Junctional expression of the prion protein PrP^C by brain endothelial cells: a role in trans-endothelial migration of human monocytes

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Summary

The conversion of prion protein (PrP^C) to its protease-resistant isoform is involved in the pathogenesis of prion diseases. Although PrP^C is highly expressed in neurons and other cell types, its physiological function still remains elusive. Here, we describe how we evaluated its expression, subcellular localization and putative function in brain endothelial cells, which constitute the blood-brain barrier. We detected its expression in microvascular endothelium in mouse brain sections and at intercellular junctions of freshly isolated brain microvessels and cultured brain endothelial cells of mouse, rat and human origin. PrP^C co-localized with the adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM-1); moreover, both PrP^C and PECAM-1 were present in raft membrane microdomains. Using mixed cultures of wild-type and PrP^C-deficient mouse brain endothelial cells, we observed that PrP^C accumulation at cell-cell contacts was probably dependent on homophilic interactions between adjacent cells. Moreover, we report that anti-PrP^C antibodies unexpectedly inhibited transmigration of U937 human monocytic cells as well as freshly isolated monocytes through human brain endothelial cells. Significant inhibition was observed with various anti-PrP^C antibodies or blocking anti-PECAM-1 antibodies as control. Our results strongly support the conclusion that PrP^C is expressed by brain endothelium as a junctional protein that is involved in the trans-endothelial migration of monocytes.

Key words: Prion protein, Brain endothelial cell, Junctional protein, Transmigration, Monocytes

Introduction

Prion protein (PrP^C) is highly expressed in brain, particularly by neurons, and in several other tissues such as lymphoid organs and intestine. Conversion of PrP^C to the scrapie, protease-resistant isoform PrP^{Sc}, followed by its accumulation as amyloid fibrils, is required for the pathogenesis of prion diseases, including bovine spongiform encephalopathy and Creutzfeldt-Jakob disease in humans (Dormont, 2002; Prusiner, 1982). Because direct interaction between PrP^{Sc} and PrP^C is believed to be important for conformational conversion of PrP^C and disease progression, unraveling the unclear physiological function of PrP^C would help understand how loss or alteration of function might contribute to prion pathology.

PrP^C is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein that, like most GPI proteins, is found in membrane raft microdomains. Several signaling molecules, such as transmembrane receptors and Src-family kinases, are also found in these microdomains, suggesting that PrP^C might be part of a signaling complex at the cell surface. This hypothesis was first strengthened by the observation that PrP^C was functionally coupled to the activation of Src-family kinases and ERK pathways in neuronal cell lines (Mouillet-Richard et al., 2000; Schneider et al., 2003). In addition, other signaling and/or adhesion molecules were also proposed as potential cellular partners of PrP^C: laminin (Graner et al., 2000), laminin receptor precursor (Rieger et al., 1997), heparan sulfate proteoglycans (Hundt et al., 2001; Snow et al., 1990), neural cell adhesion molecule (Schmitt-Ulms et al., 2001), neuropilin (West et al., 2005) and stress-inducible protein-1 (Zanata et al., 2002). In addition, the copper-binding activity of PrP^C and its role in copper metabolism has long been recognized, and this activity has been proposed to participate directly in neuronal resistance to oxidative stress: loss of function of PrP^C in prion diseases would therefore be associated with extensive oxidative neuronal damage (Brown and Sassoon, 2002).

Within the central nervous system, PrP^C is expressed not only in neurons, but also in glial cells (Moser et al., 1995), and the contribution of each cell type to prion replication and pathogenesis has recently been evaluated (Jeffrey et al., 2004; Prinz et al., 2004). In addition, overexpression of PrP^C was detected in vivo in brain endothelial cells in a rat model of

cerebral ischemia (Shyu et al., 2005). This recent observation confirmed and extended earlier reports that PrP^C was expressed by brain endothelial cells (Deli et al., 2000), as well as by minor subtypes of peripheral microvascular (intestinal and renal capillaries) (Lemaire-Vieille et al., 2000) and macrovascular endothelial cells (umbilical vein and aorta) (Simak et al., 2002; Starke et al., 2002). However, the physiological relevance of these observations still remains elusive.

Brain endothelial cells display a unique phenotype compared with peripheral endothelial cells, characterized by the presence of intercellular tight junctions and the polarized expression of numerous membrane transporters (Engelhardt, 2003). They constitute the blood-brain barrier (BBB), which actively contributes to cerebral homeostasis and strictly controls leukocyte migration and pathogen invasion from the blood to the brain (Ballabh et al., 2004; Nassif et al., 2002). In the present study, we provide more information about the subcellular localization of PrP^C and its putative function in brain endothelial cells: we report that PrP^C is expressed by vascular endothelium in mouse brain sections and is essentially present at endothelial cell-cell junctions in freshly isolated brain microvessels, as well as in cultured brain endothelial cells of various origins (mouse, rat, human). We provide evidence that this junctional localization is probably dependent upon homophilic interactions between PrP^C molecules on adjacent cells. Moreover, we unexpectedly observed a role of PrP^C in the transmigration of human monocytes through human brain endothelial cells.

Results

PrP^C is expressed by brain endothelial cells at cell-cell junctions and is localized in raft-like membrane microdomains

To investigate the expression of PrP^C in brain endothelial cells, we first conducted an immunohistological analysis of mouse brain sections. Blood vessels in mouse striatum, identified by the junctional expression of the tight-junction-associated zonula occludens protein-1 (ZO-1), were clearly found to express PrP^C (Fig. 1). As a control for staining specificity, only cerebral blood vessels of wild-type (WT) mice, but not of PrP^C-deficient knockout (KO) mice, were positively stained with anti-PrP^C antibodies, whereas ZO-1 staining was positive in both mouse strains. Interestingly, both microvessels (Fig. 1, insets) and larger vessels were stained with anti-PrP^C antibodies in WT brain sections.

To confirm that PrP^C cerebrovascular expression was localized to the endothelium, we performed an immunohistological analysis of freshly isolated brain microvessels from two-week-old rats as previously described (Perrière et al., 2005). By immunofluorescence confocal microscopy, expression of PrP^C was detected at cell-cell contacts between adjacent endothelial cells, similar to the junctional proteins ZO-1 and platelet endothelial cell adhesion molecule-1 (PECAM-1) (Fig. 2A). The preferred junctional expression of PrP^C by brain endothelium was further demonstrated using primary cultures of rat brain endothelial cells, using anti-ZO-1 and anti-PECAM-1 antibodies as controls for junctional staining (Fig. 2B). Similar observations were made using the rat (RBE4) and human (hCMEC/D3) brain endothelial cell lines (Fig. 3A), which we and others previously characterized extensively as in vitro models of brain endothelium (Friedrich et al., 2003; Reichel et al., 2002; Roux and Couraud, 2005; Roux et al., 1994; Weksler et al., 2005). Moreover, RT-PCR analysis unambiguously identified PrP^C transcripts in both brain endothelial cell lines, using primary rat astrocytes and human astrocytoma (U373) cells as positive controls (Fig. 3B) (Castelnau et al., 1994). Taken together, these observations show that brain endothelial cells express PrP^C and suggest that PrP^C might be part of the endothelial junctional complexes, like PECAM-1 and ZO-1 (Bazzoni and Dejana, 2004), which contribute to the integrity of endothelial monolayers.

