Cellular prion protein is released on exosomes from activated platelets

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Cellular prion protein (PrP<sup>C</sup>) is a glycosphingolipid-anchored protein, of unknown function, found in a number of tissues throughout the body, including several blood components of which platelets constitute the largest reservoir in humans. It is widely believed that a misfolded, prion-protective, prion-resistant form of PrP<sup>C</sup>, PrP<sup>Sc</sup>, is responsible for the transmissible spongiform encephalopathy (TSE) group of fatal neurodegenerative diseases. Although the pathogenesis of TSEs is poorly understood, it is known that PrP<sup>C</sup> must be present in order for the disease to progress; thus, it is important to determine the physiologic function of PrP<sup>C</sup>. Resolving the location of PrP<sup>C</sup> in blood will provide valuable clues as to its function. PrP<sup>C</sup> was previously shown to be on the alpha granule membrane of resting platelets. In the current study, platelet activation led to the transient expression of PrP<sup>C</sup> on the platelet surface and its subsequent release on both microvesicles and exosomes. The presence of PrP<sup>C</sup> on platelet-derived exosomes suggests a possible mechanism for PrP<sup>C</sup> transport in blood and for cell-to-cell transmission. (Blood. 2006;107:3907-3911)

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Introduction

Cellular prion protein (PrP<sup>C</sup>) is a membrane-bound, glycosphingolipid-anchored protein found primarily in lipid rafts on the cell membrane of neuronal and non-neuronal cells, including tonsils, spleen, and of the secretory granules of epithelial cells in the stomach, as well as in cultured cells. Although PrP<sup>C</sup> has been shown to be present on the surface of a number of peripheral blood cells, the relative levels on individual cell types have been contentious. Individual studies have reported that the majority of PrP<sup>C</sup> is associated with both platelets and red blood cells. In the former case the surface expression of PrP<sup>C</sup> is increased following stimulation, suggesting an additional internal membrane source of the protein, recently shown to be alpha granule membranes. Furthermore, platelet activation is associated with the accumulation of PrP<sup>C</sup> in releasates, and in platelet concentrates, stored for up to 10 days, there is an increase in initially the microsomal, and plasma levels of PrP<sup>C</sup>. Transmissible spongiform encephalopathies (TSEs) are a family of neurodegenerative disorders, including Creutzfeldt-Jakob disease (CJD), Gerstmann-Strassler-Scheinker syndrome, and familial insomnia in humans; scrapie in sheep; and bovine spongiform encephalopathy (BSE) in cattle. They are all characterized by the accumulation of a prion-protective isomer (PrP<sup>Sc</sup>) of PrP<sup>C</sup> in the brain of affected individuals. It is generally considered that PrP<sup>Sc</sup> acts as a template inducing the same structural changes within other normally folded PrP<sup>C</sup> molecules on contact, thus propagating the misfolded state of the protein.

The CNS is the site at which TSE pathogenesis is apparent in prion infections; however, the agent must first replicate and be transported to the CNS after peripheral infection. The spread of PrP<sup>Sc</sup> has been tracked from the gastrointestinal tract and the spleen to the CNS. The lymphoreticular system (LRS) is believed to be an important site of prion replication, and an accumulation of PrP<sup>Sc</sup> is apparent in spleen and lymph nodes after peripheral infection. Indeed, neuroinvasion is delayed without a functional LRS.

A new variant of CJD (vCJD) identified in humans in the United Kingdom is almost certainly the result of infection with the BSE agent. Patients infected with vCJD, in contrast to those with classic CJD, have been shown to have widespread deposition of PrP<sup>Sc</sup> in the LRS. Immune cells, in particular B cells and follicular dendritic cells, have been identified as harboring infectious PrP<sup>Sc</sup> in the LRS. However PrP<sup>Sc</sup>, the standard biochemical marker used for diagnosis of TSEs, cannot be detected by current technology in circulating lymphocytes or whole blood. Bioassays are a more sensitive assay for infectivity, and a number of studies have demonstrated that the infectious agent is present in blood and blood components, buffy coats, plasma, and platelets in animal models. The removal of all white cells by standard leukoreduction reduced infectivity by only 42%, suggesting that other blood components carry PrP<sup>Sc</sup>. There is therefore a significant concern that blood transfusions may represent a portal for the transmission of TSEs. Indeed since 2004, 3 apparent cases of transmission of vCJD by transfusion have been reported in the United Kingdom.

To understand the transmission of the disease by PrP<sup>Sc</sup>, it is important to determine the physiologic behavior and function of normal PrP<sup>C</sup> in blood cells and plasma. In the current study the cellular localization, and stimulus-induced redistribution, of PrP<sup>C</sup> in platelets has been examined.

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Materials and methods

The protocols of this study were approved by the Human Research Ethics Board of the University of Manitoba.

