Loss of expression of neutrophil proteinase-3: a factor contributing to thrombotic risk in paroxysmal nocturnal hemoglobinuria

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ABSTRACT

Background
A deficiency of specific glycosylphosphatidyl inositol-anchored proteins in paroxysmal nocturnal hemoglobinuria may be responsible for most of the clinical features of this disease, but some functional consequences may be indirect. For example, the absence of certain glycosylphosphatidyl inositol-anchored proteins in paroxysmal nocturnal hemoglobinuria cells may influence expression of other membrane proteins. Membrane-bound proteinase 3 co-localizes with glycosylphosphatidyl inositol-linked neutrophil antigen 2a, which is absent in patients with paroxysmal nocturnal hemoglobinuria.

Design and Methods
We compared expression of proteinase 3 and neutrophil antigen 2a by flow cytometry and western blotting in normal and paroxysmal nocturnal hemoglobinuria cells and measured cytoplasmic and soluble levels of proteinase 3 by enzyme-linked immunosorbent assays in controls and patients with paroxysmal nocturnal hemoglobinuria. Finally, we studied the effects of proteinase 3 on platelet activation using an *in vitro* aggregometry assay and flow cytometry.

Results
We showed that membrane-bound proteinase 3 is deficient in patients' cells, but invariantly present in the cytoplasm regardless of disease phenotype. When we isolated lipid rafts from patients, both molecules were detected only in the rafts from normal cells, but not diseased ones. Membrane-bound proteinase 3 was associated with a decrease in plasma proteinase 3 levels, clone size and history of thrombosis. In addition, we found that treating platelets *ex vivo* with proteinase 3, but not other agonists, decreased the exposure of an epitope on protease activated receptor-1 needed for thrombin activation. Conversely, treatment of whole blood with serine protease inhibitor enhanced expression of this epitope on protease activated receptor-1 located C-terminal to the thrombin cleavage site on platelets.

Conclusions
We demonstrated that deficiency of glycosylphosphatidyl inositol-anchored proteins in paroxysmal nocturnal hemoglobinuria results in decreased membrane-bound and soluble proteinase 3 levels. This phenomenon may constitute another mechanism contributing to a prothrombotic propensity in patients with paroxysmal nocturnal hemoglobinuria.

Key words: PR3, paroxysmal nocturnal hemoglobinuria, thrombosis, CD177.

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The online version of this article has a Supplementary Appendix.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder characterized by hematopoietic stem cells that have a somatic mutation in the X-linked *PIG-A* gene[1,2] involved in the biosynthesis of the glycosylphosphatidylinositol (GPI)-anchor. As a consequence, all progeny derived from the mutant stem cell lack the entire class of GPI-anchored proteins (GPI-AP) on their surface.[3] Characterization of the function of GPI-AP has elucidated the pathophysiology of certain aspects of PNH. For example, absence of GPI-linked complement regulatory proteins CD59/CD55 explains intravascular hemolysis.[4-6] However, the relationship between a deficiency of GPI-AP and the inherent apoptotic resistance of PNH cells and the link between PNH and aplastic anemia remain unexplained. Thrombosis is the most frequent complication leading to death in PNH.[7,8] The size of the PNH clone and thereby the severity of hemolysis are related to the risk of thrombotic complications.[9] For example, in PNH patients with a granulocyte clone size of greater than 50%, the cumulative lifetime risk is 44%, compared to 6% in those with a clone size of less than 50%.[10]

While the pathogenesis of the thrombophilia in PNH has not been clarified, a number of potential mechanisms have been proposed, including episodic hemolysis with release of pro-coagulant microparticles,[11-13] complement-mediated platelet activation,[9,12,14,15] and defective fibrinolytic activity secondary to loss of leukocyte expression of the GPI-linked urokinase-type plasminogen activator receptor (uPAR).[16-20] However, none of these hypotheses alone adequately explains the marked degree of hemostatic activation that results in a strikingly higher incidence of thromboembolic complications in PNH.

CD177, also known as glycoprotein NB1, is a neutrophil-specific GPI-AP belonging to the *Ly-6* superfamily that also includes uPAR and CD59.[21] Glycoprotein NB1 surface expression is associated with membrane expression of proteinase 3 (PR3), a non-GPI-linked serine protease[22,23] that may regulate platelet activation through cleavage and inactivation of thrombin receptor.[24,25] CD177 was recently shown to function as a novel heterophilic counter-receptor for the endothelial junctional protein PECAM-1 (CD31), involved in neutrophil transmigration.[26]

As for membrane-bound PR3 (mPR3), the percentage of neutrophils with membrane-bound NB1 in healthy individuals ranges from 0% to 100% and is genetically predetermined.[21,27] Various NB1 and PR3 polymorphisms have been described[28-31] and defective splicing was proposed as a mechanism explaining the phenotype in NB1-null subjects.[26] However, abnormal differential expression of both molecules has been observed in several clinical conditions without a definitive link with any of these polymorphisms.[29,32-34]

In this study, we hypothesized that loss of NB1-dependent presentation of mPR3 may contribute to the thrombophilia of PNH. Consequently, we compared expression of mPR3 and NB1 in normal and PNH cells, measured cytoplasmic and soluble levels of PR3 in control and PNH patients and studied the effects of PR3 on platelet activation.

