoprecipitation experiments showed that cell surface PDI is associated with TF on the surface of these cells. The association of PDI with TF is regulated by dynamic changes induced by cell-cell contact and PDI plays a role in switching TF function from coagulation to PAR2-dependent cell signalling in a thiol-disulfide exchange dependent manner, probably involving S-nitrosylation and/or S-glutathionylation of the TF allosteric disulfide bond. Downregulation of PDI expression in this primary keratinocyte model by siRNA technology increases basal TF PCA independent of TF protein levels [34], a finding confirmed with endothelial cells overexpressing TF [75]. Thus, in a more reducing environment, PDI may suppress cellular TF procoagulant function by facilitating breaking of the Cys¹⁸⁶-Cys²⁰⁹ allosteric disulfide bond, and this inhibitory effect can be reversed with oxidants that are also known to release NO from protein substrates [34,78]. In addition, PDI inhibition on endothelial cells results in increased PS membrane exposure through effects on both flippase and floppase activities [75]. Taken together, PDI appears to control both critical determinants for TF PCA, i.e. TF structure and the cell surface phospholipid environment.

It is obvious from these experiments that extracellular localization of PDI alone cannot serve as a predictor of the enzyme's effects on TF function. Rather the rapid changes in cell surface redox potential need to be taken into consideration to evaluate cell type- and context-dependent functions of PDI in haemostasis and thrombosis. For instance, using an *in vivo* model of macrovascular thrombosis, Reinhardt et al. [55] have suggested that soluble PDI released from activated platelets or injured cells of the vessel wall can promote fibrin deposition by converting TF (expressed on leukocytes, platelets, or MPs) from the latent to the coagulant active form. From a set of *in vitro* experiments they concluded that reduced PDI was required for deglutathionylation of TF residue Cys²⁰⁹, followed by catalytic attack of the TF-PDI mixed disulfide by the free thiol at position 186 resulting in an intact Cys¹⁸⁶-Cys²⁰⁹ disulfide bond. In this system, instead of inhibiting TF-dependent coagulation, extracellular soluble PDI rather acts as a prothrombotic injury response signal [55]. Although thiol-disulfide exchange reactions are a central functional property of PDI, certain aspects of TF activation require the enzyme's chaperone activity which prevents protein aggregation during folding [79,80].

Studies in mouse models of thrombosis with an inhibitory antibody or with small-molecule antagonists have provided further evidence for a prothrombotic role of PDI [13,55,81–83]. PDI not only regulates TF function, but also platelet, leukocyte, and endothelial integrins and platelet-dependent thrombin generation [84–87]. Platelet PDI plays an important role in thrombosis [86] and it is difficult to define the relative effects of PDI on TF decryption and PS availability in the context of platelet activation during thrombus formation *in vivo*. Blocking PDI attenuated fibrin deposition at sites of endothelial cell injury, even when fibrinogen-mediated platelet aggregation was prevented [55], but these experiments did not control for leukocyte recruitment to the sites of injury [17,85]. Endothelial and platelet-derived β_3 integrins appear to be required for the capture and localization of released PDI

into the developing thrombus [84]. In addition, other thiol isomerases contribute to platelet activation [88], and a complicated picture of potential reciprocal influences between members of this protein family is emerging in regulating thrombus formation and coagulation.

PDI-dependent TF activation couples inflammation with thrombosis

Specific pathways of cellular TF activation involving PDI illustrate additional and novel links in the coupling of inflammation and coagulation. On TF expressing macrophages and smooth muscle cells, ATP-triggered stimulation of the purinergic P2X7 receptor efficiently decrypts TF PCA and results in the shedding of procoagulant TF-positive MPs [13]. This mechanism involves both production of reactive oxygen species and PDI-dependent extracellular thiol-disulfide exchange. Because ATP is released from injured cells as a result of mechanical stress, inflammation, or ischemia, the P2X7 receptor pathway directly links tissue damage to cellular TF activation. The prothrombotic response to this danger signal may play a critical role in protecting the local tissue microenvironment from invading microorganisms, but intravascularly this pathway promotes thrombosis. Remarkably, a unique activating PDI antibody that mimics the effects of P2X7 stimulation on TF decryption and MP release is capable of restoring normal macrovascular thrombosis in otherwise protected P2X7-deficient mice [13]. Surface-expressed PDI thus regulates a critical P2X7 receptor-dependent signalling pathway on vascular and myeloid cells that generates prothrombotic TF with potential implications for antithrombotic therapy.