Antibodies and reagents

Monoclonal antibody (Ab) 308, raised against amino acids 106 to 126 of human PrP(C), was purchased from Cayman Chemical, Ann Arbor, MI, and the polyclonal Ab FL253, raised against the full-length PrP(C), was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-CD62P Ab (D541) was a generous gift from Dr Sara Israels, Manitoba Institute of Cell Biology, Winnipeg, MB. Polyclonal Ab to human fibrinogen was purchased from Calbiochem-Novabiochem, San Diego, CA. Horseradish peroxidase– and FITC-conjugated Abs were purchased from DakoCytomation, Mississauga, ON. Secondary Abs conjugated to gold, along with bovine thrombin, protease inhibitors, and all other reagents were purchased from Sigma-Aldrich Canada, Oakville, ON, and were of the highest grade available.

Preparation of washed platelets

Blood was collected following informed consent, by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks. The blood was drawn into syringes containing acid citrate dextrose anticoagulant (ACD; 3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 1.8 mL plasma-free platelet suspensions were prepared and incubated with agonist, or saline control, for the times indicated. The samples were fixed by the addition of an equal amount of 4% paraformaldehyde, then incubated with anti-PrP(C) Ab 308 diluted in PBS/0.1% BSA. Following washing 3 times in PBS/0.1% BSA, the samples were incubated in a FITC-conjugated secondary Ab. The samples were finally washed 3 times in PBS and resuspended in PBS. Flow cytometry was carried out on a Becton Dickinson (Mississauga, ON, Canada) FacsCalibur flow cytometer with forward and side scatter set to a logarithmic scale.

Results

PrP(C) is present on platelet alpha granule, but not dense granule, membranes

Frozen sections of quiescent human platelets showed the characteristic platelet intracellular architecture (Figure 1A). Immunoelectron microscopy studies of these platelets using an anti-PrP(C) Ab, FL253, followed by gold-conjugated Protein G, confirmed the association of PrP(C) with intracellular granules (Figure 1B-C) and the membranes of the open canicular system (Figure 1C). A polyclonal Ab to fibrinogen was used to identify alpha granules (Figure 1D), and double staining of these sections using Abs to PrP(C) and fibrinogen was consistent with both proteins localizing to the same organelle (Figure 1E-F).

PrP(C) translocates to and is released from the platelet surface following activation

Lysates from thrombin-stimulated platelets (1 U/mL; 0-120 seconds) were immunoblotted with anti-PrP(C) Ab 308. There was a decrease in PrP(C) levels associated with the platelet pellet over time (Figure 2A). Although there was significant donor variability in the time course of the loss of PrP(C) from the pellet, densitometric analysis indicated that by 120 seconds of stimulation the intensity...
of the PrP<sup>C</sup> band from the platelet pellet had diminished to about 50% of control (Figure 2B). This decrease in platelet-associated PrP<sup>C</sup> from activated platelets was accompanied by a corresponding accumulation of PrP<sup>C</sup> in the releasate (Figure 2A-B).

**PrP<sup>C</sup> is present on platelet microvesicles**

Flow cytometry, using anti-PrP<sup>C</sup> Ab 308, demonstrated the presence of PrP<sup>C</sup> on the surface of unstimulated platelets (Figure 3). Following thrombin stimulation (1 U/mL) there was a transient increase in the expression of PrP<sup>C</sup> on the platelet surface, followed by its release into the supernatant. There was a corresponding shedding of microvesicles from the platelet surface in response to thrombin; however, the level of PrP<sup>C</sup> on the surface of the microvesicles was low (< 10% of total released PrP<sup>C</sup>) and remained constant with time (Figure 3). Platelet activation with A23187 (20 μM) was accompanied by increased levels of microvesicle production when compared with thrombin; however, the level of PrP<sup>C</sup> associated with the microvesicles remained low (data not shown).

Immunogold labeling demonstrated the presence of PrP<sup>C</sup> on the surface of unstimulated platelets (Figure 4A). Following activation with thrombin, PrP<sup>C</sup> was observed around the periphery of the platelet and at the tips of pseudopods (Figure 4B-C). In addition, PrP<sup>C</sup> was associated with small (< 100 nm) membranous vesicles released from the platelets (Figure 4D-E).

**PrP<sup>C</sup> is present on platelet exosomes**

Previous studies have shown that, in addition to microvesicles, thrombin stimulates the release of exosomes from platelets. Given the relatively low levels of PrP<sup>C</sup> on the surface of released microvesicles (Figure 3) and its presence on the surface of smaller membrane fractions (Figure 4D-E), the possible association of PrP<sup>C</sup> with exosomes was examined.

Exosomes were prepared by differential centrifugation, and separation through a sucrose gradient, of the releasate of thrombin-stimulated platelets. Immunoblotting using anti-PrP<sup>C</sup> Ab 308 was consistent with the presence of PrP<sup>C</sup> in these exosome fractions (Figure 5A).

