PROTEIN DISULFIDE ISOMERASE CAPTURE DURING THROMBUS FORMATION IN VIVO DEPENDS ON THE PRESENCE OF \( \beta_3 \) INTEGRINS

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SUMMARY

Extracellular protein disulfide isomerase (PDI) is required for platelet thrombus formation and fibrin generation following arteriolar wall injury in live mice. PDI is secreted from platelets and endothelial cells upon cellular activation, but the mechanism of capture of secreted PDI within the injured vasculature is unknown. We establish that, like the endothelial β3 integrin αVβ3, the platelet integrin αIIbβ3 binds PDI and also binds to recombinant β3. Using intravital microscopy, we demonstrate that PDI accumulation at the site of laser-induced arteriolar wall injury is markedly reduced in β3-null (β3-/-) mice, and neither a platelet thrombus nor fibrin are generated at the vessel injury site. The absence of fibrin following vascular injury in β3-/- mice is due to the absence of extracellular PDI. To evaluate the relative importance of endothelial αvβ3 versus platelet αIIbβ3 or αVβ3, we performed reciprocal bone marrow transplants on wild type and β3-/- mice. PDI accumulation and platelet thrombus formation were markedly decreased after vessel injury in wild type mice transplanted with β3-/- bone marrow or in β3-/- mice transplanted with wild type bone marrow. These results indicate that both endothelial and platelet β3 integrins contribute to extracellular PDI binding at the vascular injury site.
INTRODUCTION

Protein disulfide isomerase (PDI), a prototypic thiol isomerase, catalyzes formation and isomerization of protein disulfide bonds.\(^1\) Despite the presence of an endoplasmic reticulum retention sequence,\(^2\) PDI is detected outside the cell following platelet and endothelium activation and secretion.\(^3-6\) We have shown that extracellular PDI accumulates on the injured luminal aspect of the arteriolar wall in a live mouse and is required for both fibrin formation and platelet thrombus formation.\(^7\) In vivo studies indicate the early appearance of endothelial cell-derived PDI, which is rapidly followed by platelet PDI during thrombus formation.\(^6,7\) Despite high shear rates in the arteriolar circulation, secreted PDI remains associated with the injured vessel wall and the developing thrombus.

The molecular basis for the role of PDI in thrombus formation remains unclear. In vivo studies have shown that PDI participates in both fibrin generation and platelet thrombus formation, regardless of whether experimental injury is induced via laser injury, ferric chloride treatment, Rose bengal oxidation or mechanical disruption and regardless of the vascular bed—cremaster, mesentery or carotid.\(^6-8\) Although its mechanism of action is unknown, PDI is required for tissue factor expression.\(^7\) Human umbilical vein endothelial cells in culture, when laser-activated while bathed in plasma, generate extracellular fibrin.\(^9\) The appearance of fibrin is inhibited by blocking antibodies to PDI and also by blocking antibodies to tissue factor.\(^6,9\) PDI also plays an important role in platelet aggregation and is thought to exert its effect on the integrin \(\alpha_{\text{IIb}}\beta_3.\(^{10}\) Several studies have demonstrated that specific disulfide bonds in the EGF domains and the \(\beta\)-tail domain of \(\beta_3\) are critical for the activation of \(\alpha_{\text{IIb}}\beta_3.\(^{11-13}\) The cleavage of these bonds might be mediated by PDI.\(^{10}\)

Integrins are heterodimeric transmembrane receptors consisting of non-covalently linked \(\alpha\)- and \(\beta\)-subunits.\(^{14}\) These receptors regulate cell-cell and cell-matrix protein interactions. The \(\beta_3\) subunit, one of eight mammalian \(\beta\) subunits, is associated with \(\alpha_{\text{IIb}}\) and \(\alpha\) subunits.
While αIIbβ3 integrin is exclusively expressed on megakaryocytes and platelets, αvβ3 integrin is widely expressed on various cell types including platelets and endothelial cells. The physical interaction between the β3 integrin and PDI remains controversial. Interaction of PDI with αvβ3 integrin on the surface of endothelial cells results in conversion of the integrin to the active state. In contrast, platelet β3 integrins were reported not to be physically associated with PDI during platelet activation.

To investigate whether β3 integrins play a role in PDI binding during thrombus formation, we examined PDI accumulation in mice lacking β3 in either the platelet or endothelial cell compartment, or both. The β3 integrin, in the form of αIIbβ3, is abundant on platelets, αVβ3 contributes a smaller component of the β3 integrin on the platelet surface. Endothelial cells express αVβ3 but not αIIbβ3. In our studies, PDI accumulation and fibrin generation were greatly reduced or eliminated in β3-/- mice following vessel wall injury. Analysis of thrombus formation in chimeric mice generated by reciprocal bone marrow transplantation between wild type and β3-/- mice revealed that both endothelial and platelet β3 integrins contribute to extracellular PDI interaction and accumulation.
METHODS

