Expression of cellular prion protein on platelets from patients with gray platelet or Hermansky–Pudlak syndrome and the protein's association with α-granules

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The cellular prion protein (PrPc) is a membrane glycoprotein expressed on many human cells including platelets. We investigated the cellular localization of platelet PrPc. In resting platelets most PrPc was localized inside the cells. The correlation of PrPc and P-selectin surface up-regulation after platelet activation suggested its association with α-granules. This was confirmed by normal expression of PrPc on Hermansky-Pudlak syndrome platelets, which lack dense granules, and failure of gray platelet syndrome platelets, which lack α-granules, to up-regulate PrPc. Our results warrant further studies on the role of platelet PrPc in the transmission of prion diseases by blood transfusion.

Key words: prion protein, PrPc, platelets, α granules.

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Two recent cases of probable variant Creutzfeldt-Jacob disease (vCJD) transmission by transfusion of non-leukodepleted packed red cells donated by asymptomatic vCJD infected donors[1,2] emphasize the necessity for detailed understanding of blood-related prion pathogenesis. The central role of cellular prion protein (PrPc) expression in the disease process was demonstrated by the resistance of PrP-negative mice to prion infection.[3] Prions seem to be composed mainly, if not entirely, of a conformationally changed isoform of prion protein (PrPsc) which can, upon physical contact, initiate a similar change in the secondary structure of normal PrPc.[4] Thus, molecules of PrPc on the cell membrane may serve not only as a substrate for conversion, but also as a cellular receptor for PrPsc. The level of PrPc expression by cells may influence the distribution of prions in blood and affect their fate in the organism.[5] Studies in laboratory animals demonstrated the presence of roughly equal amounts of infectivity in the plasma and cellular fraction of blood.[6,7] Cell-associated infectivity seems to be enriched in the buffy coat. Very little infectivity was recovered in purified, washed platelets of scrapie-infected hamsters.[8] However, hamster as well as mouse platelets do not express PrPc.[9,10] In contrast, most cell- associated PrPc in human blood seems to be present in platelets,[11] making these a possible target for binding of intravenously introduced prions. PrPc is expressed by $CD34^+$ hematopoietic cells and its expression has been shown to be higher in megakaryocytes.[12] Activation of human platelets leads to a more than doubling of PrPc molecules on the platelet membrane, demonstrating the existence of a significant intracellular pool of PrPc.[13,14] The aim of the present study was to investigate the intracellular localization of PrPc in human platelets and to confirm these observations through experimentation with platelets from patients with hereditary defects of platelet storage granules: Hermansky-Pudlak syndrome (HPS) in which there is a lack of dense granules) and gray platelet syndrome (GPS) in which α-granules are lacking.[15]

Design and Methods

Subjects

Donor blood samples were obtained from Departments of Transfusion Medicine of the Institute of Hematology and Blood Transfusion in Prague and the National Institutes of Health in Bethesda. In addition, blood samples from two patients with HPS and two with GPS were studied (provided by Dr. Gahl, NICHD, NIH, Bethesda, USA). Type 1 HPS patients were of Puerto Rican origin and had a 16-bp duplication in exon 15 of the *HPS-1* gene.[16] GPS patients were two siblings of Moslem Bedouin origin.[17] Blood was collected at a ratio of 9:1 into 3.8% sodium citrate and processed within 2 hours. The study was conducted in accordance with the Helsinki Declaration. Samples were obtained following informed consent under a protocol approved by the Institutional Review Board of the NICHD, NIH in Bethesda.

Quantification of platelet PrPc intracellular pool by proteinase K protection assay

Donor platelets were isolated by gel filtration into Tyrode's/ HEPES buffer (THB). The platelet suspension was supplemented with 2 mM EDTA and one half was activated with 20 μM thrombin activating peptide (TRAP) at 37°C for 10 minutes. Aliquots of resting and activated platelets were either treated with 250 μg/mL proteinase K on ice for 1 hour or left untreated. The control aliquots of both resting and activated platelets were solubilized with 1% Triton X-100 before proteinase K treatment. The proteinase K treatment was stopped by addition of 5 mM phenylmethylsulfonyl fluoride. Proteins were precipitated by cold methanol at -20°C and sedimented by centrifugation. The supernatant was removed and the pellet was resuspended in sodium dodecyl sulfate sample buffer. Samples of the intact and treated platelets were analyzed by western blotting using a mixture of monoclonal antibodies 6H4 (1:5000, Prionics AG) and AG4 (1:2000, TSE RC). Binding of monoclonal antibodies was visualized by anti-mouse IgG goat $F(ab)_2$ linked to alkaline phosphatase (Biosource International) with a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate and quantified by densitometry.

