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The intersection of protein disulfide isomerase and cancer associated thrombosis

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ABSTRACT

The mechanisms underlying the hypercoagulability of cancer are complex and include the upregulation coagulation factors or procoagulant proteins, shedding of microparticles, and direct activation of vascular cells. Protein disulfide isomerase (PDI) is a thiol isomerase secreted from activated platelets and endothelial cells and plays a critical role in both platelet aggregation and fibrin generation. A number of potential intravascular targets of PDI have been identified including cell surface receptors (e.g. β-integrins and glycoprotein Ib), receptor ligands (e.g. fibrinogen and von Willebrand factor), serine proteases (e.g. cathepsin G and kallekrein-14), and coagulation factors (e.g. factor XI and factor V). Recent clinical studies demonstrated that a small molecule inhibitor of PDI, isoquercetin, decreases platelet-dependent thrombin generation and PDI activity in plasma following oral administration. This review explores the mechanistic overlap between the molecular drivers of cancer associated thrombosis and the potential roles PDI plays in mediating thrombosis. These molecular insights provide rationale for clinical trials targeting PDI to prevent thrombosis in cancer patients.

1. Introduction

Cancer is associated with an increase in both venous and arterial thrombosis [1–4]. Proposed mechanisms underlying the hypercoagulability of cancer include modulation of coagulation factor activity, increased adhesion of platelets, and elaboration of prothrombotic proteins or microparticles. Protein disulfide isomerase (PDI) is a thiol isomerase secreted by platelets and endothelial cells and plays a critical role in thrombus formation in vivo [5–7]. A number of extracellular substrates of PDI have been identified [8–16]. The mechanisms underlying the regulatory role of PDI in thrombus formation appear to intersect with the dysregulated thrombotic pathways of the malignant state. Clinical studies are underway to determine whether targeting extracellular PDI will prevent thrombosis in advanced cancer populations.

1.1. PDI and thrombus formation

PDI is the most critical, and most extensively studied, thiol isomerase [17–26]. PDI is the head of the PDI superfamily, which currently includes 21 different thiol isomerases, all of which contain at least one thioredoxin-like domain [17–19,25,26]. PDI is a 57 kD protein, structured in an a-b-b'-x-a'-c confirmation, displayed in Fig. 1. PDI contains two active sites in the a and a' domains, and two protein-substrate binding sites in the b and b' domains [19,26]. The x linker region allows flexibility of the a' domain, motion which is critical for catalysis [27,28]. Both active site domains consist of the classical Cys-X-X-Cys motif, and the intervening sequences vary between the family members [17,18,20]. In PDI, both active sites have a Cys-Gly-His-Cys motif, which is common among several thiol isomerases expressed in humans [17].

PDI acts as a necessary protein folding catalyst, with classic chaperone activity toward nascent peptides [20,21,29]. The removal of PDI at the transcriptional or translational levels is lethal because of its importance in protein folding in the endoplasmic reticulum (ER) [21]. While PDI is crucial for protein folding, it also is capable of carrying out multiple enzymatic functions. PDI can also serve as a thiol oxidoreductase and isomerase, and is capable of further post-translational cysteine modifications such as S-nitrosylation or S-glutathionylation [20,22,29–31]. For oxidoreductase activity, the CXXC motif will cycle between a reduced state containing two free thiols (-SH), and an oxidized state in which both cysteines are linked via a disulfide bond (S-S) as shown in Fig. 2. During reduction, the N-terminal cysteine (N-Cys) acts as the nucleophile to begin reducing a disulfide bond on another protein [22,23]. This is possible because the local pK_a of the active site causes the N-Cys to be a stable thiolate anion (S-), instead of a true free

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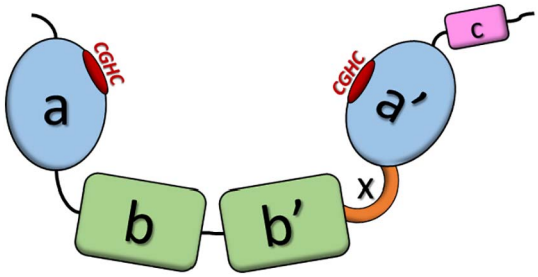

Fig. 1. Schematic Representation of Protein Disulfide Isomerase The a-b-b'-x-a'-c structure of PDI, with the CGHC active sites displayed in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

thiol (-SH). The disulfide-linked PDI-substrate complex shuffles its electrons among the four cysteine residues, until the C-terminal free thiol of PDI (C-Cys) forms a disulfide bond with its active site partner. This results in an effective disulfide bond transfer between substrate and PDI. The oxidation reaction takes place similarly but in reverse, effectively transferring a disulfide bond from PDI to a substrate protein which requires oxidation [22,23,29].