Whereas GPI-anchored proteins are well known to be highly

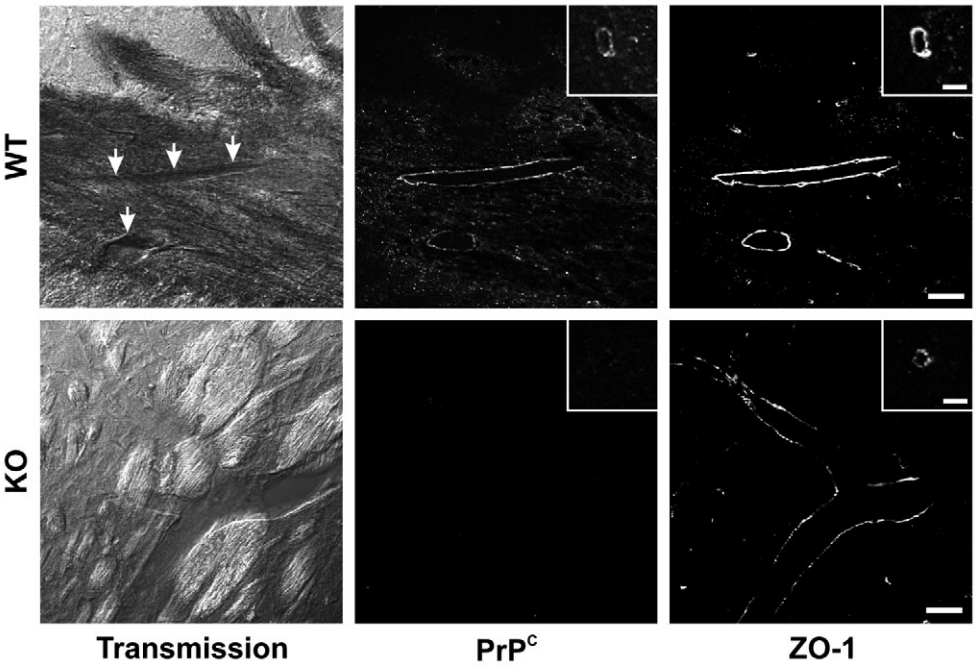

Fig. 1. Expression and localization of PrP^C in brain blood vessels. Transmission and immunofluorescence labeling of PrP^C and ZO-1 of brain sections from wild-type (WT) and PrP^C-deficient knockout (KO) mice. Sections of striatum were incubated with anti-PrP^C monoclonal antibodies (SAF32 plus SAF61, both at 1 μg/ml) and anti-ZO-1 polyclonal antibodies (5 μg/ml) for 16 hours at 4°C, then with Alexa Fluor 488-conjugated anti-mouse antibodies and Cy3-conjugated anti-rabbit antibodies for 45 minutes at 25°C. Images were collected with a confocal fluorescence microscope. Arrows in the left panel indicate blood vessels; insets show microvessels (chosen from a different field). Bars, 40 μm or 10 μm (inset).

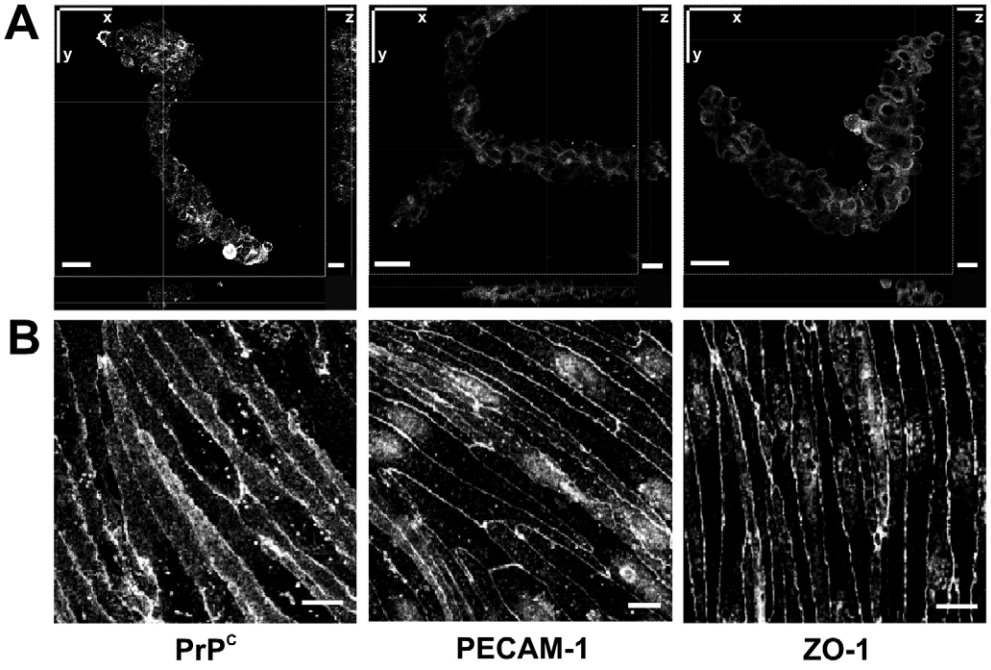

Fig. 2. Expression of PrP^C at the cell-cell contacts of primary brain endothelial cells. Immunofluorescence labeling of PrP^C, PECAM-1 and ZO-1 in freshly isolated brain microvessels. Cells were incubated with anti-PrP^C (SAF32, 2 μg/ml), anti-PECAM-1 (3A12, 2 μg/ml) or anti-ZO-1 (1 μg/ml) antibodies for 16 hours at 4°C, then with Cy2-conjugated anti-mouse antibodies or Cy3-conjugated anti-rabbit antibodies for 45 minutes at 25°C. Images were collected with a confocal fluorescence microscope. Bars, 20 μm (A, xy), 10 μm (A, z), 10 μm (B).

expressed in cholesterol-rich, raft membrane microdomains (also called caveolae-like microdomains) at the apical surface of polarized cells, some junctional proteins, including PECAM-1 (Gratzinger et al., 2003) and ZO-1, but not E-cadherin (Nusrat et al., 2000), were more recently reported to be expressed in raft microdomains. We compared the subcellular localization of the GPI-anchored protein PrP^C and the junctional protein PECAM-1 in RBE4 brain endothelial cells by cell fractionation on a 5-30% discontinuous sucrose gradient. As shown in Fig. 4A, raft microdomains were collected in the low-sucrose-density fractions (fractions 4-6), identified by the expression of caveolin-1, the prototype marker of raft/caveolae-like microdomains in endothelial cells; by contrast, the majority of membrane proteins, like the cadherin-associated junctional protein β-catenin, were recovered in the high-sucrose-density fraction (fraction 10). Co-expression of PrP^C and PECAM-1 was detected in the caveolin-1-rich fraction 4 (Fig. 4A). PrP^C appeared as a typical smear owing to the PNGase-sensitive complex pattern of *N*-glycosylation of its mature form, which was similarly detected in whole cell lysate (Fig. 4B) (Rudd et al., 2002). In addition, PrP^C was also detected in the high-sucrose-density fraction 10, with a distinct banding pattern that probably corresponds to differentially glycosylated forms of PrP^C (Fig. 3A) (Sarnataro et al., 2002). These results suggest that most of the mature form of PrP^C is co-localized with PECAM-1 in raft/caveolae-like microdomains at the cell-cell junctions of brain endothelial cells in culture.