Flow cytometry evaluation of dose-dependent PrPc up-regulation on platelet membrane

Donor platelets were activated by ADP (1-50 μM) or TRAP (20 μM) for 10 minutes at room temperature and labeled with fluorescein-conjugated monoclonal antibodies against LAMP-3 (CD63, CLBGran/12, Immunotech) and phycoerythrin-conjugated anti P-selectin (CD62P, AC1.2, Becton Dickinson) or biotinylated anti-prion monoclonal antibodies 3F4 (CD230, a gift from Dr. Kascsak) followed by phycoerythrin - streptavidin (Caltag Laboratories). Samples were analyzed by a FACScan flow cytometer (Becton Dickinson) equipped with CELLQuest™ software. The mean fluorescence of resting platelets labeled with each monoclonal antibodies was assigned as 0%, and the mean fluorescence of fully TRAP-activated platelets was 100%. The relative increment of expression of specific glycoproteins on the platelet surface was calculated after activation with different concentrations of ADP.

Flow cytometry study of PrPc expression on patients' platelets

Donor and patient platelet-rich plasma was prepared by layering 0.5 mL of citrate anticoagulated blood on 1 mL of Ficoll-Hypaque (Amersham Biosciences) and sedimenting red blood cells at 1 g. Platelet-rich plasma was diluted 10 times with THB and an aliquot of platelets activated with 20 μM TRAP (10 minutes, room temperature). Resting and activated platelets were labeled with phycoerythrin-conjugated anti P-selectin, or fluorescein-conjugated monoclonal antibodies against LAMP-3 or PrPc (1562, Chemicon International or 6H4, Prionics) and analyzed by flow cytometry.

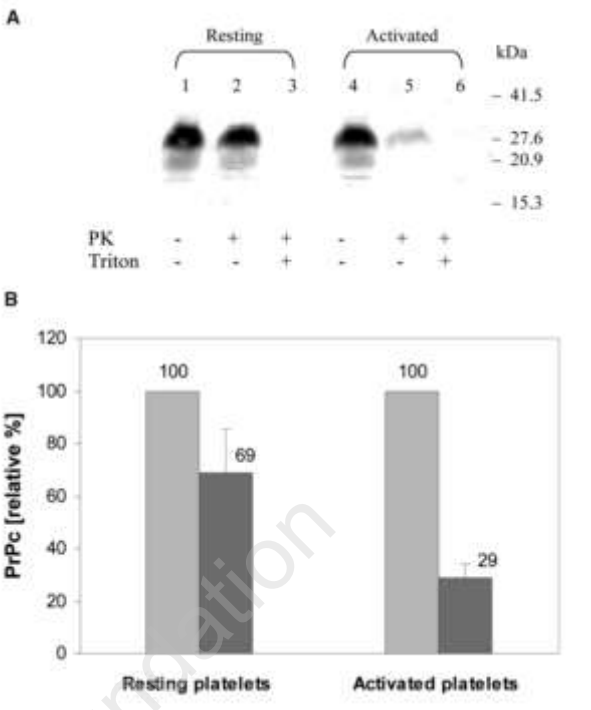

Figure 1. Most platelet PrPc resides in the intracellular pool. Intact resting and TRAP-activated platelets or platelets solubilized by Triton X-100 were treated with proteinase K (PK) to cleave accessible PrPc. The presence of PrPc was analyzed by western blotting using a mixture of monoclonal 6H4 and AG4 (A). Most PrPc in resting platelets was protected against proteolysis (line 2). Platelet activation led to translocation of intracellular PrPc onto the cell membrane and increased the proportion of PrPc accessible to PK (line 5) while solubilization of platelet membranes by Triton X-100 led to complete cleavage of PrPc (lines 3, 6). The blot is representative of five independent experiments used for densitometric quantification of PrPc (B). Light gray bars – non-treated platelets, dark gray bars – PK-treated platelets. Approximately 70% and 30% of platelet PrPc reside in the intracellular pool of resting and activated platelets, respectively.