Antibodies and reagents: Rat monoclonal anti-mouse P-selectin antibody conjugated to phycoerythrin and rat anti-mouse GPIbβ antibody conjugated to DyLight 649 were from Emfret (Eibelstadt, Germany). Human thrombin, ADP, neomycin, and rabbit polyclonal anti-bovine PDI antibody were purchased from Sigma (St Louis MO). Recombinant human PDI was obtained from Prospec Bio (Ness-Ziona, Israel). Alternatively, recombinant PDI was expressed in E. coli, then purified by sequentially chromatography using a Ni-NTA column and Superdex 200 column via FPLC. Platelet-derived full-length αIIbβ3 was obtained from Abcam. Recombinant β3 was expressed in Drosophila S2 cells and purified to homogeneity with by sequentially chromatography using a Ni-NTA column and Superdex 200 column via FPLC. A rabbit anti-PDI antibody, DL11, against a peptide was provided by Sigma (St. Louis MO) free of albumin. Another rabbit anti-PDI antibody cross-reacted with human and mouse PDI and was immunopurified using bovine PDI covalently linked to CNBr-activated Sepharose, as previously described. A mouse monoclonal anti-fibrin-specific antibody was derived from a hybridoma cell line 59D8.23 Immunoaffinity-purified rabbit anti-PDI antibody, mouse anti-fibrin antibody, and nonimmune control IgGs were labeled with Alexa 488 or Alexa 647 according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The molar ratio of Alexa to protein was varied from 2.5 to 3.0, as determined spectrophotometrically. Cephalin was purchased from Diagnostica Stago (Parsippany NJ). A23187 and PPACK (D-Phe-Pro-Arg-chloromethylketone) were from EMD Chemicals (Gibbstown NJ).

Cells: Chinese hamster ovary cells expressing P-selectin were generated as described previously24 while those expressing αIIbβ3 and αVβ3 were a generous gift from Drs. H. Kato and S. Shattil (UC San Diego). These cells were grown in Dulbecco’s modified Eagle medium.
containing 200 mM glutamine, 10% FBS, 1% NEAA, and 1% penicillin/streptomycin solution at 37°C in a 5% CO₂ incubator.

Flow cytometry: For analysis of the interaction of PDI with the cell surface proteins, CHO cells expressing αIIbβ3, αVβ3 or P-selectin were resuspended in PBS containing 10 mM Mn²⁺, unless otherwise described. Cells were then incubated with Alexa 488-labeled PDI for 10 minutes and analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson). For analysis of surface platelet PDI expression, wild type and β3⁻/⁻ mouse platelets in HEPES-Tyrode buffer (10:1) containing 0.1% fatty acid-free bovine serum albumin (3 x 10⁸ platelets/ml) were incubated with or without 0.3 U/ml of human thrombin or both 10 μM ADP and 5 μM A23187 for 5 min at 37°C. PPACK (50 μM) was added to the platelet suspension after thrombin activation. Platelets were incubated with either nonimmune rabbit IgG or rabbit anti-PDI antibodies conjugated to Alexa 488 (30 μg/ml) for 30 min. Platelets were fixed with 2% paraformaldehyde for 10 min and analyzed by flow cytometry. Data were analyzed using CellQuest software. The expression of surface PDI was obtained from subtraction of the geometric mean intensity value of Alexa 488-labeled rabbit IgG from the geometric mean intensity value of Alexa 488-labeled rabbit anti-PDI. Data were expressed as mean ± S.D. The Student’s t test was used for comparison of wild type and β3⁻/⁻ mouse platelets. Differences were taken to be significant at P<0.05.

Surface plasmon resonance: Direct binding of PDI to αIIbβ3 and to β3 was measured by surface plasmon resonance using a Biacore T100. Purified full length αIIbβ3 (Abcam), isolated from platelets, or recombinant β3, were attached to the chip. Purified recombinant PDI at different indicated concentrations in 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.05% P20 with or without 2 mM MnCl₂ was infused over the chip. Binding data were analyzed using Biacore T100 evaluation software.
Mice: The β3 integrin-null mice in a mixed C57BL/6 and 129Sv background have been previously described. These mice and matched wild type control mice were obtained from Jackson Laboratory (Bar Harbor ME). The Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee approved all animal care and experimental procedures.

Intravital microscopy in a mouse model of laser-induced arteriolar thrombosis: Intravital videomicroscopy of the cremaster muscle microcirculation was performed as previously described. Mice were anesthetized with intraperitoneal Ketamine (125 mg/kg, Fort Dodge) and Xylazine (12.5 mg/kg, Lloyd Laboratories). A tracheal tube (PE90, Becton Dickinson) was inserted and the mouse was maintained at 37°C on a thermo-controlled rodent blanket. To maintain anesthesia, Nembutal (Ovation Pharmaceuticals) was administered through a cannulus (PE10) placed in the jugular vein. After the scrotum was incised, the testicle and surrounding cremaster muscle were exteriorized onto an intravital microscopy tray. The cremaster preparation was superfused with thermo-controlled (37°C) and aerated (95% N2, 5% CO2) bicarbonate-buffered saline throughout the experiment. Alexa- or DyLight-labeled antibodies were infused into mice prior to arteriolar wall injury.