The activities of PDI are known to extend beyond the ER as PDI is required for thrombus formation in vivo. Following a laser-induced vascular injury in an mouse model of thrombosis, PDI will accumulate rapidly at the site of injury in a growing thrombus [7]. The enzymatic activity of PDI, and not just its localization, is essential, because blocking PDI activity with inhibitory antibody (RL90) or small molecule inhibitors like bacitracin, diminishes platelet accumulation and fibrin formation [7,15,32]. PDI is released from platelets, leukocytes, and endothelial cells upon activation and is capable of "binding-back" to the extracellular membrane through β_3 integrin complexes such as $\alpha_{IIb}\beta_3$ on platelets and $\alpha_V\beta_3$ on endothelial cells [14]. On the endothelium, PDI also associates with the β_1 integrin [33]. While the exact mechanism of PDI action is still controversial, multiple studies have shown interaction between a myriad of proteins known to be important for blood cell activation and thrombosis [8,10,11,34,35]. Several other thiol isomerase family members such as ERp5, ERp57, and ERp72 have also been shown to affect thrombus formation when inhibited [5,6]

To identify protein substrates of PDI or other thiol isomerases, a technique called kinetic substrate trapping has proven useful [8,11,24,25]. The majority of these studies utilized PDI variants which lack the second active site cysteine (CGHA) which forms stable covalent disulfide-linked intermediates between PDI and substrate proteins to enable isolation and identification via mass spectroscopy. One limitation of this approach is the requirement for substrates to undergo PDI-mediated disulfide reduction and not oxidation or rearrangement. Our group recently developed PDI variants capable of performing kinetic substrate trapping in both the oxidation and reduction directions [8]. These PDI variants with amino acid substitutions in the intervening sequences between the cysteines, alter the pK_a and spacial geometry of the active site to slow catalysis enough to trap the disulfide-linked intermediate complexes. This study identified several proteins as PDI substrates, from multiple classes of proteins involved with thrombosis, including coagulation factors, extracellular scaffolds, activating proteases, and cell surface receptors.

The binding substrates of extracellular PDI and how PDI regulates thrombus formation are not clearly defined. PDI binds to cell surface receptors such β-integrins, annexin V, and GP1β [8,35] as well as cell surface receptor protein ligands such as fibrinogen, fibrin, and collagen VI [8,36,37]. Previous studies identified von Willebrand's factor and thrombospondin-1 as substrates for thiol isomerases [10,12,38]. Cell surface receptors can be activated via binding, but also through cleavage by proteases; kallekrein-14 and cathepsin G are PDI substrates and are known to cleave and activate members of the PAR receptor family [8,39,40]. PDI can modulate phosphatidylserine (PS) exposure [16,41] and may regulate coagulation through disulfide regulation of tissue factor, activation of factor XI, maturation of platelet factor V, and attenuating factor XIII cross-linking activity toward fibrin [8,9,13,42,43]. More globally, PDI is believed to sense and regulate the redox state of the extracellular membrane, by interacting with small molecule effectors like glutathione and nitric oxide (NO), and modulating the redox state of other extracellular thiol isomerases [5,6,44]. Recently identified was glutaredoxin-1, a regulator of NO synthetase, and also the other thiol isomerases thioredoxin and ERp57 [8,45].

1.2. Hypercoagulability of cancer and potential interface with PDI

Certain coagulation factors and other procoagulant proteins are upregulated in expression and secretion in cancer. For example, many cancers are capable of constitutively expressing tissue factor (TF) at an elevated level [46,47]. Tissue factor initiates the extrinsic pathway of blood coagulation forming a complex with activated factor VII (VIIa), which then activates factor X. Tumor cells not only express TF on their cell surface but also generate circulating microparticles which express TF [47,48]. These tissue factor-bearing microparticles are associated with a heightened risk of venous thrombeombolism [49,50]. The regulation of tissue factor activation is complex and incompletely understood [42,51,41,52]. The reduction of an allosteric disulfide bond converting "cryptic" TF to the active form may serve as a key regulatory step [51]. This disulfide bond reduction may be mediated by PDI, which is also overexpressed and secreted by cancer cells [34,44], but this hypothesis has not been proven and remains controversial [51,53,54]. PDI can alter TF activity indirectly through PS exposure [16,41,55] or through the enzyme heparanase which increases the procoagulant activity of tissue factor by preventing the binding of tissue factor pathway inhibitor (TFPI) [52,56]. Heparanase requires disulfide bond oxidation for activation and was identified as a potential substrate for extracellular PDI [8,57]. Taken together, elevated levels of tissue factor and PDI in cancer may lead to hypercoagulability of cancer through increased TF activity and down-regulation of TFPI through heparanase activity.

Tumor cells also exert their procoagulant state by interacting with vascular cells such as endothelial cells and platelets through direct activation [46,58–60]. These platelet-tumor cell interactions are mediated through multiple receptors, most notably the P-selectin receptor and β_3 integrin [3,58,60,61]. Interaction with either of these receptors leads to platelet activation which can enhance the metastatic

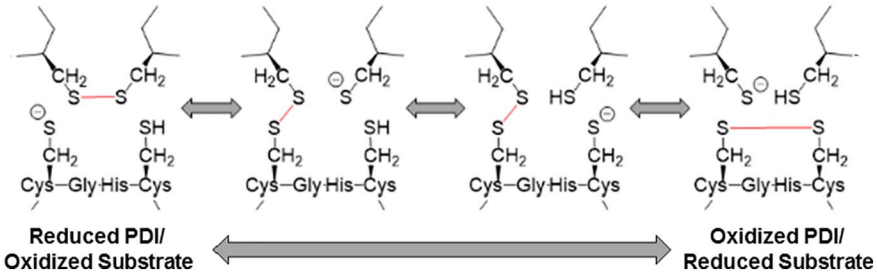

Fig. 2. Reduction/Oxidation Mechanism of Protein Disulfide Isomerase. Shown is a reaction scheme diagramming the PDI active site transitioning between a reduced (*left*) and oxidized (*right*) state performing disulfide bond reduction (*left* to *right*) or oxidation (*right* to *left*) on a protein substrate. PDI active site (*lower*) and protein substrate (*upper*) are shown.
Modified from [8]