Design and Methods

Patients

Informed consent to collection of samples was obtained from patients and controls according to protocols approved by the Institutional Review Board of the Cleveland Clinic. The diagnosis of PNH was based on clinical and laboratory parameters using previously described criteria.[35-37] The history of thrombosis was obtained through retrospective chart review and the thrombus was detected by spiral computed tomography, magnetic resonance imaging or Doppler ultrasound, wherever appropriate. For co-expression analysis, 19 patients with various sizes of PNH clone were tested, whereas plasma specimens from 40 patients were tested by enzyme-linked immunosorbent assay (ELISA). The clinical features of the patients whose samples were tested by ELISA are summarized in *Online Supplementary Table S1*. We selected patients with a history of thrombosis and matched them to those with a comparable clone size without thromboembolic complications.

Reagents

PR3 was detected using clone H-60 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit antiserum to human PR3 (Elastin Products Company, Owensville MO, USA). NB-1 was detected using monoclonal antibodies MEM-166 (eBioscience Inc, San Diego, CA, USA) and N-17 (Santa Cruz Biotechnology, Inc.). All secondary antibodies for western blotting were from Cell Signaling Cell Technology, Inc. (Danvers, MA, USA). Purified human PR3 was purchased from Elastin Products Company; tumor necrosis factor-α (TNF-α) was obtained from R&D Systems, Inc. (Minneapolis, MN, USA), phenylmethanesulphonyl fluoride (PMSF) and phospholipase C from Sigma-Aldrich (St. Louis, MO, USA). Flow cytometry antibodies anti-CD16, anti-CD66b, anti-CD55, anti-CD59, anti-CD41, anti-CD62P, anti-thrombin receptor and all isotype controls were from Beckman Coulter Inc. (Miami, FL, USA). By flow cytometry, PR3 was detected using PR3G-2 (Santa Cruz Biotechnology, Inc) and NB-1 using clone MEM-166 from GeneTex, Inc. (San Antonio, TX, USA). For platelet activation studies, α-thrombin (Sigma-Aldrich), thrombin receptor activating peptide (TRAP) (AnaSpec, San Jose, CA, USA), and ADP (Bio/Data Corporation, Horsham, PA, USA) were used.

Isolation of neutrophils and platelets from controls and patients with paroxysmal nocturnal hemoglobinuria

Neutrophils were separated from peripheral blood by Ficoll-Hypaque density gradient sedimentation (Mediatech, Inc, Manassas, VA, USA) following hypotonic erythrocyte lysis. Cell viability was assessed by trypan blue exclusion and exceeded 97% (Vi-CELL XR, Beckman Coulter, Inc.). Platelet-rich plasma and washed platelets were prepared by centrifugation of citrated blood from healthy volunteers and PNH patients. Only freshly obtained blood samples were used.

Flow cytometry

Isolated neutrophils were immunophenotyped with a panel of monoclonal antibodies including anti-CD55, anti-CD59, anti-CD16, anti-CD66b, anti-NB-1, and anti-PR3 according to the manufacturer's instructions. Multiparametric flow cytometry was performed using a FC500 flow cytometer (Beckman Coulter) and at least 20,000 events were acquired for each sample. Intracellular antigens were detected with a Fix/Perm Intracellular kit (Caltag Laboratories/Invitrogen Carlsbad, CA, USA). If indicated, cells

were stimulated with TNF-α (10 ng/mL) or incubated with 1 unit of phosphatidylinositol-specific phospholipase C for 30 min at 37°C. For inhibition of PR3, fresh whole blood and platelet-rich plasma were treated with PMSF (1 mM for 30 min at 37°C). To evaluate membrane protein expression on resting and activated platelets, a panel of anti-CD41, anti-CD61, anti-CD55, anti-CD62-P, anti-thrombin receptor, and anti-PR3 was used. If indicated, cells were activated with thrombin (0.05-0.5 U/mL), TRAP (12.5 μM), or ADP (2 μM).

Platelet aggregation

Platelets were prepared by centrifugation (75xg for 20 min) of citrated blood from healthy volunteers. Washed platelets were obtained by centrifugation of the platelet-rich plasma in washing buffer containing prostaglandin-I_2. Platelets were finally resuspended in Hank's balanced salt solution with $CaCl_2$ and adjusted to $2x10^8$/mL. Platelet aggregation of washed platelets was measured at 37°C under continuous stirring in a Chrono-Log Lumi aggregometer (Chrono-log, Havertown, PA, USA). In some experiments, platelets were incubated for 15 min with PR3 (400 nM) before addition of thrombin (0.2 U/mL).

Western blotting

Cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% SDS, 0.5% sodium deoxycholate; 1% Igepal CA630; 0.5 mM EDTA; 0.1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 10 mM NaF, 0.5 mM Na_3VO_4). Protein content was determined using the Bradford method (BioRad, Hercules, CA, USA). Equal amounts of proteins were separated by NuPage Novex 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). Following separation, proteins were transferred to nitrocellulose membranes (Invitrogen) and blocked for 1 h at room temperature with 5% BLOTTO in Tris-Buffered Saline Tween-20 (TBST) buffer (Pierce, Rockford, IL, USA). Incubation with primary antibody was conducted overnight at 4°C, followed by exposure to secondary antibody for 1 h at room temperature. Enhanced chemiluminescence detection was then performed with Pierce Supersignal Chemiluminescence substrate (Pierce, Rockford, IL, USA).

Detergent-resistant membrane isolation and western blotting

Following separation of wild type (WT) and GPI-AP$^-$ cells using anti-CD55, DRM were isolated by sucrose density gradient ultracentrifugation.[38,39] Briefly, the detergent-resistant membranes (DRM) were separated from the detergent-soluble material by flotation during centrifugation. After centrifugation, 11 fractions were collected from the top of the tube and were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblotting. Fractions 8-11 corresponded to Brj58-soluble material, remaining in the lower part of the gradient, while lipid rafts were found to have floated into the upper fractions (1-7). The low buoyant density DRM fractions corresponding to lipid rafts isolated from PNH patients and healthy donors, and the non-DRM fractions were then subjected to immunoblot assays using antibodies to PR3, CD55, and CD177.