Arteriolar wall injury was induced with a Micropoint Laser System (Photonics Instruments) at the vessel wall as described previously. One or two pulses with an equal setting of laser power were employed to induce vessel wall injury. Approximately 10 thrombi were generated in a single mouse, with new thrombi formed proximally of previous thrombi. Microvessel data were obtained using an Olympus AX microscope with a 60 x 0.9 NA water immersion objective. Digital images were captured with a Cooke Sensicam CCD camera in 640 x 480 format. Image analysis was performed using Slidebook v4.2 (Intelligent Imaging Innovations). Brightfield and widefield fluorescence images were captured at exposure times of 10 and 50 ms, respectively. Data captured digitally were collected for 4-5 min following vessel wall injury. Representative images were binarized to facilitate visualization but the median
curves include the full range of data. The kinetics of PDI accumulation, fibrin generation, and/or platelet thrombus formation were analyzed by determining median fluorescence values over time in approximately 19-28 thrombi.\textsuperscript{7}

**Preparation of washed mouse platelets:** Sodium citrate-treated mouse blood (0.8 ml) obtained from wild type or \(\beta_3\)-/- mice (6-8 weeks old) was centrifuged at 200 xg for 10 minutes. The plasma (150 \(\mu\)l) and buffy coat (150 \(\mu\)l) were transferred to a separate tube and re-centrifuged at 200 xg for 3 minutes. The platelet-rich plasma was collected and centrifuged at 600 xg for 3 minutes in the presence of 0.5 \(\mu\)M PGE1. The pellet was suspended in HEPES-Tyrode buffer containing 10% sodium citrate solution and 0.25 \(\mu\)M PGE1 and centrifuged at 600 xg for 3 minutes. Platelets were resuspended in HEPES-Tyrode buffer.

**Secretion of PDI from wild type and \(\beta_3\)-/- mouse platelets:** Mouse platelets in HEPES-Tyrode buffer (1 x 10\(^9\) platelets/100 \(\mu\)l) were incubated with human thrombin (0.3 U/ml) for 5 min at 37\(^\circ\). PPACK (50 \(\mu\)M) was added to quench thrombin activity followed by centrifugation at 600 xg for 5 min. The supernatant was collected and re-centrifuged at 1000 xg for 5 min to eliminate any residual platelets. The pellet was solubilized with 100 \(\mu\)l of SDS lysis buffer (Tris-HCl, pH 7.4, containing 150 mM NaCl, 4% SDS, 6 M urea, 2 mM EDTA and a protease inhibitor cocktail). The releasate and lysate of mouse platelets, 5 \(\mu\)l, were analyzed by SDS-PAGE under reduced conditions and immunoblotted with a rabbit polyclonal anti-PDI antibody.

**Coagulation assay:** Mouse plasma was obtained from sodium citrate-treated blood (0.8 ml) by centrifugation at 1000 xg for 10 min. The plasma was further centrifuged at 2000 xg for 5 min to eliminate any residual cells. Cephalin (120 \(\mu\)l) was added to 120 \(\mu\)l of the pre-warmed mouse plasma. After incubation for 5 min at 37\(^\circ\), CaCl\(_2\) was added to a final
concentration of 10 mM to initiate plasma clotting. The clotting time was measured using a platelet aggregometer (Chrono-log).

**Bone marrow transplantation and genotype analyses:** Bone marrow cells of wt and β3-/- male mice (6-8 weeks old) were harvested from both femurs of donor mice that were euthanized. Red blood cells in bone marrow cells were lysed with a lysis buffer (Sigma), and marrow cells were washed 3 times with ice cold RPMI 1640, 2.1 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 U/ml heparin, and 20 mM HEPES, pH 7.4. Recipient wt and β3-/- male mice (6-8 weeks old) were fed with sterile food and acidic water, pH 3.0, containing 0.1 mg/ml of neomycin 1 week prior to irradiation. The recipient mice underwent 2 cycles of radiation (650 rads; 6.5 Gy) given 3 hours apart before receiving 5 x 10^6 bone marrow nucleated cells in 0.2 ml of RPMI via a tail vein injection. Recipient mice were fed with sterile food and acidic water containing neomycin for 2 weeks. After that, the mice were provided with acidic water without the antibiotic. Seven to eight weeks after transplantation, genotype studies were performed on blood from the transplanted mice. DNA isolated from blood cells was genotyped by PCR using a common forward primer 1 (5'-CTTAGACACCTGCTACGGGC-3') and reverse primers 2 (5'-CACGAGACTAGTG AGACGTG-3'); and 3 (5'-CCTGCCTGAGGCTGAGTG-3'). PCR products were 538 base pairs for β3-/- mouse blood and 446 base pairs for wt mouse blood.25 PCR was performed for 35 cycles using the Advantage 2 PCR kit (BD Biosciences).