Cell-cell interaction is required for PrP^C junctional expression

To investigate whether PrP^C might be involved in interactions between adjacent cells, non-confluent RBE4 cell cultures were analyzed for PrP^C localization by indirect immunofluorescence and compared with confluent cultures (Fig. 3A). Whereas PrP^C was diffusely expressed over the

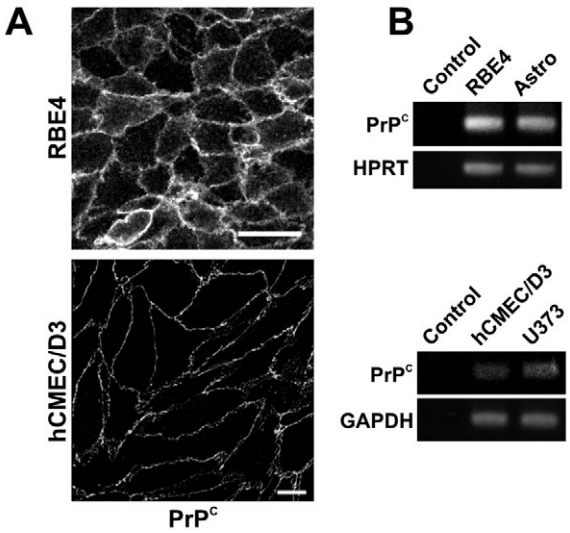

Fig. 3. Expression of PrP^C in brain endothelial cell lines. (A) Immunofluorescence labeling of PrP^C in rat (RBE4) and human (hCMEC/D3) brain endothelial cell lines. Cells were incubated with anti-PrP^C (SAF32, 2 μg/ml) monoclonal antibodies for 16 hours at 4°C, then with Cy2-conjugated anti-mouse antibodies for 45 minutes at 25°C. Images were collected with a confocal fluorescence microscope. Bars, 20 μm. (B) RT-PCR detection of PrP^C transcripts in rat (RBE4) and human (hCMEC/D3) brain endothelial cells; primary rat astrocytes (Astro) and the human U373 astrocytoma line (U373) were used as positive controls, respectively. Rat HPRT or human GAPDH were used for standardization. The size of the transcripts (rat: 244 bp; human: 243 bp) was as expected. Control lanes show no amplified fragments in the absence of cDNA.

entire cell surface in isolated cells, translocation of PrP^C to cell-cell contacts was clearly observed between two individual cells (Fig. 5A, arrow). A similar observation was

made regarding PECAM-1 expression (Fig. 5B, arrow), which is stabilized at cell-cell contacts by homophilic interactions between adjacent endothelial cells (Sun et al., 1996). To establish whether PrP^C junctional expression might similarly be dependent upon homophilic interactions between adjacent cells, brain endothelial cells were isolated from wild-type (WT) and PrP^C-deficient (KO) mice, grown to confluence in mixed cultures and analyzed for PrP^C distribution. Adherens junction complexes could form at the interface between WT and KO endothelial cells, as shown by junctional expression of the cadherin-associated protein β-catenin in endothelial cells of both origins (Fig. 6A). In contrast to β-catenin, PrP^C was only observed at cell-cell contacts between two WT cells (Fig. 6B, arrow), but not at contacts between WT and KO cells (Fig. 6B, arrowhead). Altogether, these results strongly suggest that PrP^C is involved in homophilic interactions between adjacent cells and that these interactions are required for its junctional localization in brain endothelial cells.

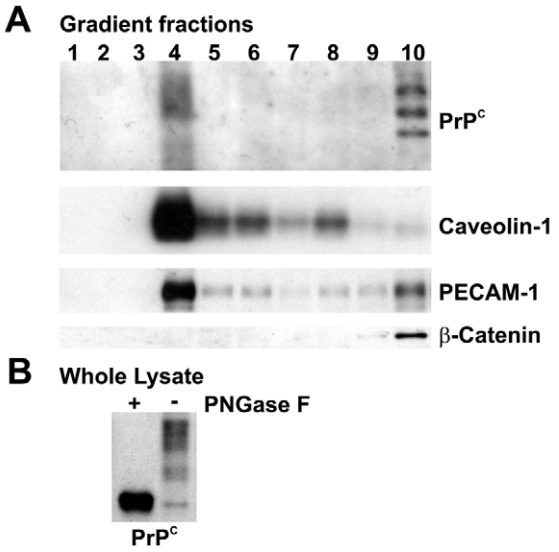

Fig. 4. Co-localization of PrP^C and PECAM-1 in raft/caveolae-like microdomains in brain endothelial cells. (A) RBE4 cells were harvested by scraping and submitted to subcellular fractionation in the presence of sodium carbonate as described in the Materials and Methods. Fractions (1-10) were collected from the top of the gradient and proteins in each fraction were concentrated by precipitation with 10% trichloroacetic acid. Equal volumes (15 μl) of concentrated fractions were analyzed by SDS-PAGE and processed for immunoblot analyses. Membranes were incubated with antibodies to caveolin-1 (0.5 μg/ml), PrP^C (SAF32, 1 μg/ml), PECAM-1 (M20, 1 μg/ml) or β-catenin (1 μg/ml). PrP^C and PECAM-1 are detected in fraction 4 containing raft/caveolae-like microdomains; PrP^C appears as a typical smear, as seen in the whole cell lysate (panel B). Other forms of PrP^C were detected in fraction 10, which contains the majority of cellular proteins, including β-catenin; these forms of PrP^C probably correspond to intermediate glycosylation forms. (B) Whole RBE4 cell lysate was treated (+) or not (–) with PNGase F and processed for western blot analyses using SAF32 anti-PrP^C antibodies (1 μg/ml). Following PNGase F treatment (+) a single band of approximately 25 kDa is detected, corresponding to unglycosylated PrP^C. The results are representative of three independent experiments.

Involvement of PrP^C in trans-endothelial migration of U937 human monocytic cells

It is well established that homophilic interactions between PECAM-1 molecules expressed at the surface of endothelial cells and monocytes directly contribute to the trans-endothelial migration of monocytes (Muller et al., 1993). We therefore investigated whether PrP^C might also be involved in this process, using the human brain endothelial cell line hCMEC/D3 (Weksler et al., 2005) and U937 human monocytic cells. By FACS analysis (Fig. 7), we first confirmed that U937 cells express PrP^C, PECAM-1 and VLA-4, the latter being the integrin receptor for vascular cell adhesion molecule-1 (VCAM-1) involved in monocyte adhesion to endothelial cells.

We previously documented that hCMEC/D3 cells express, either constitutively or upon treatment by the inflammatory cytokines tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), several surface molecules (PECAM-1, ICAM-1 and VCAM-1) known to be involved in adhesion and/or trans-endothelial migration of leukocytes (Weksler et al., 2005). Here, we present evidence that adhesion of U937 monocytic cells to hCMEC/D3 cells was strongly induced by endothelial pre-treatment with TNF-α and IFN-γ (Fig. 8A), and was drastically inhibited (89±2%) in the presence of anti-VLA-4 blocking antibodies (Fig. 8B). By contrast, antibodies to junctional proteins not involved in leukocyte adhesion (e.g. PECAM-1) (Allport et al., 2000), had no inhibitory effect compared with irrelevant anti-transferrin receptor (CD71) antibodies. In these conditions, none of the four anti-PrP^C antibodies tested showed any inhibitory activity (Fig. 8B).

We then observed that trans-endothelial migration of U937 cells through hCMEC/D3 monolayers was, as expected, dependent upon TNF-α and IFN-γ pre-treatment of endothelial cells and the presence of a concentration gradient of the chemokine stromal-derived factor-1α (SDF-1α) (Fig. 9A). Unexpectedly, all four anti-PrP^C antibodies tested (SAF32, SAF34, SAF61 and 6H4 antibodies), when pre-incubated with U937 and hCMEC/D3 cells for 1 hour prior to the assay,

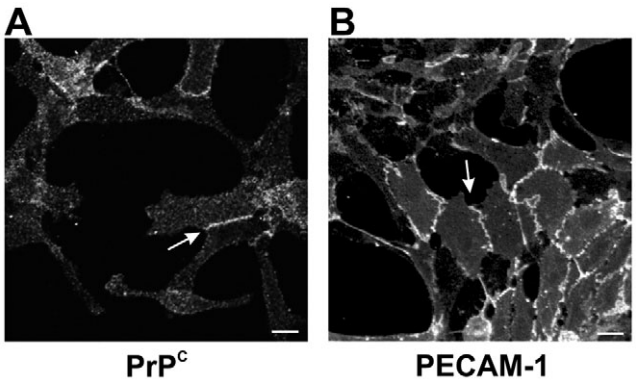

Fig. 5. Translocation of PrP^C to intercellular junctions is induced by cell-cell contacts. Immunofluorescence labeling of PrP^C and PECAM-1 in a pre-confluent culture of RBE4 cells. Non-permeabilized cells were incubated with anti-PrP^C (SAF32) (A) or anti-PECAM-1 (3A12) antibodies (B) at 2 μg/ml for 16 hours at 4°C, then with Cy2-conjugated anti-mouse antibodies for 45 minutes at 25°C. Images were collected with a confocal fluorescence microscope. Arrows indicate PrP^C (A) or PECAM-1 (B) accumulation at cell-cell contacts. Bars, 20 μm.