Enzyme-linked immunosorbent assays

Plasma PR3 levels were measured by an ELISA assay. Microtiter plates (Immulon 4HBX, Thermo Fisher Scientific Inc., Milford, MA, USA) were coated overnight with purified monoclonal PR3 antibody (1B10, 6 μg/mL, Abcam Inc., Cambridge, MA, USA). Following blocking, plasma samples diluted to 1/20 in sample buffer were added and the plates were incubated for 2 h. After washing, bound PR3 was detected by incubation for 2.5 h with affinity-purified rabbit anti-PR3 diluted to 1/300 in sample buffer followed by addition of alkaline phosphatase-conjugated swine anti-rabbit IgG (Sigma). The assay was developed using p-nitrophenyl phosphate tablets (p-NPP) (Sigma) and absorbances were read at 405 nm using an automated microtiter plate reader (Versamax, Molecular Devices, Sunnyvale, CA, USA). Automated washes were performed after each step using a Microplate Strip Washer (Elx50, BioTek, Winooski, VT, USA). Standard curves were generated with purified human PR3 (Elastin Company).

Results

Neutrophils from patients with paroxysmal nocturnal hemoglobinuria have decreased expression of membrane-bound PR3

We studied neutrophil expression of mPR3 and NB1 in 19 PNH patients and 5 healthy controls. Figure 1 shows representative flow cytometry histograms from a healthy control individual (A) and a PNH patient (B) with 67% PNH cells. In healthy individuals nearly all neutrophils expressed the GPI-AP marker NB1 and this corresponded with mPR3 expression. In patients with PNH, GPI-AP$^-$ cells (as determined by lack of CD16 or CD59 expression) did not express either NB1 or mPR3. As shown in Figure 2A, among all donors the overall percentages of mPR3$^+$ and NB1$^+$ cells were similar, regardless of the absolute proportion of mPR3$^+$ cells (r=0.986, n=24). Similarly, neutrophils from subjects with an NB1-null phenotype (3% of the Caucasian population),[21,40] also demonstrated deficiency of mPR3 (*data not shown*). Consistent with the requirement of surface NB1 for mPR3 expression, removal of GPI-AP from the plasma membrane by treatment with phosphatidylinositol-specific phospholipase C resulted in loss of mPR3 expression (Figure 2B). Conversely, treatment of the cells with TNF-α increased expression levels of both NB1 and mPR3 on GPI-AP$^+$ cells but not in NB1-null cells or GPI-AP$^-$ PNH cells (Figure 2C).

Cytoplasmic PR3 is normally expressed in patients with paroxysmal nocturnal hemoglobinuria

Since PNH subjects showed significantly fewer mPR3$^+$ cells than controls and since neutrophil PR3 is stored in intracellular granules,[41] we also assayed intracellular PR3 and NB1 by flow cytometry in PNH patients and controls. All neutrophils expressed cytoplasmic PR3 independently of GPI-AP membrane expression, but intracellular NB1 was only detected in GPI-AP$^+$ cells (Figure 3A). Thus, whereas mPR3 staining was observed only on GPI-AP neutrophils positive for NB1 there was no disparity in the intracellular pool of PR3 between the cell types. The percentages of cells positive for surface and cytoplasmic NB1 were similar since NB1 was not present in the cytoplasm of either cells from the NB1-null phenotype subjects or GPI-AP$^-$ cells derived from patients with PNH (Figure 3A). Similar results were obtained by immunoblotting (Figure 3B); cytoplasmic fractions of lysates prepared from neutrophils from either control or PNH patients revealed PR3 whereas mPR3 was seen only in GPI-AP$^+$ cells.

Membrane-bound PR3 and NB1 are expressed in lipid rafts

The notion that both PR3 and NB1 co-localize in the plasma membrane was further supported by the isolation