**Blood counts:** Sodium citrate-treated blood (0.8 ml) was collected from anesthetized wild type, β3-/-, and transplanted chimeric mice (4-5 months old). Complete blood counts were determined using an automated cell counter (Hemavet 850 FS, Drew Scientific).
RESULTS

We have previously observed the appearance and accumulation of endothelial cell-derived PDI followed by platelet-derived PDI in the injured arteriole during thrombus formation.\textsuperscript{6,7} We hypothesized that a mechanism must exist for the injured vasculature to bind the secreted PDI and prevent its removal from the injury site by blood flow. Given that β3 integrins may bind to PDI in vitro,\textsuperscript{16} and are expressed on both endothelial cells and platelets, we reexamined whether β3 integrins bind to PDI on the cell surface.

PDI interacts with activated α\textsuperscript{IIb}β\textsuperscript{3} and α\textsuperscript{V}β\textsuperscript{3}. To demonstrate the interaction of PDI and β3 occurs on the cell surface of platelets and endothelial cells, we examined the interaction of purified Alexa-488 hydrazide-labeled human PDI with CHO cells that expressed the major platelet β3 integrin, α\textsuperscript{IIb}β\textsuperscript{3}, or the endothelial β3 integrin, α\textsuperscript{V}β\textsuperscript{3}. Recombinant human PDI conjugated to Alexa 488 hydrazide retained full enzymatic activity, as monitored in the insulin reductase assay. The labeled PDI was added to CHO cells expressing recombinant α\textsuperscript{IIb}β\textsuperscript{3}, α\textsuperscript{V}β\textsuperscript{3} or P-selectin. Cells were incubated with Mn\textsuperscript{2+} to activate the integrins. In the presence of Mn\textsuperscript{2+}, PDI bound to the cells expressing either α\textsuperscript{IIb}β\textsuperscript{3} and α\textsuperscript{V}β\textsuperscript{3} integrins whereas no binding was observed to CHO cells expressing P-selectin (Figure 1A). This interaction is enhanced by the presence of Mn\textsuperscript{2+}, although some binding of PDI to α\textsuperscript{IIb}β\textsuperscript{3} and α\textsuperscript{V}β\textsuperscript{3} was observed in its absence. This is likely due to ambient metal contamination since the addition of EDTA completely inhibited PDI binding to both α\textsuperscript{IIb}β\textsuperscript{3} and α\textsuperscript{V}β\textsuperscript{3} (Figure 1B).

The direct interaction of purified α\textsuperscript{IIb}β\textsuperscript{3} with purified PDI was further studied by surface plasmon resonance. In the presence of Mn\textsuperscript{2+}, a Kd of 0.67 μM was measured in one experiment and 1.1 μM in a separate experiment (Figure 2A). These results are within experimental error of this methodology.
Similar results were obtained for the interaction of purified recombinant β3 with purified PDI by surface plasmon resonance. In the presence of Mn$^{2+}$, a Kd of 1.2 μM was measured in one experiment and 2.4 μM in a separate experiment (Figure 2B). These results are consistent with the primary importance of β3 in this interaction with PDI.

Swiatkowski et al$^{16}$ have described the interaction of PDI and αvβ3 also using surface plasmon resonance. The apparent Kd for an experiment of six minutes, similar to our conditions and performed in the presence of Mn$^{2+}$, revealed a value of 0.017 μM.

**PDI activity is not required for PDI-β3 integrin interaction.** To determine whether PDI enzymatic function is required for PDI-β3 interaction, Alexa-488 hydrazide-labeled human PDI was incubated with the blocking antibody RL90, then exposed to CHO cells expressing α$\text{IIb}$$\beta_3$ or αvβ3 in the presence of Mn$^{2+}$. Antibody concentrations of up to 10-fold molar excess over integrin did not interfere with the binding of PDI to β3 integrins (Figure S1). We labeled recombinant PDI with Alexa 488-maleimide to covalently modify free sulhydryl groups in the active site. Alexa 488-maleimide-labeled PDI bound to α$\text{IIIb}$$\beta_3$ and αvβ3 expressed on CHO cells (Figure S2) in a manner parallel to Alexa 488-hydrazide-labeled PDI. Thus, integrin activation, but not PDI activity, is required for the interaction of PDI and β3 integrins.

**Characterization of laser-induced thrombus formation in β3-/- mice.** β3-/- mice are resistant to FeCl$_3$-induced carotid arterial thrombosis.$^{25,26}$ To confirm that β3-/- mice are protected from platelet thrombus formation at the site of laser-induced arteriolar wall injury used in these studies, intravital microscopy was employed in wild type and β3-/- mice to monitor platelet accumulation. Platelet thrombi were monitored using rat anti-mouse GPIbβ antibody conjugated to DyLight 649 (red). The expression levels of GPIbβ on the surfaces of wild type and β3-/- mouse platelets were equivalent, as determined by flow cytometry (Figure S3). As previously observed,$^{27}$ β3-/- mice did not form a platelet thrombus at the site of vessel wall injury.
(Figure 3A and 3B). In wild type mice, initial platelet accumulation was observed at 15-30 sec and the maximum thrombus was detected at 90-100 sec after vascular injury. Any variability in comparison of these experiments with our earlier experiments can be ascribed to: (1) operator variation, where the time from laser injury to the recording of video may vary; (2) the use of anti-platelet antibodies with different fluorescence intensities; we have switched to a CD42 antibody in the current study after using an Fab of a CD41 antibody previously; these antibodies use different fluorochromes and have different fluorescent intensities; (3) antibody dosage. These differences are not significant, and the temporal pattern of the appearance of PDI, then platelets, then fibrin is routinely observed in all of our experiments with wild type mice. These results on β3-/- mice confirm that platelet β3 integrins are required for platelet thrombus formation at the site of arteriolar wall injury.