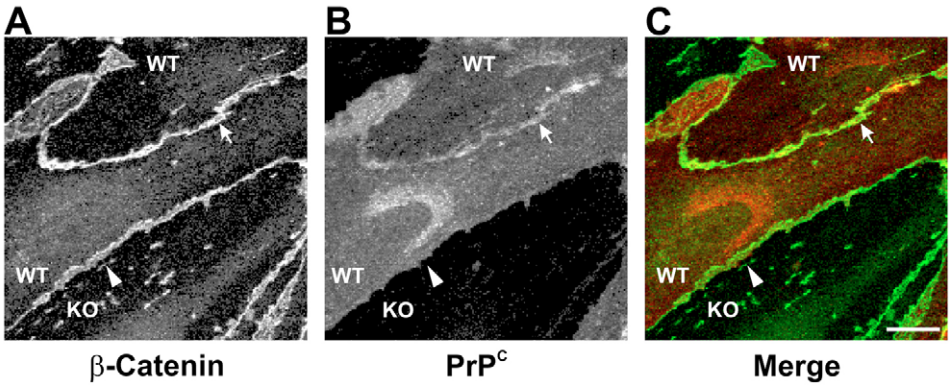

Fig. 6. Translocation of PrP^C to intercellular junctions is dependent on PrP^C expression by adjacent cells. Double immunofluorescence labeling of PrP^C and β-catenin in a mixed primary culture of wild-type (WT) and PrP^C-deficient knockout (KO) mouse brain endothelial cells. Cells were permeabilized with 0.05% saponin for 1 hour before incubation with anti-PrP^C (SAF32, 2 μg/ml) monoclonal antibody plus anti-β-catenin rabbit polyclonal antibodies (1 μg/ml) for 16 hours at 4°C. β-catenin and PrP^C were revealed using Cy2-conjugated anti-rabbit antibodies (A) and Cy3-conjugated anti-mouse antibodies (B), respectively. Images were collected with a confocal fluorescence microscope. Arrows indicate a junction between two WT cells showing a positive staining for β-catenin (A) and PrP^C (B), arrowheads indicate a junction between one WT cell and one KO cell showing a positive staining for β-catenin (A) but no staining for PrP^C (B). A merged image of the same field is presented in the right hand panel (merged), with PrP^C staining in red and β-catenin staining in green: co-localization (yellow) of PrP^C and β-catenin is observed only at junctions between two adjacent WT cells. Bar, 20 μm.

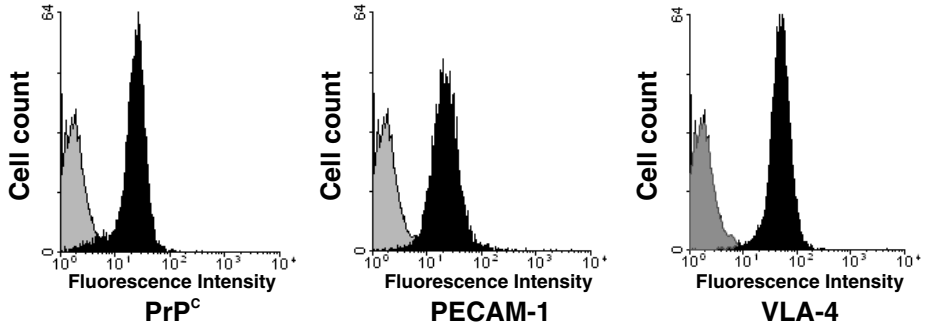

Fig. 7. Expression of PrP^C, PECAM-1 and VLA-4 by U937 human monocytic cells. U937 cells were incubated with anti-PrP^C (SAF32), anti-PECAM-1 (HEC-7) or anti-VLA-4 antibodies (2 μg/ml) for 10 minutes at 25°C, then with FITC-conjugated anti-mouse antibodies. Samples were analyzed by flow cytometry and at least 5000 cells were counted. Black areas represent staining profiles with the indicated antibodies, gray areas represent staining profiles with first antibodies omitted as negative control.

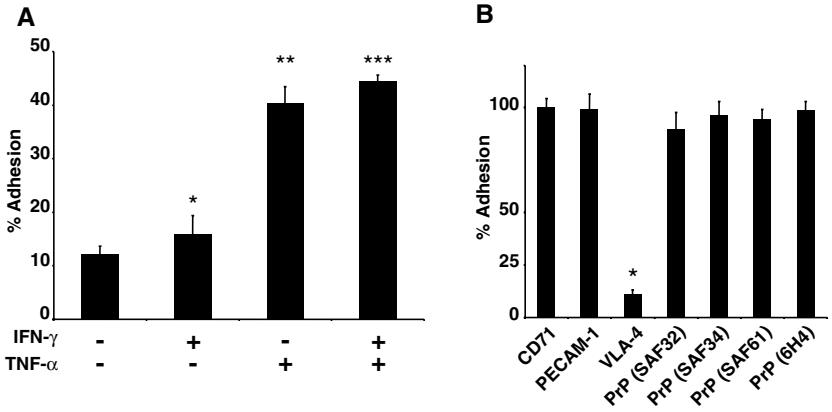

Fig. 8. Adhesion of U937 monocytic cells to human brain endothelial cells is not affected by anti-PrP^C antibodies. (A) hCMEC/D3 cells were grown to confluence and activated (+) or not (–) with TNF-α (200 U/ml) and/or IFN-γ (200 U/ml) for 24 hours. CMFDA-labeled U937 monocytic cells were then added and allowed to adhere for 30 minutes at 37°C. Numbers of adherent U937 cells are presented as percentages (%) of the total number of incubated cells. Average values (± s.e.m.) from one representative experiment out of three independent experiments performed in quadruplicate are presented (*P<0.1, **P<0.05 and ***P<0.01 versus non-activated cells: left-hand bar). (B) hCMEC/D3 cells were grown to confluence and activated with TNF-α (200 U/ml) and IFN-γ (200 U/ml) for 24 hours; activated hCMEC/D3 cells and CMFDA-labeled U937 monocytic cells were separately incubated with the indicated antibodies at 20 μg/ml for 1 hour before the adhesion assay (30 minutes), as above. Antibodies tested were specific for the irrelevant membrane protein CD71, the junctional adhesion molecule not involved in leukocyte adhesion PECAM-1 (HEC-7 antibody), the monocyte integrin VLA-4 known to mediate monocyte adhesion, or PrP^C (SAF32, SAF34, SAF61, 6H4 antibodies). Results are presented as percentages (%) of adherent U937 cells following incubation with the indicated antibodies, 100% being the number of adherent U937 cells following incubation with anti-CD71 irrelevant antibodies. Average values (± s.e.m.) from one representative experiment out of three independent experiments performed in quadruplicate are presented (*P<0.01).

