Inflammatory mediators reduce surface PrPc on human BMVEC resulting in decreased barrier integrity

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Abstract

The cellular prion protein (PrPc) is a surface adhesion molecule expressed at junctions of various cell types including brain microvascular endothelial cells (BMVEC) that are important components of the blood brain barrier (BBB). PrPc is involved in several physiological processes including regulation of epithelial cell barrier function and monocyte migration across BMVEC. BBB dysfunction and disruption are significant events in CNS inflammatory processes including HIV neuropathogenesis. TNF-α and VEGF are two inflammatory factors that have been implicated in the processes that affect BBB integrity. To examine the effect of inflammation on PrPc expression in BMVEC, we used these mediators and found that TNF-α and VEGF decrease surface PrPc on primary human BMVEC. We also showed that these factors decrease total PrPc protein as well as mRNA, indicating that they regulate expression of this protein by de novo synthesis. To determine the effect of PrPc loss from the surface of BMVEC on barrier integrity, we used small hairpin RNAs to knockdown PrPc. We found that the absence of PrPc from BMVEC causes increased permeability as determined by a FITC dextran permeability assay. This suggests that cell surface PrPc is essential for endothelial monolayer integrity. To determine the mechanism by which PrPc downregulation leads to increased permeability of an endothelial monolayer, we examined changes in expression and localization of tight junction proteins, occludin and claudin-5, and found that decreased PrPc leads to decreased total and membrane associated occludin and claudin-5. We propose that an additional mechanism by which inflammatory factors affect endothelial monolayer permeability is by decreasing cell associated PrPc. This increase in permeability may have subsequent consequences that lead to CNS damage.
Introduction

The cellular prion protein (PrP<sub>c</sub>) is the non-pathogenic cellular isoform of human prion protein that is constitutively expressed in CNS cells. PrP<sub>c</sub> is an adhesion molecule and has several proposed functions including facilitating monocyte transmigration across endothelium and intracellular signal transduction. It is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein found in membrane raft microdomains with several signaling molecules including Src-family kinases, suggesting that it might be part of a signaling complex. This protein is present in unglycosylated, monoglycosylated, and diglycosylated forms and can have approximately sixty different sugars attached to it, enabling it to interact with several ligands for diverse functions.

In the CNS PrP<sub>c</sub> is abundantly expressed in neurons, microglia, and cells of the blood brain barrier (BBB), including astrocytes and brain microvascular endothelial cells (BMVEC). It is present on brain endothelial cells of mouse, rat, and human origin, localized in raft/caveolae-like membrane microdomains and concentrated at intercellular junctions of cells. It is hypothesized that the junctional localization of PrP<sub>c</sub> is dependent upon homophilic interactions between PrP<sub>c</sub> on two adjacent cells. The interaction of PrP<sub>c</sub> between two endothelial cells as well as between endothelial cells and monocytes was also shown to be essential for the migration of monocytes across an endothelial monolayer.

BMVECs are a major component of the BBB. The integrity of the BBB is essential to CNS homeostasis by controlling the transmission of biochemical signals and the transmigration of leukocytes from blood into the CNS in a regulated manner. The BBB also excludes certain soluble factors from the CNS while allowing specific nutrients to transport in and out of the brain. During neuroinflammation, the BBB responds by remodeling of junctional proteins in response to intracellular signaling that will result in endothelial retraction. Disruption of the BBB leads to increased transmigration of leukocytes, impaired CNS homeostasis, and pathogen entry that can lead to neurologic compromise.

In enterocytes, PrP<sub>c</sub> co-localizes with E-cadherin and interacts with several desmosomal proteins, suggesting that it contributes to the adhesion and barrier function of intestinal epithelial cells. Studies in these cells demonstrate that the absence of this protein leads to the mislocalization of tight junction (TJ) proteins that is accompanied by increased paracellular permeability.

In this study we examined the effect of TNF-α and VEGF on BMVEC expression of PrP<sub>c</sub> and how changes in PrP<sub>c</sub> expression altered endothelial monolayer integrity and permeability. TNF-α is associated with BBB disruption and barrier permeability. It is increased in the CNS in response to various pathogens as well as in neurodegenerative diseases including Alzheimer’s (AD), Parkinson’s (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and HIV infection of the CNS. TNF-α increases production of the cytokines IL-6, CCL2, and IL-8 from CNS cells and these dysregulate adhesion protein expression and increase leukocyte transmigration across the BBB and accumulation within the CNS. In endothelial monolayer BBB models, TNF-α has been
shown to cause increased permeability by activating NF-κβ signaling, resulting in claudin-5, occludin and ZO-1 downregulation.  