Results and Discussion

The quantity of PrPc molecules per cell expressed on the platelet membrane was reported to be between 300 and 1800 for resting and between 600 and 4800 for activated platelets.[12-14] However, none of these studies addressed the size of the platelet intracellular PrPc pool. The proteinase K protection assay applied to resting human platelets demonstrated that the majority of PrPc (69%) is not accessible to the protease (Figure 1). This indicates that the amount of PrPc on membranes of resting platelets is substantially smaller than that in the intracellular pool. Treatment of activated platelets with proteinase K led to cleavage of a greater part of platelet PrPc (71%), although 29% of the molecules remained protected against proteolysis. This suggests that not all intracellular PrPc is up-regulated on the platelet surface

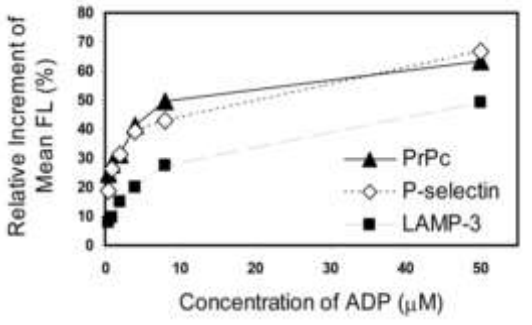

Figure 2. PrPc is up-regulated on the surface of activated platelets together with an α-granular marker P-selectin. Platelets were activated with ADP or TRAP, fixed and analyzed for expression of P-selectin, LAMP-3 and PrPc using flow cytometry. ADP induced co-expression of P-selectin and PrPc on the platelet surface at lower concentrations than those required for expression of LAMP-3, suggesting that PrPc associates with α-granules, but not with lysosomes. Data are presented as the relative increase in comparison to the level of expression on platelets fully activated with TRAP (100 %). Data from three independent experiments are presented in the graph.