Immunoelectron microscopy of fractions 3 and 4 using anti-PrP<sup>C</sup> Abs (308 or FL253) demonstrated the presence of PrP<sup>C</sup> on vesicles ranging from 40 to 100 nm (Figure 5B), the size being consistent with previous reports of platelet-derived exosomes. Double-labeling these fractions with anti-CD62 Ab D541 confirmed that these exosomes were derived from alpha granules (Figure 5C).
The current study localizes PrP<sub>C</sub> to platelet alpha granule, but not dense granule, membranes, confirming a recent study by Starke et al. Thus, PrP<sub>C</sub> is present with proteins such as the αIIb/β3 integrin, CD62 (P-selectin), CD36, and the GPIb/V/IX complex inherent in the alpha granule membrane, and, in common with these other proteins, there is an activation-mediated increase in expression of PrP<sub>C</sub> on the external platelet surface. The function of PrP<sub>C</sub> in platelets is unknown; preincubation with anti-PrP<sub>C</sub> Abs has a limited effect on agonist-induced aggregation (Robertson et al., unpublished); therefore, it is unlikely that PrP<sub>C</sub> plays a significant role in either of these platelet functions. In contrast to the expression of other activation-associated proteins, the thrombin-induced expression of PrP<sub>C</sub> in platelets is unknown; preincubation with anti-PrP<sub>C</sub> Abs has a limited effect on platelet adhesion to a variety of matrices but no effect on agonist-induced aggregation (Robertson et al., unpublished); therefore, it is unlikely that PrP<sub>C</sub> plays a significant role in either of these platelet functions. In contrast to the expression of other activation-associated proteins, the thrombin-induced expression of PrP<sub>C</sub> on the platelet surface was transient and was followed by its release. Previous studies have shown that PrP<sub>C</sub> is present in platelet releasates; however, the current study demonstrates that the released PrP<sub>C</sub> is associated with membranes, initially in small quantities on microvesicles and subsequently in higher levels on exosomes.

Exosomes are small (40-100 nm), membrane-bounded vesicles which are released from a variety of cells following exocytosis and are present in human plasma. Denzer et al. reviewed a large number of proteins and lipids that are associated with exosomes, which include members of the tetraspanin protein family, the immunoglobulin supergene family, as well as GPI-anchored proteins and cytosolic proteins. Exosomes have been implicated in cell-to-cell communication mechanisms by transferal of proteins directly from the exosomes to target cells, in a manner similar to the movement of GPI-anchored proteins from the plasma membrane of red blood cells to endothelial cells. Furthermore, exosomes have been implicated in the activation of the immune system, including the stimulation of T lymphocytes and a potential interaction with follicular dendritic cells. Reticulocyte-derived exosomes may participate in complement regulation. Interestingly, Whiteside has recently proposed that exosomes play a role in the evasion of tumor cells from the immune system.

Studies in platelets have shown the release of alpha granule membrane–derived exosomes following exocytosis. Therefore, the presence of PrP<sub>C</sub> on exosomes is entirely consistent with the alpha granule membrane source of these vesicles. The function of platelet-derived exosomes is unknown, although the low binding of factor X, prothrombin, and annexin V to their surface suggests that they do not have the same procoagulant activity as platelet-derived microvesicles. The expression of CD62 on the surface of platelet-derived exosomes points to a possible role in adhesion, or cell-to-cell transfer of adhesive properties, because CD62 is known to mediate adhesion between leukocytes and endothelial cells.

The presence of prion protein on exosomes has recently been highlighted by Fevrier et al., who reported the presence of infectious PrP<sub>Sc</sub> in exosomes derived from cultured epithelial and neuroglial cell lines after infection with scrapie. They subsequently proposed that exosomes may provide a vehicle for transport of PrP<sub>Sc</sub> from cell to cell, thus providing a mechanism for transmission of infectious proteins in the body. The current finding that PrP<sub>C</sub> is present on platelet-derived exosomes strengthens the hypothesis that exosome release is a general mechanism for transport of proteins and inferentially pathogen transmission.
including prions, between cells. Platelets contain a large proportion of circulating PrPSc; therefore, platelet-derived exosomes could potentially act as an important source of protein for prion replication. In addition, the transfer of exosomes containing PrPSc to cell types in which it is normally absent may confer susceptibility to infection with prions. To date, this has not been addressed.

Although there is no biochemical evidence for the presence of PrPSc on platelets, a recent study by Cervenakova et al.13 identified platelet-derived exosome infection in the platelet and plasma fractions of murine blood from mice infected with mouse-adapted vCJD. The present finding that PrPSc is released on exosomes from activated platelets therefore raises the possibility that PrPSc is similarly released from platelets. Although this has not been addressed in the current study, it is clearly plausible that the generation of PrPSc-containing platelet exosomes during preparation of blood products could account for the transmission of variant CJD by blood transfusion. Leukoreduction of plasma, a process which would not remove exosomes, reduced infectivity by only 42%24 and, when taken in concert with the current study, suggests that further investigation into the possible role of platelet-derived exosomes as vehicles for prion transmission is clearly warranted.

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References