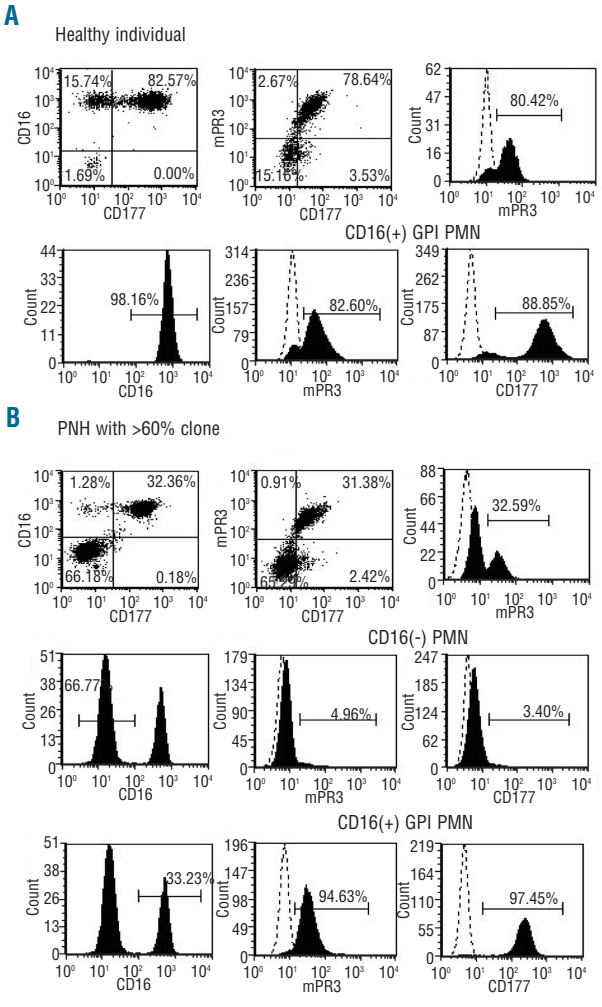

Figure 1. NB1 and PR3 surface expression in controls and patients with PNH. Freshly obtained neutrophils were stained using monoclonal antibodies directed against NB1 (CD177), PR3 and CD16. The panels represent flow dot blots/histograms (A) from a healthy individual and (B) from a PNH patient with a PNH clone size of 67% of granulocytes determined by expression of the GPI-AP CD16. In healthy subjects, CD16 is expressed on the majority of neutrophils; the NB1 surface staining is bimodal and coincides with mPR3 expression. In patients with PNH, CD16 GPI$^-$ cells expressing NB1 and mPR3 were not found. In contrast, co-expression of NB1 and mPR3 was found in GPI$^+$ cells of the same patient.

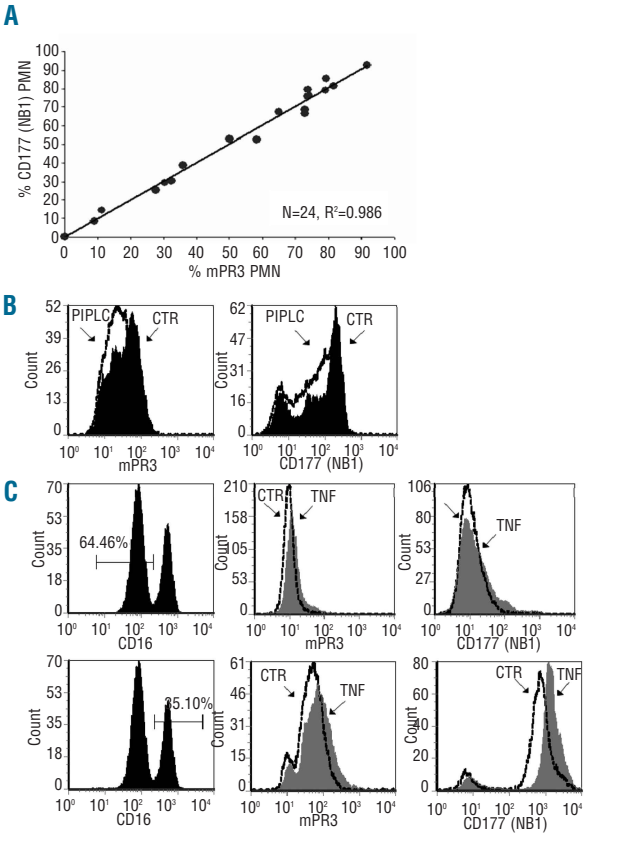

Figure 2. PR3 neutrophil membrane expression requires expression of GPI-linked NB1. (A) The percentage of mPR3-expressing cells is plotted as a function of the percentage of NB1 expressing cells. Percentages were nearly identical in all donors, independently of the proportion of mPR3$^+$ cells (r=0.986, n=24). (B) Loss of GPI-AP proteins after cleavage with phosphatidylinositol-specific phospholipase C (PI-PLC) (1U; 30 min at 37°C) results in decreased membrane expression of NB1 accompanied by a similar reduction of mPR3 expression. (C) Treatment of GPI-AP$^+$ neutrophils (as determined by CD16 expression) from a PNH patient with TNF-α induced up-regulation of mPR3 and surface NB1 whereas no such increase was detected when GPI-AP$^-$ cells were stimulated by TNF-α.

of DRM from PNH patients and controls. For these studies highly purified GPI-AP$^-$ and GPI-AP$^+$ cells derived from neutrophils of PNH patients and controls were used. Following sucrose density gradient separation by ultracentrifugation, low buoyant density DRM fractions corresponding to lipid rafts (enriched in fractions 3-4) and non-DRM enriched proteins (enriched in fractions 9-11) were compared by immunoblot analysis. All other fractions represented migration of buoyant rafts. CD55 was detected in soluble form (non-DRM enriched proteins) of GPI-AP$^-$ and GPI-AP$^+$ cell types, but only in GPI-AP$^+$ cells was it found in the DRM fraction (fractions 3-4; Figure 4A, top blots). Similarly, GPI-AP$^+$ cells also showed PR3 and NB1 expression in the DRM fractions (Figure 4A, middle and bottom blots), while these proteins were not detected in the DRM fractions from the GPI-AP$^-$ cells. Consistent with the flow cytometry and immunoblot data (Figure 3), intracellular PR3 was detected in both types of cells (middle blots in Figure 4A and 4B). These data suggest a physical interaction between NB1 and PR3 in the DRM of neutrophils.

Patients with paroxysmal nocturnal hemoglobinuria have decreased circulating levels of PR3

We next investigated whether deficient mPR3 expression on GPI-AP$^-$ cells is associated with elevated levels of soluble PR3 in plasma of patients with PNH. Plasma concentrations were measured by ELISA in healthy controls (n=49), subjects with unrelated hematologic disorders (n=18), and patients with PNH or PNH/aplastic anemia syndrome (n=40). Plasma PR3 was significantly decreased in the PNH group (mean±SD, 201±79 ng/mL) compared to in the other two groups; mean levels were 256±91 ng/mL in healthy blood donors and 311±87 ng/mL in patients with other hematologic disorders (Figure 5C; unpaired t-test, P=0.0038 and P<0.0001, respectively). Analysis of clinical parameters, including white blood cell, absolute neutrophil, and platelet counts, did not reveal significant

