TISSUE FACTOR COAGULANT FUNCTION IS ENHANCED BY PROTEIN DISULFIDE ISOMERASE INDEPENDENT OF OXIDO-REDUCTASE ACTIVITY

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Protein disulfide isomerase (PDI) switches tissue factor (TF) from coagulation to signaling by targeting the allosteric Cys$^{186}$-Cys$^{209}$ disulfide. Here, we further characterize the interaction of purified PDI with TF. We find that PDI enhances VIIa-dependent substrate factor X activation 5- to 10-fold in the presence of wild-type, oxidized soluble TF, but not TF mutants that contain an unpaired Cys$^{186}$ or Cys$^{209}$. PDI-accelerated FXa generation was blocked by bacitracin, but not influenced by inhibition of vicinal thiols, reduction of PDI, changes in redox gradients or covalent thiol modification of reduced PDI by NEM or MMTS which abolished PDI oxidoreductase, but not chaperone activity. PDI had no effect on fully active TF on either negatively charged phospholipids or in activating detergent, indicating that PDI selectively acts upon cryptic TF to facilitate ternary complex formation and macromolecular substrate turnover. PDI activation was reduced upon mutation of TF residues in proximity to the macromolecular substrate binding site, consistent with a primary interaction of PDI with TF. PDI enhanced TF coagulant activity on microvesicles shed from cells, suggesting that PDI plays a role as an activating chaperone for circulating cryptic TF.

Tissue Factor (TF) initiates coagulation by binding and catalytically activating coagulation factor VIIa (VIIa) for efficient turnover of macromolecular substrate factor X (FX). TF plays an essential role in hemostasis (1) by serving as a hemostatic envelop around blood vessels and tissues (2). In addition, TF is present in the blood and microparticle-associated TF can be localized to thrombi and contribute to local fibrin formation (3;4). TF also has non-hemostatic, signaling functions in angiogenesis, cancer progression and inflammation that involve regulation of integrins and direct TF-VIIa signaling through protease activated receptor (PAR) 2 (5-7). We recently showed that cell surface protein disulfide isomerase (PDI) is associated with inactive TF and targets the solvent-exposed, allosteric Cys$^{186}$-Cys$^{209}$ disulfide bond in TF to inhibit coagulation while maintaining direct TF-VIIa signaling (8). The thiol dependence of TF activation under these conditions (8;9) indicated that oxidation or isomerization by PDI switches TF from a non-coagulant, reduced molecule to an active molecule with an oxidized allosteric disulfide.

However, TF’s procoagulant activity is regulated at multiple levels, including rapid feedback inhibition by TF pathway inhibitor (TFPI) (10) that directs TF to low density microdomains (11). A relatively small portion of total cellular TF is sufficient for procoagulant activity and various amounts of non-coagulant or cryptic cell surface TF are present dependent on cell type, cellular differentiation and proliferation (8;12;13). Location of TF to rafts or caveolae attenuates coagulant activity of TF (11;14-16). On certain
cells, TF may predominantly activate coagulation in cholesterol-rich microdomains (17). Release of TF on microvesicles appears to involve raft domains and is cholesterol-dependent, but TF requires activation dependent on fusion of microvesicles with activated platelets (16). In this and other systems (13), exposure of phosphatidylserine (PS) and increased availability of procoagulant lipids are important factors that contribute to coagulation that is initiated by TF. However, interference with redox and thiol pathways can enhance TF activity independent of PS (9;18).

Mutational elimination of the allosteric Cys\textsuperscript{186} - Cys\textsuperscript{209} disulfide abolishes TF coagulant function (8;19). This disulfide is partially exposed to solvent and becomes buried under the Gla-domain of VIIa when the TF-VIIa complex forms (20). The Cys\textsuperscript{186}-Cys\textsuperscript{209} disulfide further stabilizes an extended loop that contains several residues involved in the binding of macromolecular substrate FX (21). Together these structural features explain why affinity for VIIa is somewhat reduced and substrate FX activation is severely impaired upon breaking of the disulfide (8;19). The activation of macromolecular substrate FX involves binding to an extended exosite that is formed by TF and a region in the VIIa protease domain distant from the catalytic center (21-23). Because these extended contacts are largely unaffected by the zymogen activation of FX to FXa (21), FXa retains a relatively high affinity for TF-VIIa (24;25). As a consequence, product release can be a rate limiting factor in substrate turnover by TF-VIIa. The rate enhancing effect of PS on these reactions may be caused by facilitated substrate release (13;26).

In addition to changes in PS exposure, TF activity in thrombosis may be regulated by PDI, because PDI is released from activated platelets and found associated with the platelet surface (27). PDI, a 64 kD protein normally found in the endoplasmic reticulum (ER), has both oxidoreductase and isomerase activity, thus facilitating thiol/disulfide exchange reactions during protein folding (28). In addition, PDI has chaperone function (29) and PDI has denitrosylase (30) and transnitrosylase activity that mediates cellular uptake of NO (31;32). Cell surface PDI furthermore regulates platelet integrin activation (33-36). Because of the unique extracellular redox environment, cell surface PDI likely performs specific functions that are distinct from ER-localized PDI (37).