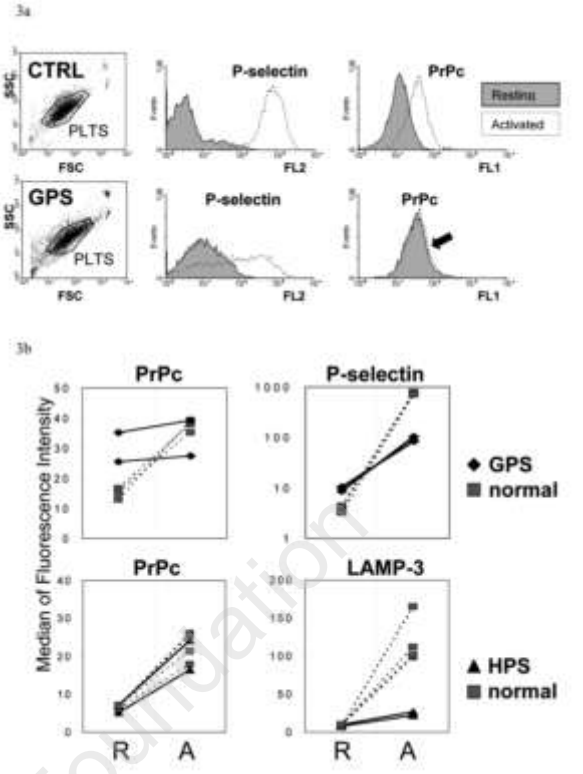

Figure 3. Platelets from patients with gray platelet syndrome (GPS) patients, but not those from Hermansky-Pudlak syndrome (HPS), fail to up-regulate PrPc after activation. The expression of P-selectin, LAMP-3 and PrPc was studied by flow cytometry on resting and TRAP-activated platelets from two GPS patients and three normal donors. Platelets from GPS patients have decreased numbers of α-granules. Resting GPS platelets expressed high levels of α-granular P-selectin and similarly increased levels of PrPc when compared with normal donor platelets (CTRL) (A). Activation of GPS platelets led to an aberrant up-regulation of P-selectin and almost no up-regulation of PrPc expression (arrow) which suggests that PrPc is located in α-granules. Typical scattergrams with gated platelets (PLTS) from CTRL (upper part) and GPS patients (lower part) are shown in 3A. Histograms are representative of P-selectin and PrPc expression on resting (filled peak) and activated (dashed line) platelets. A similar study was performed on platelets from two HPS patients and four normal donors. HPS platelets have decreased numbers of δ granules and this correlated with a decreased expression of LAMP-3 on activation. The expression of PrPc in resting and activated platelets from HPS patients was normal. GPS (upper part), HPS (lower part) and CTRL platelet fluorescence is shown in 3B. Data are presented as medians of fluorescence intensity. Patients: GPS: black diamonds; HPS: black triangles; healthy donors: gray rectangles; R: resting platelets; A: activated platelets.

or shed from platelets[18] after activation. Furthermore, solubilization of the platelet membranes with Triton X-100 led to complete degradation of platelet PrPc by proteinase K, demonstrating that intracellular PrPc is sensitive to proteolysis. This PrPc distribution is in agreement with results of our previous study revealing the incomplete translocation of PrPc from the organelle fraction to the membrane fraction in activated platelets.[13]

In order to elucidate the intracellular localization of platelet PrPc we conducted immunoelectron microscopy studies with a mixture of anti-PrP monoclonal antibodies and a gold-labeled secondary antibody. Gold particles were found to be associated with plasma membranes, membranes of the open canalicular system and α-granules (*KH and Dr. Michael Jarnik, Fox Chase Cancer Center, Philadelphia, unpublished results*). However, the signal was not strong enough to allow conclusive evaluation of the intracellular PrPc distribution. Recently, Starke *et al.*[12] and Robertson *et al.*[18] reported a similar intracellular distribution of platelet PrPc, determined by immunoelectron microscopy with polyclonal anti-PrP antibodies P3 and FL253, respectively.

To learn more about the intracellular localization of PrPc in platelets we used flow cytometry to follow the correlation of an agonist dose-dependent membrane up-regulation of PrPc, P-selectin (α-granular protein) and LAMP-3 (dense granular and lysozomal protein) on platelets from normal donors (Figure 2). The concentrations of ADP necessary to induce co-expression of P-selectin and PrPc on the platelet surface were lower than those required for expression of LAMP-3 (e.g. 5 μM ADP vs. 50 μM ADP to reach 40% of maximal expression). This suggests that PrPc is up-regulated from the same compartment as P-selectin, but from a different compartment than LAMP-3.

To further address the question of the origin of intraplatelet PrPc, we evaluated the expression of platelet PrPc in two patients with HPS and two patients with GPS. HPS platelets have a low number of dense granules, but normal numbers of α-granules.[15] The expression of LAMP-3 was equivalent on resting control and HPS platelets, but was substantially decreased on HPS platelets after full platelet activation (Figure 3B). In comparison, similar levels of PrPc and α-granular P-selectin were expressed on normal and HPS activated platelets demonstrating that a lack of dense granules does not affect PrPc up-regulation (Figure 3B). Platelets

from patients with GPS are deficient in α-granules.[15] Resting GPS platelets demonstrated higher expression of P-selectin and PrPc than normal platelets (Figure 3). This difference may represent a redirection of proteins from insertion into membranes of absent α-granules to platelet cytoplasmic membranes. In contrast to normal platelets, GPS platelets failed to up-regulate P-selectin and PrPc with agonist-induced activation illustrating that intact α-granules are essential for normal up-regulation of these proteins (Figure 3).