correlations with PR3 levels. When PNH patients were sub-grouped according to the percentage of GPI-AP-deficient granulocytes (data not available for 5 patients at the time of sampling), those with 60% or more PNH cells had significantly lower PR3 plasma levels (171±52 ng/mL; n=17; *P*=0.013) compared to those with smaller PNH clones (235±89 ng/mL; n=18). Overall, a negative correlation was seen with mPR3$^-$, GPI-AP$^-$ neutrophils (r=-0.42; *P*=0.0093; Figure 5A). Interestingly, seven of the eight patients with a history of thrombosis had a high percentage of PNH granulocytes and low NB1/mPR3 expression, but reduced levels of soluble PR3 (160±38 ng/mL; n=8). Those patients without a history of thrombosis had plasma PR3 levels of 213±84 ng/mL (n=31; *P*=0.078, Figure 5D). Detailed information on the patients and their thromboses, including the time elapsed from diagnosis to thrombosis, the site of thrombosis and treatments, is presented in *Online Supplementary Table S1*.

It is possible that the lower concentrations of plasma PR3 seen in PNH patients could be due to the general decrease in membrane PR3 expression. We, therefore, analyzed plasma PR3 levels in individuals with the NB1-null phenotype and in patients with unrelated hematologic disorders characterized by decreased mPR3 expression, including myelodysplastic syndrome and myeloproliferative neoplasms. Unlike what was seen in PNH patients, we did not find any correlation between the surface expression and soluble PR3 (r=-0.32, *P*=0.19). Similarly, there was no relationship between plasma PR3 and percentage of mPR3/NB1-expressing neutrophils in controls (r=-0.064, *P*=0.77) (Figure 5B).

PR3 inhibits activation of platelets by thrombin

Protease activated receptor-1 (PAR-1), the predominant human platelet thrombin receptor, has been shown to be a substrate for PR3.[24,25] We, therefore, hypothesized that thrombophilia in patients with PNH could be caused by an increased propensity for platelet activation due to impaired down-regulation of the activity of platelet PAR-1 by PR3. Since PR3 bound to the surface of neutrophils, like other membrane-bound proteases, is not readily inhibited by circulating physiological proteinase inhibitors,[42] we speculated that specific loss of mPR3 along with the modest decrease in plasma PR3 associated with PNH could contribute to the pro-thrombotic phenotype. We pretreated platelets with purified PR3 and used a monoclonal antibody against WEDE, an epitope located downstream (*i.e.* C-terminal) to the thrombin activation site, to detect receptor cleavage. Loss of expression of this epitope would be associated with loss of availability of the thrombin cleavage site on the receptor. As shown in Figure

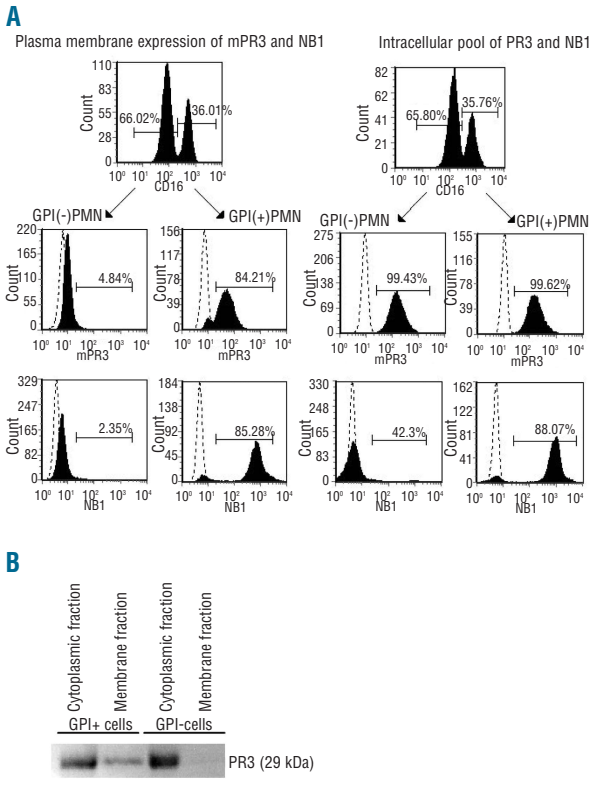

Figure 3. Cytoplasmic PR3 is expressed normally in patients with PNH (A) Double immunofluorescence staining of permeabilized (right group of histograms) and non-permeabilized (left group) neutrophils using antibodies to PR3 and NB1 along with GPI-linked surface molecule CD16. All neutrophils contain intracellular PR3, independent of the status of GPI-AP expression. In contrast, NB1 was only detected in GPI-AP-expressing cells. (B) Western blot analysis showing PR3 in the membrane fraction of GPI-AP$^+$ cells, but not GPI-AP$^-$ cells.