In this study we show that PDI enhances TF procoagulant activity on microvesicles generated \textit{in vitro}. In a defined system with purified proteins, we further demonstrate that PDI accelerates macromolecular substrate FX activation of soluble TF that is known to have low procoagulant activity (38). Our data show that PDI acts on oxidized TF independent of PDI’s oxidoreductase activity. Whereas PDI on cells inactivates TF’s procoagulant activity by nitric oxide (NO)-dependent pathways, the data presented here indicate that PDI also participates in activating cryptic TF through PDI’s chaperone activity. This study provides new insight into the interaction of PDI with the ternary TF initiation complex.

**EXPERIMENTAL PROCEDURES**

**Materials** - Bovine PDI, reduced (GSH) and oxidized (GSSG) glutathione, phenylarsenic oxide (PAO), methyl-methanethiosulfonate (MMTS), N-ethylmaleimide (NEM) and bacitracin were purchased from Sigma-Aldrich (St. Louis, MO). Bacitracin was repurified by gel filtration to eliminate enzymatic activity that degrades PDI (39). The NO-donor SNP was from Alexis (Carlsbad, CA) and N-(3-maleimidylpropionyl)biocytin (MPB) was from Invitrogen (Carlsbad, CA). The glutathione-reactive antibody (αGSH) was from Virogen (Watertown, MA).

**Proteins** – Monoclonal antibodies 5G9 and 10H10 to TF and polyclonal rabbit anti-TF cytoplasmic domain antibody have been previously described (8;40;41). Isotype-matched control antibody TIB115 to the viral large T antigen was obtained from ATCC. Soluble TF (TF\textsubscript{1-218}) was expressed in \textit{E. coli}, as previously described (42;43). Briefly, protein was prepared from inclusion bodies, solubilized, reduced and refolded in 0.8 M guanidine HCl, 0.3 M NaCl, 50 mM Tris-HCl, pH 8.0, in a redox gradient of 2.5 mM reduced glutathione (GSH) and 0.5 mM oxidized glutathione (GSSG). Mutants with an unpaired Cys\textsuperscript{186} or Cys\textsuperscript{209} were refolded in the same buffer.
in the presence of SNP added to 2.5 mM at the beginning of the folding reaction and again 2 hours later at the same concentration, based on initial observations that indicated better yield of monomeric mutants under these conditions. Refolded protein was recovered after extensive dialysis and concentrated, followed by gel filtration to separate monomeric mutant from higher molecular weight aggregates and dimers. Removal of the expression tag by thrombin was omitted to avoid degradation of the mutants. Wild-type soluble TF with an attached expression tag were used as controls. The TF \( \text{K}^{149}\text{A/D}^{150}\text{A} \) mutant was expressed as a fusion protein with a carboxy-terminal leucine zipper domain (43) which was chosen to improve poor refolding efficiency of the mutant. Characterization of this mutant was in comparison to the same construct of wild-type TF. The \( \text{VII}^{a} \text{D}^{\text{VQ}^{a}} \) mutant was kindly provided by Dr. Lars Petersen, Novo Nordisk. FX was purified from plasma (44) and VII contamination was eliminated by a subsequent immunopurification step, as previously described (22).

Detection of free thiols in wild-type soluble TF-
Free thiol content of soluble wild-type was determined by the Ellman reaction using Cys as standard. The sensitivity of the assay would have detected the reduction of 1% of the allosteric disulfide in TF. Soluble TF was further labeled with polyethylene glycol-maleimide (PEG-mal) which reacts with free thiols to add 5 kDa per modified Cys residue on SDS-PAGE. Typically, soluble TF was labeled with 5 mM PEG-mal at 37°C for 60 minutes in the presence or absence of reducing agents or PDI. Control reactions under denaturing (0.8% SDS) conditions showed no appreciable difference in the degree of PEG-mal labeling. In order to remove excess PEG-mal, TF was precipitated with acetone and resuspended in sample buffer for SDS-PAGE and Westernblotting with polyclonal anti-TF.

Cell culture and generation of TF-positive microparticles- Human umbilical vein endothelial cells (HUVEC) were passaged in Endothelial Cell Basal Medium (EBM), prepared with endothelial medium supplement (Cambrex, Walkersville, MD), and transduced with adenovirus encoding TF and PAR2, as previously described (8). After 2 days, cells were transferred to serum-free Medium 199, supplemented with 1 mM CaCl\(_2\), L-glutamine and HEPES to collect released microparticles for 5 hrs. The supernatant was cleared from cellular debris at 14,000 g for 10 min. Cleared supernatant was used without further manipulations for FXa generation assays or immunodepletion using Dynabeads-coupled with anti-TF 5G9 or isotype-matches control TIB115. TF immunoprecipitated from 1 ml supernatant was used for Westernblotting with immunopurified, polyclonal goat anti-TF or a rabbit anti-TF cytoplasmic domain antibody (40). Blots were digitized for densitometry by using Scion Image (Scion, Frederick, MD).