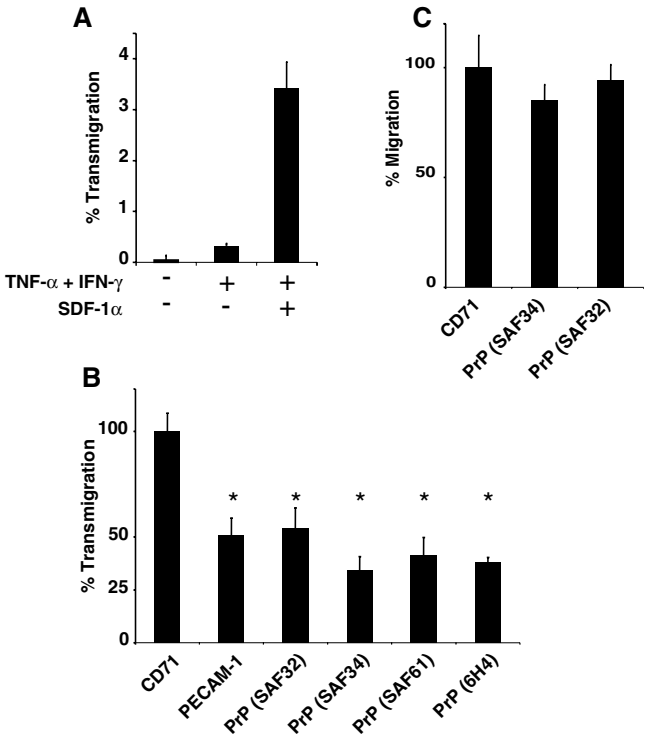

Fig. 9. Transmigration of U937 monocytic cells through human brain endothelial cells is prevented by anti-PrP^C antibodies. (A) hCMEC/D3 cells were grown to confluence on Transwell filters with 3 μm pore size and activated (+) or not (–) with TNF-α (200 U/ml) and IFN-γ (200 U/ml) for 24 hours. CMFDA-labeled U937 monocytic cells were then added and allowed to transmigrate for 16-18 hours in the presence (+) or absence (–) of SDF-1α (100 ng/ml) in basal compartments. Transmigrated U937 cells were counted by FACS analysis of basal compartments. Results are presented as percentages of transmigrated cells. Average values (± s.e.m.) from one representative experiment out of three independent experiments performed in triplicate are presented. (B) The transmigration assay was performed as above in the presence of SDF-1α (100 ng/ml), following separate incubation of activated hCMEC/D3 cells and U937 monocytic cells with the indicated antibodies at 20 μg/ml for 1 hour. Antibodies tested were specific for: the irrelevant membrane protein CD71; PECAM-1 (HEC-7 antibody), known to support monocyte transmigration; or PrP^C (SAF32, SAF34, SAF61, 6H4 antibodies). Average values (± s.e.m.) are presented of triplicates from three to five independent experiments with 100% being the number of transmigrated U937 cells following incubation with anti-CD71 irrelevant antibodies (*P<0.01 versus cells pre-treated with anti-CD71 antibody). (C) Migration of CMFDA-labeled U937 monocytic cells was performed through endothelial-cell-free filters coated with fibronectin and collagen, for 16-18 hours in the presence of SDF-1α (100 ng/ml) in basal compartments. Cells were pre-incubated with the indicated antibodies at 20 μg/ml for 1 hour before the migration assay, as above, with anti-CD71 or anti-PrP^C (SAF34, SAF32) antibodies. Results are presented as percentages (%) of transmigrated cells with 100% being the number of transmigrated U937 cells following incubation with anti-CD71 irrelevant antibodies. Average values (± s.e.m.) from one representative experiment out of three independent experiments performed in triplicate are presented. Numbers of migrated U937 cells following incubation with SAF32 or SAF34 anti-PrP^C antibodies were not statistically different from control values (migrated U937 cells following incubation with anti-CD71 antibodies), indicating that pre-incubation with the indicated antibodies did not differentially affect the migration capacity of U937 cells.

inhibited transmigration of U937 cells to a similar extent as blocking anti-PECAM-1 antibodies (49±8%) used as control: 46±10%, 66±7%, 59±8% and 62±2% inhibition was observed respectively in the presence of SAF32, SAF34, SAF61 and 6H4 (Fig. 9B). In a control experiment, anti-PrP^C (SAF32, SAF34) or anti-CD71 antibodies did not affect U937 cell migration through endothelial-cell-free Transwell filters coated in fibronectin and collagen, indicating that neither viability nor migration capacity of U937 cells was affected by antibody pre-treatment (Fig. 9C).

Involvement of PrP^C in trans-endothelial migration of freshly isolated human monocytes

We further assessed the contribution of PrP^C to leukocyte trans-endothelial migration using freshly isolated human monocytes known to express PrP^C constitutively (Dürig et al., 2000). Transmigration of freshly isolated monocytes through hCMEC/D3 monolayers was observed at a higher level under

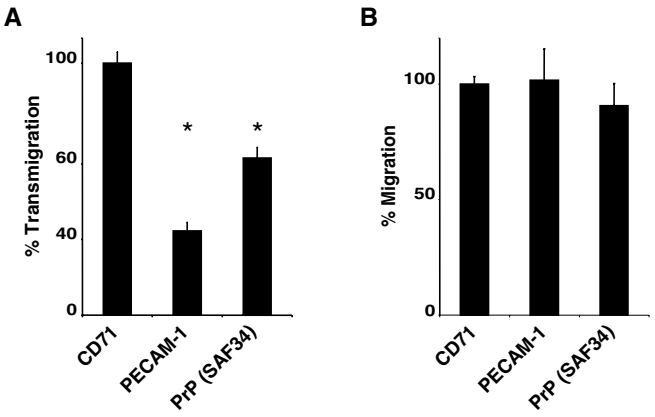

Fig. 10. Transmigration of freshly isolated human monocytes through human brain endothelial cells is prevented by anti-PrP^C antibodies. (A) hCMEC/D3 cells were grown to confluence on Transwell filters with 3 μm pore size and activated with TNF-α (200 U/ml) and IFN-γ (200 U/ml) for 24 hours. Freshly isolated and purified monocytes were labeled with CMFDA and were then added and allowed to transmigrate for 3 hours in the presence of MCP-1 (25 ng/ml) in basal compartments, following separate incubation of activated hCMEC/D3 cells and monocytes with the indicated antibodies at 20 μg/ml for 1 hour. Antibodies tested were specific for: the irrelevant membrane protein CD71; PECAM-1 (HEC-7 antibody), known to support monocyte transmigration; or PrP^C (SAF34 antibody). Average values (± s.e.m.) are presented of triplicates from three independent experiments with 100% being the number of transmigrated monocytes following incubation with anti-CD71 antibodies (*P<0.01 versus cells pre-treated with anti-CD71 antibody). (B) Migration of CMFDA-labeled monocytes was performed through endothelial-cell-free filters coated with fibronectin and collagen, for 3 hours in the presence of MCP-1 (25 ng/ml) in basal compartments. Cells were pre-incubated with the indicated antibodies at 20 μg/ml for 1 hour before the migration assay as above, with anti-CD71, anti-PECAM-1 (HEC-7) or anti-PrPC (SAF34) antibodies. The number of migrated cells following incubation with CD71 antibodies was taken as 100%. Average values (± s.e.m.) from one representative experiment out of three independent experiments performed in triplicate are presented. No significant difference was observed between the different conditions, indicating that pre-incubation with the indicated antibodies did not differentially affect the migration capacity of monocytes.

a gradient of monocyte chemoattractant protein-1 (MCP-1) (5.5±0.3%) than under a gradient of SDF-1α (1.3±0.3%) (data not shown), as previously reported (Bleul et al., 1996; Cambien et al., 2001). This process was significantly inhibited following pre-incubation of both cell types with the anti-PrP^C antibodies SAF 34 (38±4%) as well as by the control anti-PECAM-1 antibodies (68±3%) (Fig. 10A), thus confirming our data regarding U937 trans-endothelial migration. No additive effect between anti-PrP^C and anti-PECAM-1 antibodies was detected (data not shown). As reported in Fig. 11, transmigration inhibition was also observed when only one cell type was pre-treated with anti-PrP^C antibodies or with anti-PECAM-1 antibodies, further suggesting that PrP^C expressed on both cell types is involved in trans-endothelial migration of monocytes. As control, antibody pre-treatments did not affect the viability of the monocytes or their migration capacity through endothelial-cell-free Transwell filters coated in fibronectin and collagen (Fig. 10B). These results provide the first indication that PrP^C contributes to the trans-endothelial migration of monocytes.