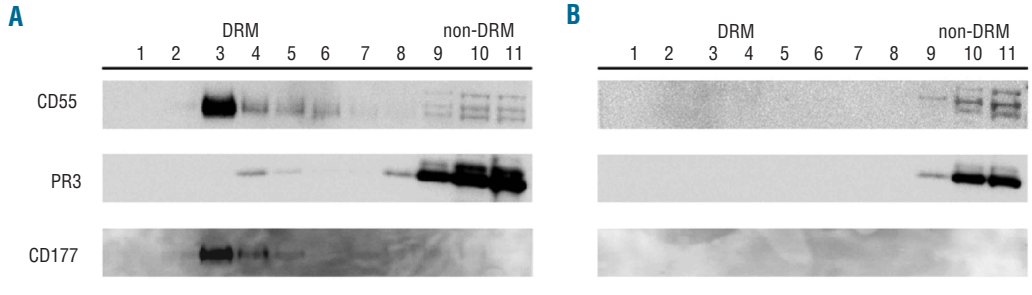

Figure 4. PR3 and NB1 localize in lipid rafts. Detergent-resistant membranes (DRM) were isolated from GPI-AP$^+$ (A) and GPI-AP$^-$ (B) neutrophils derived from a PNH patient or control and subjected to immunoblot analysis for CD55, PR3, and NB1. CD55 was seen in the DRM fractions 3 and 4 from GPI-AP$^+$ cells, but not from GPI-AP$^-$ cells and was also present in soluble form in the cellular proteome of both cell types (lane 1). Similarly, PR3 and NB1 were detected in the DRM fractions in GPI-AP$^+$ cells, but not in GPI-AP$^-$ cells (lanes 2-3). PR3 was detected in the non-DRM fractions of both cell types, while NB1 was not seen either in DRM (fractions 3 and 4) or in the cellular form (lanes 2-3). Fractions 1, 2, 3 and 4 contain DRM enriched proteins; fractions 9, 10 and 11 represent non-DRM enriched proteins. The remaining fractions (5, 6, 7 and 8) represent migration of buoyant rafts. Lipid rafts are mostly localized in fractions 3 and 4.

6A, treatment of platelets with PR3 led to an approximately 45% reduction of binding of the anti-WEDE antibody as measured by mean fluorescence intensity (MFI). Conversely, treatment of whole blood with PMSF (1mM for 30 min), resulted in a 25-30% increase in the expression of the WEDE epitope on platelets, evaluated as changes in MFI, compared to that of untreated specimens. Expression of the WEDE epitope was also examined among healthy controls, patients with PNH/aplastic anemia (neutrophil PNH clone ≤20%) and PNH patients (neutrophil PNH clone ≥80%). Among those patients with a larger PNH clone, we observed a slight increase in binding of the antibody against the WEDE epitope compared to the binding in those patients with a smaller PNH clone including patients with PNH/aplastic anemia overlap [MFI=14.5 (±3.95) *versus* MFI=12.15 (±2.36), respectively]. However, the trend was not maintained in comparison with healthy controls [MFI=17.37 (±2.69)]. PR3 treatment also significantly blocked thrombin-induced platelet activation, as measured by flow cytometric detection of surface P-selectin, a marker for granule secretion (Figure 6B shows a representative flow histogram). Studies involving seven different healthy platelet donors (Figure 6C) showed that pre-incubation with 400 nM PR3 for 5 min inhibited platelet activation by 35-75%. Purified PR3 by itself did not activate platelets (*data not shown*) and importantly did not influence platelet activation by agonists, such as ADP (Figure 6B), which do not work via PAR-1. PR3 also failed to impair platelet activation by TRAP (6.25-12.5 μM), as shown in Figure 6C, consistent with cleavage of the receptor at a site distal to the thrombin activation site, but still leaving intact the binding site for the activating peptide. To confirm the inhibitory effect of PR3 on platelet activation, we also showed that pre-incubation of washed platelets with PR3 inhibited platelet aggregation (Figure 6D) in an *in vitro* aggregometry assay. Four different donors showed a range of 10-58% inhibition in response to 0.2 units/mL thrombin.

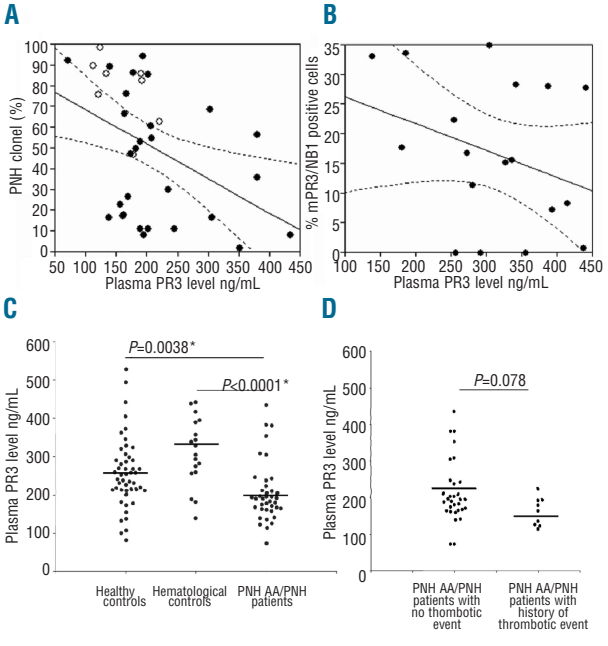

Figure 5. Circulating levels of PR3 are decreased in plasma from patients with PNH. Plasma PR3 levels were plotted as a function of PNH clone percentage (A) in patients with PNH and aplastic anemia (AA)/PNH syndrome. The percentage of GPI-AP$^-$ neutrophils was determined at the time of plasma sampling by flow cytometry. Solid circles represent patients without a history of thrombosis (n=27). Open circles represent patients with a history of thrombotic events (n=8). A negative correlation between PNH clone size and PR3 plasma level was observed, n=36, *P*=0.0093 (Spearman's non-parametric correlation coefficient was -0.42). (B) Plasma PR3 levels were plotted as a function of percentage of NB1/mPR3$^+$ neutrophils in individuals with the NB1-null phenotype and those with unrelated conditions characterized by decreased membrane-bound NB1/PR3 expression (≤mean±2 standard deviations of 50 healthy controls) (r=-0.32, n=18, *P*=0.19). The solid line represents the linear regression and broken lines show 95% confidence intervals. (C) The concentration of PR3 was measured by enzyme-linked immunosorbent assay in 49 healthy donors, 18 subjects with unrelated hematologic conditions, and 40 patients with PNH or AA/PNH syndrome. Decreased levels of PR3 were seen in PNH and AA/PNH patients (mean 201 ng/mL) compared to those in both control groups (mean 256 ng/mL for normal subjects and 311 ng/mL for those with unrelated hematologic disorders). (D) Plasma PR3 levels in patients grouped by history of thrombosis. Lower levels of PR3 were seen in those who had had thrombotic events (mean 160 ng/mL) compared to other patients (mean 213 ng/mL). Student's t test was used to determine statistical significance.