**Mouse β3-/- platelets and wild type platelets contain equivalent amounts of PDI and secrete PDI.** We investigated whether β3 deficiency influences platelet storage or secretion of PDI. The releasate and lysate of thrombin-activated wild type and β3-/- mouse platelets were subjected to electrophoresis and analyzed for PDI by immunoblotting using rabbit polyclonal anti-PDI antibodies. Equivalent amounts of PDI were detected in the lysate of wild type and β3-/- mouse platelets (Figure 4). Further, β3-/- mouse platelets released similar amounts of PDI compared to wild type platelets in response to thrombin (Figure 4).

**β3-/- plasma supports normal fibrin generation.** Since we wished to assess fibrin formation after laser-induced injury in β3-/- mice we determined that there was no unanticipated defect in fibrin generation in plasma from β3-/- mice using a modified activated partial thromboplastin time. Wild type and β3-/- mouse plasma were incubated in the presence of cephalin and Ca^{2+}. There was no significant difference in the time to clot formation between wild
type (45.6 ± 8.3 sec) and β3-/- (38.1 ± 4.2 sec) mouse plasma. Data represent mean ± SD (n=4). The P value was 0.15 versus the wild type control (Student t-test).

**PDI accumulation is greatly reduced at the site of vessel injury in β3-/- mice.** To investigate whether β3 integrins are important for PDI capture following laser-induced vessel wall injury, we compared PDI accumulation in wild type and β3-/- mice following vascular injury using intravital microscopy. Since a platelet thrombus does not form in the β3-/- mice, the effect of the absence of αVβ3 integrin on accumulation of PDI secreted by endothelial cells can be monitored in these mice. A non-inhibitory rabbit anti-PDI antibody conjugated to Alexa 488 (green) and a monoclonal anti-fibrin specific antibody conjugated to Alexa 647 (red) were infused prior to vessel injury to label PDI and fibrin respectively. In contrast to wild type mice, β3-/- mice showed minimal accumulation of extracellular PDI at the site of arteriolar wall injury (Figures 5A and 5B). Failure to observe PDI accumulation at the site of laser injury indicates a role for β3 in binding PDI in vivo at a site of vascular injury. Fibrin generation was markedly decreased after vessel injury in β3-/- mice compared to that observed in wild type mice (Figures 5A and 5C). The absence of fibrin cannot be explained by the absence of a platelet surface to support thrombin generation as we have previously shown that normal levels of fibrin are generated following laser injury in Par4-/- mice lacking the platelet thrombin receptor and in wild type mice without platelet thrombi following treatment with eptifibatide. These results indicate that β3 integrins play a role in fibrin generation at the site of vessel injury that is unrelated to the presence of activated platelets and dependent on β3 integrin-supported PDI accumulation at the site of injury.

**Chimeric mice generated by reciprocal bone marrow transplantation in wild type and β3-/- mice.** We independently studied the roles of endothelial and platelet β3 integrins in extracellular PDI accumulation after vessel injury by performing bone marrow transplantation
with wild type and β3-/- mice. We generated the following chimeric mice by reciprocal bone marrow transplantation in wild type and β3-/- mice: wild type donor bone marrow into wild type recipient mice (wt/wt); β3-/- donor bone marrow into wild type recipient mice (β3-/-wt); β3-/- donor bone marrow into β3-/- recipient mice (β3-/β3-); wild type donor bone marrow into β3-/- recipient mice (wt/β3-). In these chimeric mice, wild type mice receiving donor bone marrow from β3-/- mice have platelets lacking β3 integrins whereas β3-/- mice receiving donor bone marrow from wild type mice have endothelial cells lacking β3 integrins.

**Characterization of mice following bone marrow transplantation.** To confirm transplantation of the donor mouse hematopoietic cells into the recipient mice, we performed PCR using chimeric mouse blood and blood from β3-/- and wild type mice. In representative genotyping experiments, PCR products in wt/β3- chimeras exhibited the 446 base pair fragment observed in wild type mouse blood; β3-/wt chimeras exhibited the 538 base pair fragment observed in β3-/- mouse blood (Figure 6A). These results indicate that wt/β3- chimeras are wild type for their hematopoietic cells whereas β3-/wt chimeras are positive for the β3-/- genotype for their hematopoietic cells.

Transplanted mice had normal numbers and distribution of blood cells (Figure 6B). Blood cell counts and leukocyte differential counts in representative chimeric mice were comparable with those in control mice.

To determine whether fibrin generation was significantly affected by transplantation, we performed modified partial thromboplastin times on the plasma isolated from chimeric mouse blood. There was no significant difference in clotting times between chimeric and control mice.