Discussion

We present evidence that brain endothelial cells express PrP^C at intercellular junctions, using freshly isolated rat brain microvessels, primary cultures of mouse and rat brain endothelial cells, and rat (RBE4) and human (hCMEC/D3) brain endothelial cell lines. We show that PrP^C is co-localized with PECAM-1 in raft/caveolae-like membrane microdomains, is largely restricted to cell-cell contacts and is probably involved in PrP^C-PrP^C homophilic interactions between adjacent cells. Moreover, we believe that the most important result of the present study is the identification of a previously unsuspected functional role of PrP^C in monocyte transmigration through human brain endothelial cells.

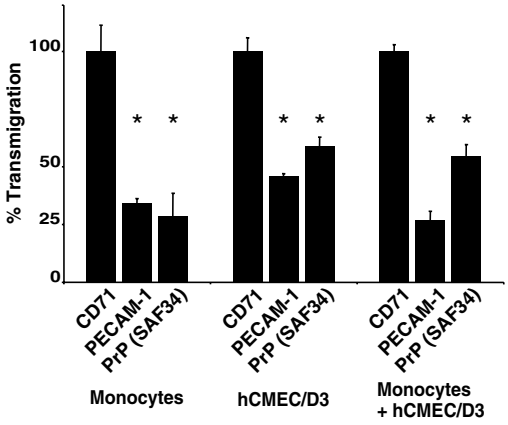

Fig. 11. PrP^C expressed by monocytes and endothelial cells is involved in transmigration of freshly isolated monocytes. Freshly isolated monocytes were labeled as above and separately incubated (monocytes) with anti-CD71, anti-PECAM-1 or anti-PrPC antibodies at 20 μg/ml for 1 hour, as indicated. Similarly, hCMEC/D3 endothelial cells, grown and activated as above, were separately incubated in the same conditions (hCMEC/D3). In parallel, both monocytes and hCMEC/D3 endothelial cells (monocytes + hCMEC/D3) were separately incubated in the same conditions. Monocytes were then added to the apical compartments of Transwell filters and allowed to transmigrate for 3 hours in the presence of MCP-1 (25 ng/ml) in basal compartments, as above. Average values (± s.e.m.) from one representative experiment out of three independent experiments are presented. The number of migrated cells following incubation with CD71 antibodies was taken as 100% in each condition (*P<0.01 versus cells pre-treated with anti-CD71 antibody).

Like other GPI-anchored proteins, PrP^C has been localized to raft/caveolae-like membrane microdomains in most cell types, including neurons and cells of the immune system (Massimino et al., 2002). However, in contrast to the majority of GPI-anchored proteins, which are sorted to the apical membrane of polarized cells, raft-associated PrP^C was shown to be restricted to the basolateral membrane (Sarnataro et al., 2002) and/or intercellular junctions of polarized epithelial cells (Morel et al., 2004). Our results extend these observations to brain endothelial cells, which constitute a polarized physiological barrier in vivo known as the BBB. Our observation that PrP^C is co-localized with PECAM-1 at intercellular junctions and in raft/caveolae-like membrane microdomains of brain endothelial cells is supported by a recent report that PECAM-1 is expressed in endothelial raft microdomains (Gratzinger et al., 2003). Together with the previous demonstration that tight junction proteins are also found in raft/caveolae-like membrane microdomains in epithelial cells (Nusrat et al., 2000), our results suggest that PrP^C is localized in junctional microdomains that are involved in the regulation of cell-cell adhesion in epithelial and brain endothelial cells.

Localization of PrP^C to cell-cell contacts in brain endothelial cells was shown here to be dependent on the expression of PrP^C by two adjacent cells. This conclusion is strengthened by two parallel observations: (1) in non-confluent cultures, PrP^C expression was diffuse at the cellular surface, but concentrated at occasional cell-cell contacts; and (2) in confluent mixed cultures of PrP^C-expressing (WT) and PrP^C-deficient (KO) brain endothelial cells, PrP^C was strictly localized to contacts between two WT cells, although adhesion complexes did form between WT and KO endothelial cells, as shown by β-catenin junctional expression. Taken together, these results strongly suggest that PrP^C localization to cell-cell contacts in brain endothelial cells is controlled by homophilic interactions between adjacent cells. As previously proposed for epithelial junctions (Morel et al., 2004), PrP^C-PrP^C interaction on two adjacent cells might involve a 'head-to-tail conformation' similar to that described for PrP^C oligomerization by in vitro modeling (Knaus et al., 2001). In addition, our observations are highly reminiscent of those previously reported with several junctional adhesion molecules, such as PECAM-1, VE-cadherin or JAM-A expressed by endothelial cells (Bazzoni et al., 2000; Lampugnani et al., 1995; Sun et al., 1996), further supporting our hypothesis of a role for PrP^C as an intercellular adhesion molecule in endothelial cells.

However, over the past ten years, alternative hypotheses have been proposed about the putative physiological function of PrP^C. In particular, there is increasing evidence supporting a functional role of PrP^C in copper binding and metabolism, and in the modulation of anti-oxidant enzyme Cu/Zn superoxide dismutase activity (Brown and Sassoon, 2002). In addition, a role has also been proposed for PrP^C in cellular adhesion to extracellular matrix and in maintenance of integrity of the physiological intestinal barrier (Graner et al., 2000; Mattei et al., 2004; Morel et al., 2004; Rieger et al., 1997).

In the present study, we report an unsuspected role of PrP^C in the trans-endothelial migration of U937 human monocytic

cells as well as freshly isolated human monocytes. Indeed, anti-PrP^C antibodies, like blocking anti-PECAM-1 antibodies used as control, were shown to prevent the trans-endothelial migration of U937 monocytic cells or monocytes. By contrast, neither anti-PrP^C antibodies nor anti-PECAM-1 antibodies affected the adhesion of monocytic cells. It is interesting to note that the present study actually points to several similarities between PrP^C and PECAM-1 localization and function: both proteins (1) co-localize to raft/caveolae-like membrane microdomains; (2) are concentrated at intercellular junctions of endothelial cells; and (3) are involved in monocyte transmigration through brain endothelium, inasmuch as their engagement with specific antibodies prevents monocyte transmigration. These data strongly suggest the involvement of PrP^C homophilic interactions between adjacent endothelial cells, as well as between endothelial cells and monocytes, which is again similar to PECAM-1 (Newman, 1997). On the basis of these parallel observations between PrP^C and PECAM-1, it could be proposed that PrP^C might interact with PECAM-1, as it does with other membrane proteins such as neural cell adhesion molecule (Schmitt-Ulms et al., 2001) or laminin receptor precursor (Rieger et al., 1997), and that anti-PrP^C antibodies might indirectly prevent monocyte migration by targeting a junctional PrP^C–PECAM-1 complex. However, we failed to demonstrate such an interaction by co-immunoprecipitation assays (data not shown), strengthening the conclusion that PrP^C might be directly involved in controlling the trans-endothelial migration of monocytes.