**Chemical modification of PDI with MMTS/NEM and PDI functional assays-** PDI (16 \( \mu \)M) was reduced with 1 mM DTT for 1 hr at ambient temperature. Reduced PDI was then reacted with 20 mM MMTS for 1 hr followed by dialysis against HBS. Efficiency of MMTS-modification was determined by labeling residual free thiols with 100 \( \mu \)M MPB for 30 min at ambient temperature, followed by Westernblotting to detect biotin incorporation. For NEM-modification, reduced PDI was reacted after 4-fold dilution with 1 mM NEM, followed by dialysis as above. Reductase activity of MMTS-treated PDI was measured by insulin turbidity assay (45). Briefly, 800 nM PDI, PDI in the presence of 100 \( \mu \)M PAO or 3 mM bacitracin, or MMTS-modified PDI were added to a solution of 100 mM K\(_2\)HPO\(_4\), 2 mM EDTA, 500 \( \mu \)M GSH, 75 \( \mu \)M insulin. Changes in insulin turbidity were measured for 20 min at 650 nm in a kinetic microplate reader. Chaperone activity of MMTS-modified PDI was determined by rhodanese aggregation assay with the following modifications (46). Rhodanese (1 mg/ml) was first denatured in 5.6 M urea and 10 mM DTT. Rhodanese was diluted 1:100 in 100 mM Tris pH 8.0 to induce protein aggregation. PDI or MMTS-modified PDI (1 \( \mu \)M) were added and protein aggregation was monitored at 405 nm over a 15 minute time period.

**FXa generation and amidolytic assays-** FXa generation assays were performed in HBS (10 mM HEPES, pH 7.4; 137 mM NaCl; 5.3 mM KCl; 1.5 mM CaCl\(_2\)). Typically 25 nM TF, VIIa and PDI were used to activate 1 \( \mu \)M substrate FX and aliquots were quenched in 20 mM Tris, pH 7.4,
150 mM NaCl, 100 mM EDTA at defined times for chromogenic assay with Spectrozyme FXa. PDI inhibitors bacitracin (3 mM) and PAO (100 μM) were added at the start of the reaction. FXa generation assays using supernatant containing microparticles were performed with the same VIIa and FX concentrations and PDI was added as indicated. Amidolytic activity of the TF-VIIa complex was assayed as described before (47). Briefly, complexes of the indicated concentrations of TF and VIIa were formed in HBS in the presence of 1 mM Spectrozyme FXa and conversion of substrate by TF-VIIa was continuously monitored in a kinetic plate reader at 405 nM. All data are presented as means and standard deviations from at least 3 repeat experiments.

**RESULTS**

**PDI enhances TF-VIIa dependent FXa generation-** In order to characterize the interaction of TF and PDI in a purified system, we expressed wild-type soluble TF or mutants that retained either Cys186 (TF C209A) or Cys209 (TF C186A) of the allosteric disulfide. TF has an additional extracellular disulfide (Cys49-Cys57) that is buried and not required for function (19). SDS-PAGE showed homogeneity of the purified proteins (Fig. 1A). Because the refolding reaction contained glutathione, we addressed whether wild-type or mutants became glutathionated during refolding. Western blotting with anti-glutathione antibody demonstrated that no glutathione was incorporated into wild-type TF or TF C209A, but detectable in TF C186A, indicating that Cys209 is prone to glutathionation (Fig. 1B).

Disulfide formation of wild-type TF was confirmed by the Ellman reaction which did not detect free thiols in wild-type TF. Reactivity of the conformation-sensitive antibody 5G9 to the carboxyl terminus of TF also indicated that the Cys186-Cys209 disulfide was intact. In addition, labeling of TF with PEG-mal, which increases the molecular weight of proteins by 5 kDa for each modified Cys residue, produced a molecular weight shift for only a small percentage of the wild-type soluble TF preparation (Fig. 1C) while 50% or more of the soluble TF preparation was modified after reduction with DTT. Note that competition of excess DTT over PEG-mal probably prevented complete modification of reduced TF. In addition, purified bovine PDI or VIIa had no appreciable effect on PEG-mal labeling of soluble TF, indicating that neither the physiological ligand nor PDI promote reduction of TF in solution.