**PDI accumulation and function in chimeric mice.** To determine the contribution of endothelial and platelet β3 integrins in PDI accumulation after vessel injury, both Alexa 488-labeled anti-PDI antibody and DyLight 649-labeled anti-GPIbβ antibody were infused into
transplanted mice prior to vessel injury to detect and quantitate PDI and platelets respectively. The representative results of studies of PDI accumulation and platelet thrombus formation are shown in Figure 7. Quantitative results of the median of multiple thrombi in each group are presented in Figure 8.

As in wild type mice (Figure 5), PDI rapidly accumulated on the vessel wall and within the developing platelet thrombus in the wt/wt control mice. A typical platelet thrombus formed at the site of vessel injury site in wt/wt mice (Figures 7 and 8A). Similarly, as in the β3−/− mice (Figures 3 & 5), PDI and platelets did not accumulate on the vessel wall in the β3−/β3− control mice. In contrast, PDI accumulation and platelet thrombus formation were significantly reduced at the vessel injury site in wt/β3− mice compared to wt/wt mice (Figure 8), indicating that endothelial αvβ3 integrin plays a role in both PDI and platelet accumulation at the vessel injury site. In β3−/wt mice, a minimal platelet thrombus formed at the injury site in the absence of β3 integrins on platelets. Significantly reduced PDI accumulation was also observed at the site of vessel injury (Figure 8B). These results suggest that both endothelial αvβ3 integrin and platelet β3 integrins are important for initial and sustained PDI accumulation as well as platelet thrombus development after vessel injury. Furthermore, these results indicate that in addition to platelet β3 integrins, the endothelial αvβ3 integrin is also important for platelet thrombus formation at the site of laser-induced vessel injury.

**DISCUSSION**

PDI is secreted first from the endothelium and then from bound platelets during cell activation after vascular injury, and is among the initial steps in thrombus generation. Inhibition of extracellular PDI following injury greatly impairs platelet thrombus formation and fibrin generation, emphasizing the functional importance of PDI.7–9 One of the primary questions
raised by these observations is the mechanism by which secreted PDI remains associated with the disrupted endothelium and developing thrombus. The capture of PDI by the endothelium and platelet thrombus requires a critical interaction between extracellular PDI and a cell surface receptor. Given the distribution of β3 integrins on both platelets and endothelium as well as literature reports indicating the interaction of PDI with the αVβ3 integrin, we explored the role of β3 integrins in the capture of PDI secreted from the endothelium and from platelets following vascular injury.

Jordan et al observed the interaction of PDI with ERp5, another thiol isomerase, but reported that they observed no interaction between PDI and β3. In contrast, Swiatkowska described the interaction of endothelial αVβ3 with PDI. This controversy over the interaction of PDI with integrins containing β3 led us to reinvestigate this question. We confirm that PDI binds directly to αVβ3, as was originally demonstrated with endothelial cells treated with Mn⁺². PDI bound to CHO cells expressing αVβ3 but not cells expressing P-selectin. Moreover, we observed interaction between PDI and platelet αIIbβ3 by analysis of binding of PDI to CHO cells expressing αIIbβ3. Measurement of the binding constant, Kd, between purified PDI and purified full length αIIbβ3 revealed a value of about 1 μM. Given the similarity of the binding affinity of PDI for αIIbβ3 and β3, we suggest that the α subunit does not contribute significantly to the binding properties of PDI to αIIbβ3. The interaction of PDI with β3 integrins required stabilization of the active conformer of the integrin with Mn⁺², but was not inhibited by either an inhibitory antibody to PDI or derivatization of free sulhydryl groups on PDI. PDI lacks the Arg-Gly-Asp sequence found in many integrin ligands that is responsible for cell adhesion and cell matrix interaction.

β3 deficiency in mice has been shown to be associated with a greatly prolonged bleeding time, impaired clot retraction and nearly absent uptake of fibrinogen on β3-/-. 
Spontaneous hemorrhage in β3-/- mice reduced survival. Unlike wild type mice, β3-/- mice were protected from hemorrhagic lesions as a complication of the local Shwartzman reaction. Carotid artery thrombosis following ferric chloride treatment was significantly impaired in β3-/- mice compared to wild type and β3+/- heterozygotes. In contrast to wild type mice in which a platelet-rich thrombus occluded the lumen, β3-/- mice showed but a single layer of platelets but no occlusive thrombus. We now extend these original findings to demonstrate the absence of fibrin generation following laser-induced vascular injury in β3-/- mice due to the lack of accumulated PDI. This raises the question of whether the deficiency in fibrin generation contributes to the hemorrhagic phenotype of patients with Glanzmann's thrombasthenia, particularly homozygotes or compound heterozygotes. Although a role for platelets in fibrin generation was proposed on the basis of a defect in thrombasthenic platelets, more recently this defect has been possibly ascribed to a release defect associated with platelet polyphosphates. However, we suggest for consideration that the procoagulant defect ascribed to Glanzmann's thrombasthenia may be due to the absence of PDI during thrombus formation rather than the failure of polyphosphate secretion since we have previously demonstrated that PDI is required for fibrin generation. In the current work we demonstrate that fibrin generation is greatly impaired in β3-/- mice, not because of the failure to form a platelet thrombus or the inability to activate platelets, but rather due to the failure to accumulate extracellular PDI at the injury site due to the absence of its cellular receptor, β3 integrin.