Several recent studies on leukocyte trans-endothelial migration described two distinct mechanisms for this process, involving either a transcellular or a paracellular route, which are proposed to be preferentially used by peripheral blood mononuclear cells and polymorphonuclear leukocytes, respectively (Barreiro et al., 2002; Carman and Springer, 2004). Because the transcellular pathway was very recently shown to depend on raft/caveolae-like membrane microdomains (Millan et al., 2006), a contribution of PrP^C to this process might be suggested. However, our data with U937 monocytic cells and freshly isolated monocytes, together with the proposition that monocytes preferentially transmigrate through a PECAM-1-dependent paracellular route, particularly upon chemokine treatment (Mamdouh et al., 2003; Muller et al., 1993; Nieminen et al., 2006), further suggest the involvement of PrP^C in a paracellular process. Moreover, our observation that anti-PrP^C and anti-PECAM-1 antibodies have no additive effect suggests that PrP^C and PECAM-1 might be involved in the same step of monocyte trans-endothelial migration. Additional experiments will be required to discriminate between these hypotheses and to unravel the precise mechanism of PrP^C contribution to monocyte transmigration.

In conclusion, the results of the present study identify PrP^C as a junctional protein in brain endothelial cells, probably involved in homophilic interactions between adjacent cells, and strongly support a role for PrP^C in the trans-endothelial migration of monocytes.

Materials and Methods

Materials

All reagents were purchased from Sigma unless otherwise stated. Dispase II was obtained from Roche Molecular Biochemicals. Collagenase type 2 was purchased from Worthington Biochemical Corporation. Endothelial Basal Medium (EBM-2) and EGM-2 BulletKit were obtained from Cambrex. RPMI 1640, αMEM and F10 media, fetal calf serum (FCS), basic fibroblast growth factor (bFGF, human, recombinant), were purchased from Invitrogen, and bovine-plasma-derived serum (BPDS) was purchased from First Link. Rat tail collagen type I was purchased from BD Biosciences, and collagen type IV from Nunc Labttek Gibco. Peptide: N-Glycosidase F (PNGase F) was from Ozyme. Tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), stromal-derived factor-1α (SDF-1α, CXCL12) and monocyte chemoattractant protein-1 (MCP-1, CCL-2) were from Euromedex.

Antibodies

Mouse monoclonal antibodies specific to PrP^C (SAF32, IgG2b; SAF34, IgG2b; SAF61, IgG2a) were purchased from SPI-BIO, mouse monoclonal antibody 6H4 (IgG1) was from Prionics. Antibodies to zonula occludens-1 (ZO-1; rabbit polyclonal) were purchased from Zymed, antibodies to β-catenin (rabbit polyclonal) and to VLA-4 (CD49d; mouse monoclonal, IgG1) were from Euromedex, mouse monoclonal antibody HEC-7 anti-PECAM-1 (IgG1) was from Perbio Science France, and mouse monoclonal antibody 3A12 anti-PECAM-1 (IgG1) was from BD Biosciences. Antibodies to platelet endothelial cell adhesion molecule-1 (PECAM-1; M20, goat polyclonal) and to caveolin-1 (mouse monoclonal, IgG1) were from TEBU. Antibodies to transferrin receptor (CD71; mouse monoclonal, IgG1) were from R&D Systems. Peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies and ECL reagents were from Amersham, Cy2- or Cy3-conjugated anti-mouse or anti-rabbit antibodies from Jackson ImmunoResearch Laboratories, and Alexa Fluor488-conjugated anti-mouse antibodies were from Invitrogen.

Animals

Rats (OFA) and wild-type (WT) mice (C57BL/6J) were purchased from Charles River laboratories, PrP$^{-/-}$ mice (C57BL/6Jx129) (Bueler et al., 1992) were from CDTA. PrP$^{-/-}$ mice were inbred for at least nine generations onto the C57BL/6J background and used in experiments with age-matched wild-type C57BL/6J mice as control.

Isolation of brain microvessels and primary culture of endothelial cells

All animals were treated according to protocols evaluated and approved by the local ethical committee of INSERM, France. Isolation of brain microvessels and primary culture of endothelial cells were performed as previously described (Perrière et al., 2005) for rat and, with minor modifications, for mouse. Briefly, 8-10 8-week-old mice were used for brain microvessel isolation. Mouse cerebral cortices free of meninges were digested in a mixture of collagenase (270 U/ml), dispase (0.1%) and DNAse (10 U/ml) for 30 minutes at 37°C, centrifuged and then incubated for another 10 minutes at 37°C with the same mixture. Endothelial cells were grown in EBM-2 medium supplemented with 20% plasma-derived bovine serum, Hepes (1 mM), glutamine (2 mM), bFGF (2 ng/ml for rat cells, 5 ng/ml for mouse cells), antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and hydrocortisone (1.4 μM); in addition, the medium was supplemented with puromycin (4 μg/ml for rat cells or 1 μg/ml for mouse cells) for the first 4 days in culture as previously described (Perriere et al., 2005). Microvessels were seeded on Thermanox coverslips coated with collagen type IV (0.1 μg/ml) for direct immunofluorescence assay. Microvessels were either fixed 1 hour after seeding or maintained in culture until a confluent monolayer of endothelial cells was obtained.

Cell cultures and isolation of human monocytes

This study used the RBE4 rat brain endothelial cell line that has been extensively characterized by us and others (Durieu-Trautmann et al., 1994; Etienne-Manneville et al., 2000; Roux et al., 1994) and the hCMEC/D3 human brain endothelial cell line we recently described (Weksler et al., 2005). Briefly, RBE4 cells were grown on plates coated with type I collagen in αMEM/F10 medium supplemented with 10% FCS, bFGF and G418; and hCMEC/D3 cells were grown on plates coated with type I collagen in EGM-2 medium. The U937 human monocytic cell line was obtained from P. Lutz (IPBS-CNRS, Toulouse, France) and grown in RPMI 1640 medium supplemented with 10% FCS.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat units, obtained from consenting donors, by gradient centrifugation on Ficoll-Paque (Amersham) using standard protocols. Monocytes were then isolated by magnetic negative cell sorting (Dynal Monocyte Negative Isolation Kit from Invitrogen), according to the manufacturer's protocol, after a 2-hour adhesion step on uncoated flasks at 37°C, 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS.

U373-MG human astrocytoma cells were obtained from the ATCC repository (LGC Promochem) and grown on DMEM with 4.5 g glucose (Invitrogen) supplemented with 10% FCS. Primary cultures of rat astrocytes were prepared as previously described (Perriere et al., 2005) and grown on DMEM with 4.5 g glucose supplemented with 10% FCS.

Immunohistochemistry

Brain tissue was obtained from 5-month-old wild-type (WT) or PrP$^{-/-}$ C57BL/6J mice. According to protocols evaluated and approved by the local ethical committee of INSERM, France, the mice were deeply anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde in 0.1 M Na_2HPO_4/NaH_2PO_4

buffer, pH 7.5. After perfusion, the brains were collected, postfixed and cut with a vibratome. Sections (30 μm) were then processed for immunohistochemistry. After H_2O_2 treatment, non-specific antibody binding was blocked with 3% normal goat serum and incubated with primary antibodies directed to ZO-1 (5 μg/ml) or PrP^C: SAF 32 (1 μg/ml) plus SAF 61 (1 μg/ml) for 16 hours at 4°C. After washing with Tris buffer saline (TBS), sections were then incubated for 45 minutes at 25°C with Cy3-conjugated anti-rabbit and Alexa Fluor 488-conjugated anti-mouse antibodies (1/400) in 3% normal goat serum. Negative controls were obtained by omission of primary antibodies. Sections were then rinsed three times in TBS and mounted under coverslips using Vectashield with DAPI (Vector Laboratories). Immunofluorescence images were collected with a confocal fluorescence microscope (LEICA TCS SP2).