The Cys186-Cys209 disulfide is necessary for binding of the VIIa Gla-domain to TF and consistently both soluble mutants showed decreased affinity for VIIa in an amidolytic assay that measures TF-dependent allosteric activation of VIIa’s catalytic activity. However, no differences were seen at saturating concentrations of mutants (data not shown). Both mutants had low activity in a macromolecular substrate FX activation assay and addition of purified PDI had no appreciable effect on FXa generation by the mutants alone or in combination (Fig. 1D), consistent with the accepted view that formation of the disulfide is required for efficient substrate FX binding. Unexpectedly, PDI enhanced substrate FX activation by wild-type soluble TF 5- to 10-fold. Elimination of VIIa or TF from the reaction showed that PDI was not contaminated with a protease or a cofactor that induced activation of FX independent of TF-VIIa (Fig. 1E).

PDI interaction with partially unfolded proteins is influenced by glycosylation and TF is glycosylated at three N-linked sites in the extracellular domain (42). In order to exclude that the observed rate enhancing effect of PDI is an artifact of the nonglycosylated preparation of E. coli-derived soluble TF, we analyzed glycosylated soluble TF secreted from yeast or mammalian Chinese hamster ovary (CHO) cell expression systems (38;42). All three preparations had the same basal activity and in
each case PDI enhanced the activity to a similar extent (Fig. 1F). Eukaryotic soluble TF was not refolded, but rather secreted from cells. These data also exclude that the refolding protocol for E. coli-derived TF introduced an artificial inactive state.

Although soluble TF has no defect in promoting the allosteric activation of VIIa’s catalytic activity towards small chromogenic substrates, macromolecular substrate binds to an exosite of the VIIa protease domain that is conformationally labile (23;48;49). One possibility was that PDI accelerated macromolecular substrate turnover by influencing allosteric activation of the VIIa exosite for macromolecular substrate. To address this issue, we first analyzed a mutant of VIIa (VIIaV21D/E154V/M156Q/K188A, VIIa DVQA) that is representative for a series of mutants that do not require allosteric activation by TF and activate FX efficiently in the absence of cofactor (50-52). PDI did not increase activity of this mutant in the absence of TF, but enhanced FXa generation when TF was added (Fig. 1G). These data show that PDI does not influence the allosteric activation of VIIa, but rather has a specific effect on TF to enhance turnover of substrate FX.

**PDI specifically targets cryptic TF**- Soluble TF has low activity that can be enhanced by phospholipids (38;53). Increasing concentrations of phospholipids accelerated FXa generation by soluble TF and progressively diminished the rate enhancing effect of PDI (Fig. 2A). In addition, PDI had no effect on the procoagulant activity of full-length TF reconstituted into procoagulant lipid vesicles (Fig. 2B). These data show that PDI specifically acts on TF with low procoagulant activity.

On cells, the majority of TF is in a cryptic conformation with low procoagulant activity. TF can be activated by exposure to detergents, such as octyl glucopyranoside (OG). OG also increased the activation of FX by soluble TF-VIIa in a dose dependent manner. The optimal concentration was 5-10 mM (not shown) which is similar to optimal OG concentrations required to activate cellular, cryptic TF (54). OG increased soluble TF activity to a similar extent as PDI and the presence of both, PDI and OG, did not produce an additive effect on FXa generation (Fig. 2C). These data further support the concept that PDI selectively acts upon cryptic TF.

In cellular systems, cryptic TF has low affinity for VIIa as compared to fully active, procoagulant TF. We tested by amidolytic assay whether PDI or OG enhance TF’s affinity for VIIa. At saturating (50 nM) concentration of TF, neither PDI nor OG had an effect on TF-VIIa amidolytic activity (Fig. 2D). However, PDI, but not OG, enhanced the amidolytic activity of 2 nM VIIa at a subsaturating (2 nM) concentration of soluble TF. These data indicate that PDI has a specific direct effect on TF which results in enhanced affinity for VIIa. However, the increase in affinity cannot account for the rate enhancing effect of PDI on FX activation, because the FXa generation assay was performed at near saturating concentrations of TF for VIIa.

The complex of VIIa and a site specific mutant of TF (TFK149A/D150A) activated FX with similar efficiency as wild-type TF when PDI was absent. However, addition of PDI produced only a <2-fold enhancement of FXa generation in the case of the mutant (Fig. 2E). Control experiments with OG confirmed that detergent activation of the mutant was unaffected, excluding the possibility that the conformation of the mutant prevents activation to full procoagulant function. Taken together these data provide evidence that PDI has a direct effect on cryptic TF to enhance FXa generation.

**PDI facilitates ternary complex formation and substrate turnover**- In a soluble system, the rate of FXa generation is dependent on the encounter of three reactants, cofactor, enzyme and substrate. Consequently, the reaction rate will increase at a fixed enzyme concentration, if cofactor concentration is raised to stoichiometry with substrate, as shown in Fig. 3A. This model predicts that addition of cofactor that is devoid of enzyme binding cannot enhance the reaction rate. Indeed, a site specific mutant of TF, TFN144A, that activates and binds VIIa poorly (55) did not enhance FXa generation when added at substrate concentration to 25 nM wild-type TF-VIIa complex (Fig. 3A). PDI may enhance ternary complex formation without accelerating dissociation of product or, alternatively, accelerate both aspects of substrate turnover. In the former...
case, one would expect that increasing TF concentrations progressively diminish the PDI effect, because ternary complex formation is close to optimal at saturating cofactor. However, the rate enhancing effect of PDI was observed throughout the TF concentration range (Fig. 3B), indicating that PDI facilitates both ternary complex formation and product release.