Decreased extracellular PDI in β3-/- mice could be due to decreased stored PDI in β3-/- platelets or decreased secretion of PDI from β3-/- platelets. We demonstrate that PDI content and PDI secretion of β3-/- platelets appear similar to wild type platelets. Therefore, we suggest that the failure of secreted PDI to remain associated with the injured vasculature and developing
thrombus is due to the absence of a counterreceptor, specifically the β3 integrins, exposed in the lumen of the vasculature.

We have previously shown eptifibitide blocks αIIbβ3-mediated platelet binding to the injured vessel wall, thus precluding the delivery of extracellular PDI; in the β3-/wt chimera, we anticipate that there would be neither αIIbβ3-mediated platelet binding nor αVβ3-mediated platelet binding to the injured vessel wall, again precluding the delivery of extracellular PDI. We might expect that the results from these two experiments would be similar. However, significantly less PDI is observed in the β3-/wt chimera than the eptifibitide-treated wild type mouse. We can speculate between two possible explanations. First, platelets from the β3-/wt chimera will be lacking αIIbβ3 and αVβ3 whereas eptifibitide-treated wild type mice will be interfering only in αIIbβ3-mediated platelet binding. If αVβ3 contributes to platelet binding in any significant way, this interaction is preserved in the eptifibitide-treated wild type mouse and will allow delivery of PDI to the wild type endothelium. Alternatively, eptifibitide, a pharmacologic agent, may allow for the coating of a monolayer of platelets in an interaction that is not dependent upon αIIbβ3. These platelets may secrete PDI upon activation.

PDI plays a central role in the initiation of thrombus formation and is important for both platelet aggregation and fibrin generation. Whether it functions as an oxidoreductase or chaperone, and whether it acts directly on platelet receptors or tissue factor, remains unknown. It has been suggested that both αIIbβ3 and tissue factor may undergo changes in their oxidation state-- αIIbβ3 by reduction of several disulfide bonds and tissue factor by formation of a disulfide bond leading to protein activation. Regardless of this mechanism, the presence of PDI at the site of vascular injury is critical for thrombus formation, and the β3 integrins are required for capture of PDI at this site.
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Author Contributions

J.C. and D.K. designed and performed the experiments, analyzed the results, and wrote the manuscript; G.M., L.L and M.H. performed the experiments and analyzed the results; and B.F. and B.C.F. designed the experiments and wrote the manuscript.

Conflicts of Interest

None
FOOTNOTES

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REFERENCES


LEGENDS TO FIGURES

Figure 1. PDI interacts with β3 integrins on cell surfaces. A. Interaction of PDI with P-selectin, αIIbβ3 or αVβ3. Histograms show fluorescence intensity of Alexa-488 labeled recombinant PDI interacting with CHO cells expressing either P-selectin (red), αIIbβ3 (green) or αVβ3 (blue). CHO cells were pretreated for 10 min in PBS with or without Mn^{2+} and incubated with PDI labeled with Alexa-488. B. CHO cells expressing either αIIbβ3 (left panel) or αVβ3 (right panel) were incubated with Alexa 488-labeled PDI in the presence of Mn^{2+} (green) or EDTA (blue). The interaction of PDI with CHO cells expressing P-selectin (red) is shown as control.

Figure 2. PDI interaction with αIIbβ3 and β3 by surface plasmon resonance. Full-length αIIbβ3 (25 µg/ml) or recombinant β3 (25 µg/ml) in 10 mM acetate buffer, pH 5 or pH 4.5 respectively, were immobilized on the surface of a Biacore CM5 chip. Different concentrations of PDI (21 (magenta), 7 (gray), 2.33 (gold), 0.78 (turquoise), 0.26 (pink), 0.09 (blue), 0.03 (green), 0 (red) µM) were injected and the experiments were performed in duplicate with the running buffer of 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.05% P20, 2 mM MnCl_{2}. The dissociation constant, Kd, was calculated based on the Kon and Koff value. (A) PDI binds to αIIbβ3 with Kd of 0.67 µM. (B) PDI binds to β3 with the Kd of 1.2 µM.

Figure 3. Platelet thrombus formation following laser-induced arteriolar wall injury in β3-/- mice. A non-blocking rat anti-mouse glycoprotein lbβ antibody conjugated to DyLight 649 (0.2 µg/g body weight) was infused into a wt or β3-/- mouse 5 min prior to laser-induced arteriolar wall injury. Representative images associated with platelets (red) are shown.
over 180 seconds following vessel injury within the context of the brightfield microvascular histology. B. The median integrated platelet fluorescence ($F_{\text{platelets}}$) for 22-23 thrombi in 2 wt or 2 β3-/- mice infused with the anti-GPIbβ antibody conjugated to DyLight 649 is presented over 250 seconds after vessel injury. wt mice, black; β3-/- mice, gray.