Immunofluorescence analysis

Immunofluorescence analyses were conducted as previously described (Cazaubon et al., 1997) with brain microvessels, primary cultures of brain endothelial cells and endothelial cell lines seeded on Thermanox coverslips. In all cases, cells were fixed with paraformaldehyde (4%) in phosphate-buffered saline (PBS) for 10 minutes, protected with glycine 0.1 M for 15 minutes and blocked with PBS containing bovine serum albumin (BSA, 2%) for 1 hour. Alternatively, for detection of intracellular antigens, cells were permeabilized with 0.05% saponin for 1 hour. The cells were then incubated for 16 hours at 4°C with monoclonal antibodies specific to PrP^C (2 μg/ml), PECAM-1 (2 μg/ml) or ZO-1 (1 μg/ml). Cy2- or Cy3-conjugated anti-rabbit or anti-mouse antibodies were used as secondary antibodies. Negative controls were obtained by omission of primary antibodies. Immunofluorescence images were collected with a confocal fluorescence microscope (LEICA TCS SP2).

RT-PCR

Total RNA was isolated from confluent monolayers of RBE4, hCMEC/D3 and U373 cells, as well as primary cultures of rat astrocytes, using Trizol (Invitrogen) according to the manufacturer's instructions. PCR amplification was carried out from 10 μg total RNA after reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen) according to standard protocols. Primers for PCR amplification of rat PrP^C were 5′-GTGCACGACTGTGTCAAT-3′ (forward) and 5′-CTCCTCATCCCACGATCAGG-3′ (reverse), yielding an expected product size of 244 bp. Primers for PCR amplification of human PrP^C were 5′-GTGCA-CGACTGCGTCAAT-3′ (forward) and 5′-CCTTCCTCATCCCACTATCAGG-3′ (reverse), yielding an expected product size of 243 bp. Standardization was performed using primers 5′-CCTGCTGGATTACATTAAAGCGCTG-3′ (forward) and 5′-CCTGAAGTGCTCATTATAGTCAAGG-3′ (reverse) for rat hypoxanthine-guanine phosphoribosyl transferase (HPRT) or primers 5′-GGAGAAGGCTG-GGGC-3′ (forward) and 5′-GATGGCATGGACTGTGG-3′ (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR amplifications. All primers were synthesized by Eurogentec. PCR conditions were as follows: denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 1 minute; 50°C (rat) or 60°C (human) for 1 minute; 72°C for 1 minute; and an extension step of 72°C for 7 minutes.

Flow cytometry

Fluorescence-assisted cell sorting (FACS) assay was performed following standard protocols. Briefly, U937 cells were washed, saturated with 2% BSA and incubated for 10 minutes at 25°C with the indicated antibodies at 2 μg/ml, followed by FITC-conjugated anti-mouse secondary antibody; omission of first antibodies was used as control. Acquisition was performed on an Epics XL cytometer (BD Biosciences).

Detergent-free purification of caveolae and western blot analysis

RBE4 or hCMEC/D3 cells grown to confluence were used to prepare caveolae fractions as previously described (Teixeira et al., 1999). Briefly, cells were washed in ice-cold PBS and scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenization was carried out using a loose-fitting Dounce homogenizer (20 strokes) and a sonicator (three bursts of 20 seconds). The homogenate was then adjusted to 40% sucrose by addition of 2 ml 80% sucrose solution prepared in 25 mM MES pH 6.5, 0.15 M NaCl (MBS buffer), placed in the bottom of an ultracentifuge tube and a 5-30% discontinuous sucrose gradient was formed above. After centrifugation at 200,000 ***g*** for 16-20 hours in an SW41 rotor (Beckman Instruments), a light-scattering band confined to the 5-30% sucrose interface was observed that contained caveolin-1. Gradient fractions were analyzed by western blotting as previously described (Cazaubon et al., 1997), using the following antibodies: anti-caveolin-1 (0.5 μg/ml), anti-PrP^C (1 μg/ml), anti-PECAM-1 (1 μg/ml) and anti-β-catenin (1 μg/ml).

Glycosidase digestion

RBE4 cells were scrapped onto 500 μl of 1% NP40 detergent lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl and protease inhibitors (leupeptine, aprotinin and pepstatin at 2 μg/ml each). Lysates were then centrifuged to remove nuclei and cell debris, and digested with PNGase F according to manufacturer's instructions. Briefly, cleared lysates (500 μl) were incubated with denaturing buffer for 10 minutes at 100°C, followed by incubation with 2500 units of enzyme in appropriate buffer for 1 hour at 37°C. As a control, lysates were incubated in parallel without enzyme. Samples (10 μl) were analysed by western blotting, as described above, using anti-PrP^C antibodies.

Adhesion of U937 monocytic cells to brain endothelial cells

U937 human monocytic cells were fluorescently labeled with 10 μM 5-chloromethylfluorescein diacetate (CMFDA) for 30 minutes at 37°C and were subsequently washed in EBM-2 medium with 0.1% BSA. Fluorescently labeled U937 cells (10^5 per well) were incubated with non-stimulated human brain endothelial cells hCMEC/D3 or hCMEC/D3 cells pre-treated for 24 hours with IFN-γ (200 U/ml) plus TNF-α (200 U/ml), and were allowed to adhere for 30 minutes at 37°C. When indicated, U937 cells and hCMEC/D3 monolayers were pre-treated for 1 hour at 37°C with various blocking or control antibodies (20 μg/ml) before the adhesion assay. After the incubation, non-adherent U937 cells were removed by washing with PBS and the adherent cells were hypotonically lysed. The proportion of adherent U937 cells was determined by quantification of the amount of fluorescence released using a FUSION fluorescent plate reader (Perkin Elmer) with an excitation wavelength of 492 nm and an emission wavelength of 517 nm.

Trans-endothelial migration of U937 cells and freshly isolated monocytes

hCMEC/D3 cells (10^5 cells/cm^2) were seeded onto type I collagen-coated 6.5 mm Transwell culture inserts with a pore size of 3 μm (Corning) and grown for 3 days in 5% CO_2 at 37°C. Prior to the assays, monolayers were stimulated with 200 U/ml IFN-γ plus 200 U/ml TNF-α for 24 hours and washed twice with EBM-2 medium with 0.1% BSA.

U937 monocytic cells or freshly isolated human monocytes were fluorescently labeled with 10 μM of CMFDA at 37°C for respectively 30 minutes or 45 minutes. Fluorescently labeled U937 cells (10^6 cells/ml in 100 μl of EBM-2 medium) or freshly isolated monocytes (2.5×10^5 cells/ml in 100 μl of RPMI medium) were added to the apical chamber. A chemotactic gradient was created by addition to the lower chamber of SDF-1α (100 ng/ml) or MCP-1 (25 ng/ml), respectively. U937 cells or monocytes were allowed to migrate at 37°C for 16-18 hours or 3 hours, respectively.

When indicated, U937 cells, monocytes and/or hCMEC/D3 monolayers were pre-incubated for 1 hour at 37°C with various blocking or control antibodies (20 μg/ml) before the migration assay. Transmigrated, fluorescently labeled U937 cells or monocytes were then recovered from the lower chambers and counted by flow cytometry, using an Epics XL cytometer (BD Biosciences).

Statistical analysis

Data are expressed as means ± s.e.m. Student's *t* or Mann-Whitney *U* tests were performed for statistical data analysis. For statistical significance (*P* value), see legend of respective figure.

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