In order to directly demonstrate the association of TF and PDI, we subjected reactions to immunoprecipitations with anti-TF. TF, PDI and FX/Xa in the immunoprecipitates were then detected by Westernblotting. PDI co-precipitated with TF at the start of the reaction, but PDI did not influence the amount of FX that was initially associated with TF (Fig. 3C). In the absence of PDI, the faster migrating FXa accumulated in the TF immunoprecipitates. In contrast, FXa appeared to be more readily released from TF in reactions with PDI. At later times, FXa association with TF increased in the PDI reactions which probably resulted from a combined effect of increased product concentration and reduced PDI binding to TF. The partial degradation of PDI in some samples suggested the possibility of PDI inactivation by prolonged exposure to proteases in the reaction. The substantial amount of FXa detected in the reactions without PDI indicates that the poor activity of soluble TF may in part be caused by slow product release. Taken together, these data support a mechanism by which PDI enhances ternary complex formation and facilitates the release of product FXa.

**PDI chaperone function is sufficient to enhance TF coagulant activity**- We next determined whether PDI-enhanced FXa generation was dependent on its oxidoreductase, isomerase or chaperone activity. Bacitracin inhibits all functions of PDI which is thought to be an effect resulting from occupancy of PDI’s hydrophobic pocket. Bacitracin completely blocked the effect of PDI on TF-VIIa mediated FX activation without influencing TF function in the absence of PDI (Fig. 4A). Vicinal thiols are typically required for reductase and isomerase activity of PDI. However, blockade of vicinal thiols with phenylarsenic oxide (PAO) did not reduce coagulant activity in the presence of PDI. In control experiments, we confirmed that PAO also was without effect when PDI was reduced immediately prior to the assay.

In order to address the oxidoreductase function of PDI in this system further, we incubated PDI for 30 minutes in reducing (4:1 GSH/GSSG) or oxidizing (1:4 GSH/GSSG) gradients of glutathione. There was no appreciable effect of redox gradients on PDI-mediated enhancement of TF-dependent FXa generation (Fig. 4B). In addition, we preincubated soluble or relipidated TF with 1 mM DTT in the presence or absence of PDI for 1 hour and determined FXa generation after dilution to 0.5 mM DTT. Reducing conditions diminished basal FXa generation for both, soluble and relipidated TF, but DTT did not inhibit the rate enhancing effect of PDI that is specific for soluble TF (Fig. 4C). PDI also maintained rate enhancing activity after reduction with 8 mM DTT and dilution to 1 mM (data not shown). Taken together, these data indicate that reductase and oxidase function are not required for the rate enhancing effect of PDI on TF.

To further address the role of free thiols in this reaction, we incubated each of the reactants separately with 20 mM methylmethanethiosulphonate (MMTS) which in our previous experiments prevented TF activation by strong oxidants on cells (8;9). After pretreatment with 20 mM MMTS, samples were diluted 100-fold into the FXa generation assay (Fig. 5A). Blocking free thiols of reactants individually did not inhibit the rate enhancing effect of PDI. We further reduced PDI with 1 mM DTT and reacted free thiols with 20 mM MMTS, followed by extensive dialysis to remove MMTS. Quantification of residual free thiols by labeling with N’-(3-maleimidyl propionyl) biocytin (MPB) showed that reduction with DTT led to a 2.5 fold increase in MPB labeling of PDI free thiols, whereas subsequent MMTS treatment abolished free thiols with >95% efficiency (Fig. 5B). MMTS-treated PDI was indistinguishable from unmodified PDI in enhancing TF-dependent FX activation (Fig. 5C). MMTS-modified PDI displayed significantly diminished reductase activity as determined by insulin denaturation assay and reductase activity of PDI was similarly inhibited by PAO or bacitracin (Fig 5D). Importantly, MMTS-modified PDI retained
chaperone activity similar to unmodified PDI towards denatured rhodanese (Fig. 5E). Because dithiol methane catalytic Cys residues in PDI rapidly convert to disulfides (56), we further covalently modified DTT-treated PDI with N-ethylmaleimide (NEM). MPB labeling confirmed that free thiols were efficiently blocked (Fig. 5F) and the rate enhancing effect of PDI was not diminished. These data provide additional evidence that oxidoreductase activity is not required for PDI’s effect on TF and suggest that PDI acts through its chaperone activity to enhance TF procoagulant activity.