Taken together, our data confirm the localization of intracellular PrPc in platelet α-granules. The potential role of platelets and platelet α-granular PrPc in transmission of prion diseases by blood transfusion remains to be investigated. Hypothetically, PrPsc present in infected donor plasma could bind to PrPc on the platelet surface of the transfusion recipient and be delivered into α-granules. This mechanism may prevent PrPsc from reaching cells which would be capable of supporting prion replication. Our results warrant further studies on the interactions of platelets with intravenously introduced prions.

KH designed and performed experiments, analyzed and interpreted the data and wrote the manuscript. HG performed and analyzed the PK protection assay. JS was involved in interpretation of flow cytometry data and revised the manuscript. JGV was involved in the design of experiments and revised the intellectual content of the manuscript.

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References

1. Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet 2004; 363:417-21.
2. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet 2004; 364:527-9.
3. Brandner S, Raeber A, Sailer A, Blattler T, Fischer M, Weissmann C, et al. Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system. Proc Natl Acad Sci USA 1996;93:13148-51.
4. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998;95:13363-83.
5. Vostal JG, Holada K, Simak J. Expression of cellular prion protein on blood cells: potential functions in cell physiology and pathophysiology of transmissible spongiform encephalopathy diseases. Transfus Med Rev 2001; 15:268-81.
6. Brown P, Rohwer RG, Dunstan BC, MacAuley C, Gajdusek DC, Drohan WN. The distribution of infectivity in blood components and plasma derivatives in experimental models of transmissible spongiform encephalopathy. Transfusion 1998;38:810-6.
7. Brown P, Cervenakova L, McShane LM, Barber P, Rubenstein R, Drohan WN. Further studies of blood infectivity in an experimental model of transmissible spongiform encephalopathy, with an explanation of why blood components do not transmit Creutzfeldt-Jakob disease in humans. Transfusion 1999;39:1169-78.
8. Holada K, Vostal JG, Theisen PW, MacAuley C, Gregori L, Rohwer RG. Scrapie infectivity in hamster blood is not associated with platelets. J Virol 2002;76:4649-50.
9. Holada K, Vostal JG. Different levels of prion protein (PrPc) expression on hamster, mouse and human blood cells. Br J Haematol 2000;110:472-80.
10. Barclay GR, Houston EF, Halliday SI, Farquhar CF, Turner ML. Comparative analysis of normal prion protein expression on human, rodent, and ruminant blood cells by using a panel of prion antibodies. Transfusion. 2002;42:517-26.
11. MacGregor I, Hope J, Barnard G, Kirby L, Drummond O, Pepper D, et al. Application of a time-resolved fluoroimmunoassay for the analysis of normal prion protein in human blood and its components. Vox Sang 1999;77:88-96.
12. Starke R, Harrison P, Mackie I, Wang G, Erusalimsky JD, Gale R, et al. The expression of prion protein (PrP(C)) in the megakaryocyte lineage. J Thromb Haemost 2005;3:1266-73.
13. Holada K, Mondoro TH, Muller J, Vostal JG. Increased expression of phosphatidylinositol-specific phospholipase C resistant prion proteins on the surface of activated platelets. Br J Haematol 1998;103:276-82.
14. Barclay GR, Hope J, Birkett CR, Turner ML. Distribution of cell-associated prion protein in normal adult blood determined by flow cytometry. Br J Haematol 1999;107: 804-14.
15. Nurden AT. Qualitative disorders of platelets and megakaryocytes. J Thromb Haemost 2005;3:1773-82.
16. Gahl WA, Brantly M, Kaiser-Kupfer MI, Iwata F, Hazelwood S, Shotelersuk V, et al. Genetic defects and clinical characteristics of patients with a form of oculocutaneous albinism (Hermansky-Pudlak syndrome). N Engl J Med 1998; 338:1258-64.
17. Falik-Zaccai TC, Anikster Y, Rivera CE, Horne MK 3rd, Schliamser L, Phornphutkul C, et al. A new genetic isolate of gray platelet syndrome (GPS): clinical, cellular, and hematologic characteristics. Mol Genet Metab 2001;74: 303-13.
18. Robertson C, Booth SA, Beniac DR, Coulthart MB, Booth TF, McNicol A. Cellular prion protein is released on exosomes from activated platelets. Blood 2006 (Epub ahead of print).