More recently, we have delineated a mechanism of rapid monocyte TF decryption that is initiated by ATG-induced complement activation [68]. Monocytes are capable to translocate PDI onto their surface and TF activation in these cells critically depends on intact surface PDI activity. TF activation by ATG required oxidation of membrane-expressed PDI downstream of C5 cleavage, probably due to the engagement of complement regulatory proteins with subsequent depletion of reductive equivalents such as Trx-1 [89]. Complement activation also leads to PS exposure following C5b-7 membrane insertion, but full assembly of the terminal membrane attack complex was not required for providing optimal amounts of procoagulant PS [68]. In this model, PDI-dependent thiol-disulfide exchange closely cooperates with extracellular PS to generate procoagulant TF. It is tempting to speculate that ATG-mediated targeting of complement activation to the cryptic pools of TF initiates a spatially and temporarily orchestrated sequence of molecular events in which thiol-disulfide exchange reactions fold TF in vicinity of newly externalized PS. This pathway may require disruption of other TF regulatory structures such as lipid rafts and TF homo- or heteromeric complexes with PDI or integrins [34,90], which could explain why ATG induces TF activation on THP1 cells more potently than non-specific oxidants like HgCl₂, despite producing only limited PS exposure [Figure 2]. ATG-induced PCA is markedly inhibited by annexin V, but TF-dependent FXa generation is only marginally reduced by PS neutralization [Figure 3]. The pronounced effect on PCA is consistent with availability of PS to support prothrombinase activity in the clotting assay. However, PS exposure alone can apparently not explain TF activity.

It is important to note that molecular probes do not exist that would allow detection of changes in the TF allosteric Cys¹⁸⁶-Cys²⁰⁹ disulfide bond on these primary cell models with exceedingly low TF expression. Considering the multiple interactions of PDI with the redox machinery of cells, one should also not limit further studies on the interaction between TF and PDI itself and consider other redox partners up- or downstream of PDI in the cellular regulation of TF activity. For example, the Trx/thioredoxin reductase system or additional thiol-regulated proteins such as integrins may be directly linked to these pathways through interaction with TF and/or PDI [72,85,90,91]. Moreover, even though encryption/decryption appears to be a universal mechanism by which TF expressing cells regulate their procoagulant phenotype [33], the underlying molecular pathways and/or their relative contributions may well differ between cell types [68–70]. Rather than depicting TF regulation as a two state process of reduced and oxidized TF, TF encryption/decryption should be envisioned as a dynamic and reversible process regulated by various cellular processes and oxidative stress [Figure 4].

Pathophysiological scenarios of complement-dependent TF decryption

Our observations of ATG-induced TF activation may have pathophysiological implications for several prothrombotic disorders characterized by deregulated complement activation, one of them being paroxysmal nocturnal haemoglobinuria (PNH). In PNH, evolution and expansion of a myeloid cell clone deficient in glycosylphosphatidylinositol-anchored complement regulatory membrane proteins renders erythrocytes susceptible to haemolysis. Patients with PNH have a high risk of thrombosis, which may at least partially result from increased TF expression by complement-injured CD55- and CD59-deficient monocytes/macrophages and an increased shedding of TF-positive MPs [92,93]. Although the latter is not directly correlated with haemolysis [93], release of haem(oglobin) upon red blood cell destruction may function as a positive feedback loop, because haem(oglobin) has been shown to induce TF expression in vascular cells and to fix activated complement components on cell membranes [94–96]. This positive feedback loop could also be relevant for the prothrombotic state in sickle-cell disease, another haemolytic disorder characterized by activation of the TF-dependent coagulation pathway [97].

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Figure 1. Saturation with FVIIa does not interfere with ATG-mediated cellular TF activation
Monocytic THP1 cells were preincubated with buffer (control) or 100 nM recombinant human activated FVII (rFVIIa) for 30 min at 37°C. Following loading with rabbit IgG or ATG, cell-associated PCA was measured by single-stage clotting assay as described in Langer et al. [68]. A representative experiment is shown.

Figure 2. TF activation on THP1 cells by HgCl₂ or ATG is poorly correlated with extracellular PS

Activation of monocytic THP1 cells by ATG (100 µg/ml) or HgCl₂ (100 µM) was carried out using the conditions described in Langer et al. [68]. (A) PCA was measured by single-stage clotting assay and (B) PS exposure by flow cytometry using fluorescently labelled annexin V. Representative experiments are shown.

Figure 3. Saturating concentrations of annexin V only partially inhibit TF-dependent FXa generation on ATG-activated THP1 cells

Effect of annexin V on (A) PCA and (B) TF-dependent FXa generation on ATG-activated THP1 cells (means $\pm$ SD, n=3). Inhibitory TF antibody (α TF) was used for comparison.

Figure 4. Mechanisms of thiol-disulfide exchange-dependent cellular TF activation involving cell surface PDI

On intact cells, TF is maintained in a predominantly non-coagulant or cryptic state by sequestration of PS to the inner membrane leaflet and by a reduced or broken membrane-proximal Cys¹⁸⁶-Cys²⁰⁹ allosteric disulfide bond. Experiments provided evidence for modification of this bond by reduction, S-nitrosylation/-glutathionylation of the cysteine at position 209, or by the formation of mixed TF disulfides with other thiol-regulated proteins such as thioredoxin (Trx), integrins, or PDI. ATP-triggered activation of the purinergic P2X7 receptor or complement activation with subsequent C5b-7 membrane insertion results in PDI-dependent thiol-disulfide exchange with optimal TF folding and oxidation in the context of simultaneously exposed PS.