**DISCUSSION**

We had identified PDI as a regulator of TF functions on cells, but how PDI contributes to activation of cryptic, non-coagulant forms of TF remains incompletely understood. Here, we show in a purified protein system that PDI can enhance coagulant activity of oxidized TF. Blockade of PDI free thiols, which are essential for PDI’s oxidoreductase and isomerase function, did not reduce PDI chaperone function. Nevertheless, MMTS- and NEM-modified PDI fully enhanced TF-dependent FXa generation, indicating that chaperone function of PDI is sufficient for enhancement of TF’s coagulant activity. The rate enhancing effect of PDI is dependent on specific TF residues that either directly or indirectly support the interaction with PDI. Unlike detergent activation, PDI not only facilitates substrate turnover, but also enhances affinity for VIIa. High affinity for VIIa and efficient FX turnover are hallmarks of procoagulant TF. Thus, these data strongly implicate PDI as a physiologically relevant regulator for the conversion of cryptic to coagulant TF.

**PDI enhances microparticle TF procoagulant activity** - PDI specifically enhanced the activity of cryptic TF. TF in microvesicles has low activity and TF in such a membrane environment may represent a likely target for PDI. To test this hypothesis, we generated microparticles under serum free conditions from human umbilical vein endothelial cells (HUVECs) in which TF was overexpressed by adenoviral transduction. Supernatants were cleared from cellular debris and TF shedding into the supernatant was confirmed by immunoprecipitation of TF by anti-TF antibody coupled to paramagnetic beads (Fig. 6A). Western blotting with an antibody against the TF cytoplasmic domain showed that full length TF is released into the supernatant. Detection of flotillin specifically in the TF immunoprecipitate is consistent with a previous study (16) that suggested a shedding mechanism of TF from raft domains.

Supernatants were used for FXa generation assay in the presence or absence of PDI. PDI dose-dependently increased FXa generation in microvesicle-containing culture supernatant (Fig. 6B). Depletion of TF from the supernatant by monoclonal antibody beads prevented FXa generation both in the absence and presence of PDI (Fig. 6C), demonstrating that shed TF is the primary target for PDI. Consistent with the data using purified soluble TF, blockade of free thiols by MMTS or of vicinal thiols by PAO did not reduce the enhancing effect of PDI on TF coagulant activity (Fig. 6D). These data further support our overall conclusion that PDI specifically regulates the activity of cryptic TF.
procoagulant PS exposure (9;18), indicating that PDI dynamically inactivates a proportion of TF through reductive modification. Consistent with this notion, blockade of free thiols with MMTS can prevent the activation of non-coagulant, PDI-associated pools of TF when the redox environment of the cell surface is changed by strong oxidants (8;9).

Using the immortalized keratinocyte HaCaT, we found that incubation with GSH did not reduce TF coagulant activity (8), indicating that PDI does not act simply as a reductase for TF on the cell surface. In the presented purified system, this concept is further supported by the finding that neither DTT nor changes in redox gradients resulted in inactivation of TF by PDI. However, TF activity on cells was diminished by incubation with GSH in the presence of the nitric oxide donor SNP, suggesting a role for S-nitrosylation to regulate TF activity. The experimental system presented here should be applicable to further studies that aim to elucidate the NO-dependent reaction by which PDI inactivates TF.

Macromolecular substrates make extended contacts with the TF-VIIa complex and conversion to product has little impact on these contact sites (21). Our data show that PDI reduces FXa that coprecipitates with TF, suggesting a mechanism of PDI action that involves facilitated dissociation of the product complex. This model is consistent with the finding that PDI has little effect towards TF on procoagulant membranes where product release is facilitated by lateral diffusion of lipids (13;26). PDI regulation of TF activity is therefore of significance only in cellular microdomains with low PS content, such as rafts, or on relative quiescent and non-apoptotic cells (11;13-15;17). In addition TF can be shed from raft domains in a poorly coagulant form that becomes active in the presence of stimulated platelets (16). Upon activation of platelets, up to 20% of total platelet PDI is locally secreted (59) and upregulation of TF activity in association with activated platelets may depend on secreted PDI.

Our data suggest a mechanism that can activate cryptic pools of TF on shed microparticles. Platelets also express PDI on the cell surface (27) which emphasizes that the activation of TF may begin prior to the exposure of procoagulant phospholipids during platelet activation. NO regulates redox pathways during platelet activation (60). The finding that PDI may inactivate TF in the presence of NO donors under reducing conditions (8) indicates additional layers of regulatory control. These may become important for the extent of TF-driven thrombosis, depending on whether platelets are activated on NO-producing endothelial cells versus exposed vessel wall matrix components. Taken together, our study provides new evidence that PDI not only switches the specificity of TF functions on cells, but can serve as a modulator in trans to activate cryptic pools of circulating TF of relevance for thrombosis and hemostasis.

