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

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' 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-negativemice to prion infection. Prions seem to be composed mainly, if not entirely, of a conformationally changed isoform of prion protein (PrPSc) which, upon physical contact, initiate a similar change in the secondary structure of normal PrPc. 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. Studies in laboratory animals demonstrated the presence of roughly equal amounts of infectivity in the plasma and cellular fraction of blood. Cell-associated infectivity seems to be enriched in the buffy coat. Very little infectivity was recovered in purified, washed platelets of scrapie-infected hamsters. However, hamster as well as mouse platelets do not express PrPc. In contrast, most cell-associated PrPc in human blood seems to be present in platelets, making this 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. 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. 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.

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

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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. GPS patients were two siblings of Moslem Bedouin origin.

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.

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.
or shed from platelets 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.

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 Janrik, 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. and Robertson et al. 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 marker) 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. 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).
from patients with GPS are deficient in α-granules.\(^9\) 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.

KJ 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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