Discussion

Thrombotic complications are a major source of morbidity and mortality in PNH. The prevalence of thrombotic complications in PNH is high compared to that in other known inherited and acquired thrombophilias. The pathogenesis is likely to be multifactorial given the increased risk of both arterial and venous events. The degree of hemolysis is a significant contributor to hypercoagulability; the rate of thrombotic events is diminished in patients who are given the complement inhibitor eculizumab, which significantly reduces intravascular hemolysis.[35,43,44] In the absence of complement blockade, the degree of hemolysis is related to the size of the PNH clone. Consequently, in an indirect fashion, the degree of deficiency in certain GPI-AP may affect the propensity to thrombosis. Previous studies investigating thrombophilia in PNH implicated several possible mechanisms; however, none of the pathways described to date provide sufficient explanation for the extraordinarily high risk of thrombosis in these patients.[11-20] For example, abnormalities of the fibrinolytic cascade mediated through deficiency of the GPI-linked uPAR normally present on platelets and leukocytes have been described. Competitive binding of pro-uPA by an excess of soluble uPAR could contribute to a deficiency of fibrinolytic activity as could the loss of active uPA normally localized to thrombi by binding to uPAR on cells within a clot. A recent study showed that factors correlating with markedly elevated levels of soluble uPAR included the absolute number and percent of GPI-negative neutrophils, and absolute neutrophil count.[20] Other investigators highlighted enhanced platelet activation and elevated levels of circulating procoagulant microparticles derived from platelets and/or endothelial cells.[12-15] It has also been suggested that PNH platelets, which likely correlate with the size of PNH clone, may trigger a prothombotic cascade due to lack of certain GPI-deficient proteins. Similarly, based on the correlation between thrombosis and severity of hemolysis, many believe that either complement activation or products of hemolysis contribute to increased platelet activation and thrombin sensitivity of platelets. In addition, endothelial cell activation was suggested to contribute to the pathogenesis of thrombosis in PNH. Eculizumab, by blocking intravascular hemolysis, induced

a significant and sustained decrease in the activation of both the plasma hemostatic system and the vascular endothelium, therefore likely contributing to the protective effect of eculizumab on thrombosis in this setting.[45]

Our study demonstrates that GPI-AP deficiency affects membrane expression of the non-GPI-linked protein PR3 and suggests that mechanisms of various clinical sequelae of PNH may involve abnormalities in a wider spectrum of proteins. PR3 and the GPI-linked NB1 glycoprotein are co-localized on neutrophil plasma membranes and NB1 is necessary for the surface display of the complex.[22,23] We, therefore, hypothesized that NB1 deficiency in PNH would be associated with loss of membrane PR3 and this could contribute to the clinical phenotype in PNH. We found that PR3 was invariantly present in the neutrophil cytoplasm regardless of GPI phenotype, but was displayed on the surface membrane only in normal cells, suggesting that the loss of surface expression is not likely to be due to effects on PR3 translation or transcription. TNF-α, a known inducer of PR3 membrane up-regulation in normal neutrophils, does not have this effect in PNH cells, suggesting that expression of GPI-AP is required for the up-regulation. When we isolated lipid rafts from PNH and normal cells, we identified NB1 and PR3 only in the rafts derived from normal cells, as confirmed by the expression of CD55/59 antigens. In contrast to other GPI-linked proteins, NB1 was not detected intracellularly in PNH cells by flow cytometry or immunoblotting. It is possible that NB1 is degraded early and efficiently or processed in such a way that antigenic determinants are lost.

Two independent groups previously demonstrated co-localization of PR3 and CD177 in the neutrophil plasma membrane. While CD177 appears to be the exclusive binding partner of mPR3,[22,23] the mechanism of PR3 presentation is not clear. However, active PR3 can bind to the surface of CD177-transfected HEK293 cells, suggesting that N-terminal processing is important for binding of PR3 to CD177.[46] While CD177, as a receptor of mPR3, accounts for substantial membrane-expression of PR3, other lower capacity binding partners, such as CD11b/CD18 and FcγRIIIb, cannot be excluded. They may be involved in the pathophysiology of activation of neutrophils by anti-neutrophil cytoplasmic antibodies (ANCA) in ANCA-associated systemic vasculitis.[47,48] For instance, neutrophils from $CD177^-$ subjects can, after priming with TNF-α, express low levels of PR3 and are rendered susceptible to PR3-ANCA-induced neutrophil activation to a similar degree as $CD177^+$ cells.[47,48] In our study, priming with TNF-α did not cause up-regulation in mPR3 expression on plasma PNH neutrophils. Others suggested that the increased membrane expression of PR3 found in ANCA-associated systemic vasculitis is dependent on CD177 expression and correlated with the transcription of the *CD177* gene and not directly linked to circulating PR3 or PR3 gene transcription.[47,48] Thus various binding partners of mPR3 might have a role in the process of PR3-ANCA induced vessel damage and venous thromboembolism common to ANCA-associated systemic vasculitis.[49] Similarly, endothelial cell activation has been suggested to contribute to thrombophilia in PNH. Such a mechanism may also involve PR3 and pathways mediated by co-expressed molecules. Indeed, it was recently shown that PR3 can play a role in enhancing endothelial cell vascular integrity and can antagonize vascular permeability induced by PAR1, possibly through regulation of RhoA activity.[50]