REFERENCES


FOOTNOTES

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Abbreviations used: DTT: Dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; HUVEC, human umbilical vein endothelial cell; MPbA, N-(3-maleimidylpropionyl)biocytin; MMTS, methyl-methanethiosulfonate; NEM, N-ethylmaleimide; PAO, phenylarsine oxide; PDI, protein disulfide isomerase; PC, phosphatidylcholine; OG, octyl glucopyranoside; PS, phosphatidylserine; SNP, sodium nitroprusside; TF, tissue factor.

FIGURE LEGENDS

Fig. 1: PDI enhances TF procoagulant activity. A, Coomassie blue-stained non-reducing SDS-gels of wild-type, TF C186A and TF C209A soluble TF 1-218. Proteins were expressed in E. coli, refolded and monomeric soluble TF was obtained by gel filtration chromatography. B, Reactivity of antibodies with purified wild-type and TF C186A and TF C209A mutant soluble TF 1-218 loaded at the indicated amounts. Blotting with a polyclonal antibody to TF 1-218 confirms equal loading and blotting with anti-glutathione antibody (αGSH) shows that TF C186A, but not TF C209A is prone to glutathionation during the refolding. Anti-TF 5G9, but not 10H10, has poor reactivity after reduction of the allosteric disulfide. C, Labeling of free thiols in soluble TF with PEG-mal. In the top panel, 200 nM soluble TF was incubated with 10 mM DTT or 150 nM PDI for 1 hour prior to labeling with 5 mM PEG-mal. In the lower panel, 200 nM soluble TF was labeled with 5 mM PEG-mal at 37°C in the absence or presence of 150 nM untreated or DTT reduced PDI. VIIa was added at 200 nM where indicated. Western blots for TF are shown. D, PDI enhances coagulant activity of wild type TF, but not single thiol mutants. Stoichiometric amounts of VIIa (25 nM) and of soluble wt TF, TF C186A or TF C209A, as well as a combination of TF C186A or TF C209A were incubated with or without 25 nM of PDI and 1 μM FX. FXa generation after 30 min is shown. E, PDI-enhanced FXa generation requires TF, VIIa and FX. FXa generation after 10 min is shown. F, PDI-enhanced TF coagulant activity is independent of TF glycosylation. PDI enhancement of FXa generation was analyzed with soluble TF (25 nM) expressed in E. coli, yeast or CHO cells with VIIa (25 nM), PDI (25 nM) and FX (1 μM). G, PDI enhances FXa generation independent of the allosteric activation mechanism of VIIa. 25 nM VIIa DVQA has similar FXa generation activity as wild-type VIIa in the presence of soluble TF, but PDI has no effect on FXa generation by the free mutant. Inset: In the presence of TF1-218, VIIa DVQA mediated FXa generation was enhanced by PDI. FXa generation after 3 min is shown.

Fig. 2: PDI targets cryptic TF. A, Addition of PC/PS vesicles overcomes PDI-mediated enhancement of TF procoagulant activity. Activation of 1 μM FX by 25 nM VIIa and TF1-218 with or without 25 nM PDI was characterized in dependence of increasing concentrations of PC/PS (80/20, w/w) vesicles. FXa generation after 2 min is shown. B, PDI does not enhance activity of relipidated, full length TF. FX (100 nM) activation by 0.1 nM TF reconstituted in 80% PC/20% PS vesicles and 10 nM VIIa was analyzed in the absence or presence of 25 nM PDI. C, PDI and activating detergent OG similarly enhance TF coagulant function. Activation of 1 μM FX by 25 nM TF1-218 and VIIa was analyzed without or with 25
nM PDI and/or 7.5 mM OG. D, PDI, but not OG increases affinity of TF for VIIa. Amidolytic activities of VIIa (2 nM) with sub-saturating concentrations (2 nM) or saturating concentrations (50 nM) of soluble TF were determined in the presence or absence of OG and/or PDI (50 nM). E, OG, but not PDI, induces activation of TFK149A/D150A. FXa generation by 25 nM VIIa and TFK149A/D150A or wild-type TF was analyzed in the presence of 25 nM PDI or 7.5 mM OG. WT and the TFK149A/D150A mutant, which shows inefficient folding, were expressed as carboxyl terminal Leu zipper fusion proteins for improved refolding. Note that the carboxyl-terminal fusion increases FX activation by ~ 5-fold relative to soluble TF1-218, as previously characterized (43).

Fig. 3: PDI associates with the ternary complex. A, Effect of saturating TF concentrations on FXa generation in solution. Activation of 1 μM FX by 25 nM VIIa was characterized at stoichiometric concentrations of TF with either enzyme or substrate using 25 or 1 μM wild-type TF1-218 or 1 μM VIIa binding-deficient soluble TFD44A (55) in the presence and absence of 25 nM wild-type TF1-218. B, PDI increases FX activation independent of the TF concentration. Activation of 1 μM FX by 25 nM VIIa with or without 25 nM PDI was determined in dependence of the TF1-218 concentration. FXa generation at 4 min is shown. C, PDI co-precipitates with a TF-FX complex. Xa generation by 25 nM VIIa and TF in the presence or absence of 25 nM PDI was stopped after 0, 5, 30, 60 and 90 min. Co-precipitation of FX/Xa and PDI with TF was assessed by Western-blotting.