**Figure 4. Comparison of PDI secretion from wild type and β3-/- mouse platelets.** A. Platelets from wild type and β3-/- mice (1 x 10⁹ platelets/100 μl) were incubated with thrombin for 5 min, and the supernatant and pellet were separated. The pellet was solubilized with 0.1 ml of SDS lysis buffer. Washed human platelets (1 x 10⁹ platelets/ml) were lysed with 0.1 ml of SDS lysis buffer. Washed human platelets (10 μl), the platelet releasates (S; 5 μl) and platelet lysates (P; 5 μl) of wt and β3-/- mouse platelets, 5 μl, were electrophoresed in an SDS-PAGE gel and immunoblotted with rabbit polyclonal anti-PDI antibodies or non-immune rabbit IgG.

**Figure 5. PDI accumulation and platelet thrombus formation in β3-/- mice.** Non-inhibitory rabbit anti-PDI antibody conjugated to Alexa 488 (0.5 μg/g body weight) and anti-fibrin-specific antibody conjugated to Alexa 647 (0.3 μg/g body weight) were infused into a wild type (wt) or β3-/- mouse 5 min prior to laser-induced arteriolar wall injury. A. Representative images of the fluorescence signals associated with PDI (green) and fibrin (red) are shown over 180 seconds following vessel injury within the context of the brightfield microvascular histology. The median integrated fluorescence associated with PDI ($F_{\text{PDI}}$) fluorescence and fibrin ($F_{\text{fibrin}}$) fluorescence after infusion of anti-PDI antibodies (B) and anti-fibrin antibodies (C) in 3 wt (n=28 thrombi) and 3 β3-/- mice (n=25 thrombi) is presented over 250 seconds after vessel wall injury. wt mice, black; β3-/- mice, gray.
Figure 6. Analysis of chimeric and homozygous mouse blood. A. PCR was performed using whole blood of β3-/−, wt and wt/β3-/− and β3-/−/wt chimeric mice. PCR products were obtained as 538 base pairs for β3-/wt and 446 base pairs for wt/ 3-/−. B. Blood cell counts of mice following bone marrow transplant. Complete blood counts were obtained, including red cells (RBC), platelets (PLT), white blood cells (WBC), neutrophils (NE), lymphocytes (LY) and monocytes (MO). Data represent mean ± S.D. of 4-5 independent experiments. **P<0.01 as compared with wt mouse platelets by Student t-test.

Figure 7. PDI and platelet accumulation in chimeric mice following vascular injury. Chimeric mice were generated by reciprocal bone marrow transplantation between wildtype (wt) and β3-/− mice. Non-inhibitory rabbit anti-PDI antibody (0.5 μg/g body weight) conjugated to Alexa 488 was infused into mice with a non-blocking rat anti-mouse GPIIbβ antibody conjugated to DyLight 649 5 minutes prior to laser-induced arteriolar wall injury. A. Representative images associated with platelets (red) and PDI (green) are shown over 180 seconds following vessel injury within the context of the brightfield microvascular histology.

Figure 8. Quantitation of fluorescence associated with PDI and platelets in four transplanted mouse cohorts. The median integrated platelet (A) and PDI (B) fluorescence after infusion of anti-GPIIb antibody and anti-PDI antibody in each of the transplanted mice is presented over 240 seconds after vessel wall injury. 1; wt/wt wt/wt (n=25 thrombi), 2: wt/β3- (n=28 thrombi), 3, β3-/wt (n=26 thrombi); 4, β3-/β3- (n=26 thrombi). (donor/recipient).
FIGURE 1

A. 

- $\text{Mn}^{2+}$  
+ $\text{Mn}^{2+}$

B. 

$\alpha_{\text{IIb}}\beta_3$  
$\alpha_{\text{V}}\beta_3$
Figure 2
Figure 3

A

wt

β3-/-

B

F platelets

0 50 100 150 200 250

Seconds

wt

β3-/-
Figure 4

For personal use only.
Figure 5

A

wt | β3-/-
---|---

B

![Graph showing F PDI over time for wt and β3-/-]  
**wt** | **β3-/-**
---|---

C

![Graph showing F fibrin over time for wt and β3-/-]  
**wt** | **β3-/-**
---|---

**Figure**

A

B

C
**Figure 6**

A

![Figure 6 Image]

B

<table>
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<tr>
<th>Mouse</th>
<th>RBC ($10^6/μl$)</th>
<th>PLT ($10^3/μl$)</th>
<th>WBC ($10^3/μl$)</th>
<th>NE ($10^3/μl$)</th>
<th>LY ($10^3/μl$)</th>
<th>MO ($10^3/μl$)</th>
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<td>wt</td>
<td>7.0 ± 0.5</td>
<td>622 ± 86</td>
<td>4.5 ± 1.7</td>
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<td>6.1 ± 1.3</td>
<td>595 ± 150</td>
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<td>β3-</td>
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<tr>
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</table>
Figure 7

wt/wt  wt/β3-  β3-/wt  β3-/β3-

0 s
15 s
30 s
60 s
90 s
120 s
180 s
Figure 8

A

B
Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of \( \beta_3 \) integrins

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