In addition, we also showed that deficiency of mPR3 in PNH was strongly associated with a decrease in plasma PR3 levels and with the size of the PNH clone, in particular in patients with a history of thrombosis. This phenomenon was limited to PNH patients since analysis of otherwise normal individuals whose neutrophils are NB1/PR3-null did not show significant differences in circulating lev-

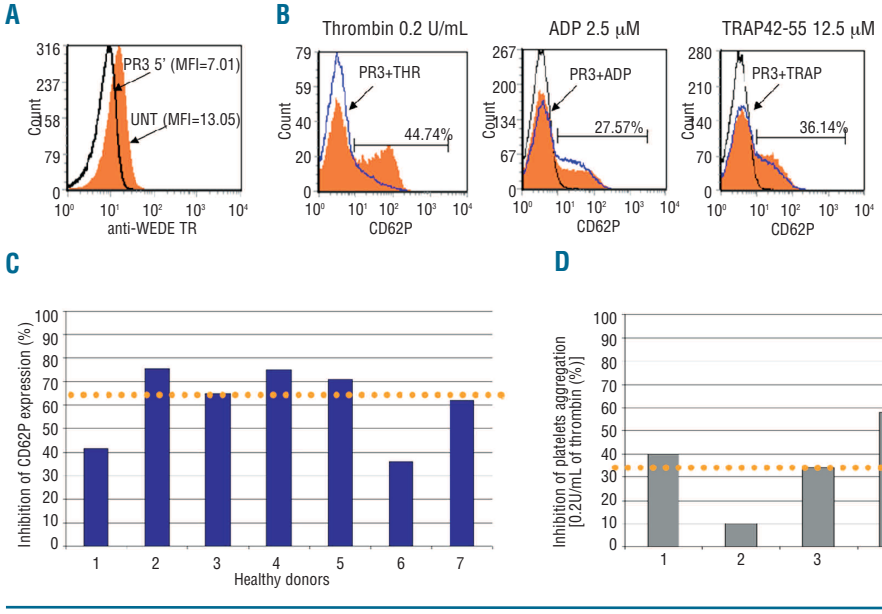

Figure 6. PR3-treated platelets show decreased activation and aggregation induced by thrombin. (A) Pretreatment of platelets with 400 nM PR3 (5 min at 37°C) resulted in significant loss of binding of a monoclonal antibody against WEDE (an epitope on PAR-1 located C-terminal to the thrombin cleavage site. (B) Representative flow cytometry histograms showing inhibition of surface expression of CD62P in PR3–treated platelets following exposure of the platelets to thrombin, ADP or TRAP. PR3 treatment (400 nM, 5 min at 37°C) led to loss of thrombin-induced CD62P expression, but did not affect ADP- or TRAP-induced expression (black line represents isotype control; blue line- PR3-treated platelets, solid line- untreated platelets). (C) Pre-incubation of platelets from seven healthy donors for 5 min with PR3 (400 nM) resulted in a 35-75% inhibition of CD62P surface expression after exposure to thrombin (0.2 U/mL). The dashed line represents the mean level of inhibition. (D) Pretreatment of washed platelets from four healthy donors with PR3, as in panel (C), inhibited thrombin-induced platelet aggregation by 10-58%. The dashed line represents the mean level of inhibition.

els of PR3. Interestingly, the concomitant decrease in both membrane and plasma PR3 is also different from that seen with deficiency of the other member of the *Ly-6* family, GPI-anchored uPAR, whose membrane levels are diminished but soluble levels increased in patients with PNH. Interestingly, lower levels of circulating PR3 were detected in two patients prior to their thrombotic events.

The lack of the NB1-PR3 membrane complex on neutrophils in PNH patients and the associated decrease in circulating PR3 levels could affect platelet activation through deficient PR3-mediated cleavage of the platelet thrombin receptor PAR-1, since PR3-mediated cleavage of PAR-1 renders the receptor insensitive to thrombin. Platelets in this context would show increased susceptibility to thrombin-mediated activation. Indeed, we found that *ex vivo* PR3 treatment of platelets decreased the amount of PAR-1 epitope needed for thrombin activation and thereby rendered platelets resistant to activation by thrombin, but not by other agonists. Incubation of whole blood with PMSF, a serine protease inhibitor, enhanced expression of an epitope located downstream of the thrombin activation site (WEDE epitope). In other words, the inhibition of PR3 with PMSF might have effects comparable to those of PR3 deficiency in PNH cells. We, therefore, hypothesized that PR3 may serve as a "natural" anti-thrombotic by limiting PAR-1 activity. While our studies do not allow for distinction as to whether the cell surface bound or the soluble form of PR3 (or both) has predominant platelet activity, cell surface-bound PR3 on neutrophils has been shown to be catalytically active,[42] and yet, unlike its soluble form, it is substantially resistant to physiological proteinase inhibitors. Although, PR3 inhibition resulted in augmentation of WEDE expression, no significant difference was observed in the expression of this epitope between patients and healthy controls. In addition to its consequences on thrombin receptors and thereby platelet activation and aggregation, PR3 deficiency in PNH may also affect flow characteristics, an effect that could be studied in future *in vitro* flow experiments.

In conclusion, we have demonstrated that GPI-anchor deficiency in PNH results in decreased membrane-bound and soluble PR3 levels. This phenomenon may constitute another mechanism contributing to the prothrombotic propensity in patients with PNH. Our study also clarified the interaction of PR3 with platelets and mechanisms of PR3 expression, thus providing insights into the function of this protein.

Authorship and Disclosures

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