Fig. 4: PDI enhanced TF activity is not dependent on oxidoreductase activity. A, Bacitracin, but not PAO, inhibits PDI-enhanced TF activity. Stoichiometric amounts of VIIa (25 nM) and soluble TF were incubated with or without 25 nM of PDI and 1 μM FX, in the presence or absence of 100 μM of the vicinal thiol blocker PAO or 3 mM of the PDI inhibitor bacitracin. B, Redox gradients do not affect PDI-mediated enhancement of TF-dependent FXa generation. PDI was preincubated 30 min in control buffer or redox gradients (6.4 mM GSH: 1.6 mM GSSG or 1.6 mM GSH: 6.4 mM GSSG) and then diluted to the indicated final concentration for a reaction with 25 nM VIIa and soluble TF with or without 25 nM PDI and 500 nM FX. FXa generation after 10 min is shown. C, Reduction does not abolished the enhancing effect of PDI on TF activity. Soluble (100 nM) or relipidated (0.5 nM) TF were incubated with 1 mM DTT in the presence or absence of PDI for 1 hour. VIIa (100 and 10 nM, respectively) and substrate FX (2 μM) were added and FXa generation was determined after 1 min.

Fig. 5: PDI enhances TF coagulant activity through chaperone function. A, PDI-enhanced FXa generation is not dependent on pre-existing free thiols in TF, VIIa, FX or PDI. Soluble TF, VIIa, PDI or FX were pretreated with 20 mM MMTS and diluted 100-fold into the final reaction. 25 nM of TF and VIIa in the absence of PDI (no PDI) or presence of PDI (control) were used to activate 1 μM FX. Reactions in the presence of PDI were also performed after pretreatment of each reactant. B, Free thiol detection in reduced and MMTS-modified PDI. PDI was reduced with 1 mM DTT and subsequently reacted with 20 mM MMTS. Free thiols were detected in non-treated, DTT- and MMTS-treated PDI by labeling with 100 μM MPB and Western-blotting with HRP-conjugated streptavidin (SA-HRP). Densitometric quantitation is shown, mean and standard deviation, n = 3. C, Effect of MMTS-modified PDI on TF-dependent FXa generation. Activation of 1 μM FX by 25 nM TF1-218 and 25 nM VIIa was analyzed in the absence or presence of 25 nM unmodified PDI, PDI reduced with 1 mM DTT, or MMTS-modified PDI. D, Oxidoreductase activity was determined by insulin turbidity assay in the presence of 800 nM MMTS-modified PDI or unmodified PDI in the absence or presence of 100 μM PAO or 3 mM bacitracin., E, MMTS-modified PDI retains chaperone activity. Chaperone activity of 1 μM PDI and MMTS-modified PDI were compared by rhodanese aggregation assay. F, Effect of blocking free thiols of PDI with NEM on TF-dependent FXa generation. Free thiols after NEM modification were quantified by densitometry of MPB labeling. Activation of 1 μM FX by 25 nM TF1-218 and VIIa was analyzed in the absence or presence of 25 nM unmodified PDI or NEM-modified PDI
Fig. 6: PDI enhanced TF coagulant activity on microparticles. A, Release of full length TF into the supernatant of HUVECs. Serum-free culture supernatant from TF transduced HUVEC was immunoprecipitated with immobilized 5G9 or isotype-matched control antibody (TIB115) and TF immunoprecipitates were analyzed by Western blotting with polyclonal anti-TF, anti-TF cytoplasmic domain antibody or anti-flotillin (FLT) antibody as a marker for raft domains. The arrow indicates the correct molecular weight of FLT (48 kD). B, PDI dose-dependently enhances TF activity on microparticles. Supernatant containing shed TF was incubated with 10 nM VIIa and 100 nM FX in the presence of increasing concentrations of PDI. FXa generation after 15 min is shown. C, PDI specifically targets TF on microvesicles. Microparticle-containing supernatants were depleted using immobilized 5G9 or control beads and analyzed for FXa generation in the presence of 100 nM PDI. D, PDI chaperone function is sufficient for microparticle TF activation. Supernatant containing TF was analyzed for FX (100 nM) activation by VIIa (10 nM) in the absence or presence of 25 nM MMTS-treated PDI or unmodified PDI with or without 100 μM PAO. FXa generation was determined after 15 min.
FIGURE 3

A

B

C

- PDI  + PDI
FIGURE 4

A

![Graph showing time vs. Xa generation (nM) for different conditions.]

B

![Bar graph showing Xa generation (nM) for different conditions.]

C

![Bar graph showing Xa generation (nM) for different conditions.]
FIGURE 6

A

B

C

D
Tissue factor coagulant function is enhanced by protein disulfide isomerase independent of oxido-reductase activity
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