Another factor implicated in BBB breakdown is vascular endothelial growth factor (VEGF). VEGF is the most potent angiogenic factor and is involved in vasculogenesis during embryonic development and vascular injury. In addition to its importance in angiogenesis, it has been shown to be neuroprotective in rodent models of neurodegeneration, indicating that its role in the CNS is complex. In the inflamed CNS, VEGF is produced by reactive astrocytes and this growth factor has been shown to contribute to loss of brain microvascular integrity by decreasing the tight junction proteins claudin-5 and occludin. VEGF promotes VE–cadherin endocytosis in a β-arrestin dependent manner as well as VE-cadherin phosphorylation, resulting in its disassembly. This adherens protein is essential for BBB integrity and its dysregulation by VEGF can lead to increased BBB permeability.

We used these inflammatory factors in our study to examine the regulation of PrP in the context of HIV CNS infection and its role in HIV neuropathogenesis. HIV enters the CNS within the first 2 weeks of primary infection mainly through infected monocytes crossing the BBB, and results in increased cytokine production, in particular TNF-α. Increased VEGF has also been shown in reactive astrocytes during HIV neuropathogenesis.

In this study, we showed that TNF-α and VEGF decrease cell surface PrP in BMVEC. These factors also decreased PrP mRNA suggesting that they regulate de novo synthesis of this protein. We hypothesized that decreased PrP expression will lead to endothelial monolayer integrity disruption and showed that PrP knockdown in BMVEC leads to increased permeability in monolayers of BMVEC lacking PrP. We also showed that loss of PrP leads to decreased expression of tight junction proteins occludin and claudin-5, suggesting a mechanism by which PrP knockdown leads to increased permeability of BMVEC monolayers. Therefore, we propose that during CNS disorders, inflammatory mediators that decrease PrP expression in BMVEC contribute to CNS damage by increasing BBB permeability.

**Materials and Methods**

**Brain microvascular endothelial cell culture and treatment**

Human brain microvascular endothelial cells (BMVEC) (Applied Cell Biology Research Institute, Kirkland, WA) were grown in M199 media, supplemented with 20% heat inactivated newborn calf serum, 1% penicillin- streptomycin (all from Thermo Fisher Scientific, Waltham, MA), 0.8% heparin (Sigma, St. Louis, MO), 5% heat inactivated human serum AB (Lonza, Walkersville, MD), 0.1% ascorbic acid (Sigma, St. Louis, MO), 0.25% endothelial cell growth supplement (Sigma, St. Louis, MO), and 0.06% bovine brain extract (Clonetics, Walkersville, MD). Recombinant TNF-α (PeproTech, Rocky Hill, NJ) and recombinant VEGF (R&D, Minneapolis, MN) were added directly to the cell culture media to a final concentration of 10 ng/ml and 100 ng/ml, respectively.
Western blotting

BMVEC were grown to confluence on 0.2% gelatin (Fisher Scientific, Pittsburgh, PA) coated 60mm tissue culture dishes. Cells were lysed with cell lysis buffer (Cell Signaling, Boston, MA) supplemented with protease inhibitor cocktail (Roche, Indianapolis, MN). Total cellular protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Forty micrograms of cell lysate were loaded onto 4–20% polyacrylamide gels (Bio-Rad), separated by electrophoresis, and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburgh, PA). Membranes were blocked for 2h at room temperature with 5% nonfat dry milk and 3% BSA in Tris-Buffered Saline and 0.1% Tween 20 (TBST). Blots were probed with antibody against PrP\(^c\) (SAF 32 clone, 1:200 dilution, Cayman Chemicals, MI) overnight at 4°C, washed with TBST, and probed with anti-mouse IgG-HRP secondary antibody, 1:2000 dilution (Cell Signaling) for 1h at room temperature. Signal was detected using Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA). Blots were stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific), and reprobed with antibody against GAPDH, 1:500 dilution, (Cell Signaling), for 1h at room temperature, washed with TBST and probed with anti-mouse IgG-HRP secondary antibody (1:2000 dilution) for 1h at room temperature. Signal was detected using Western Lightning Plus-ECL. Data were quantified by densitometry using UN-SCAN-IT software (Silk Scientific, Orem, UT).

Flow cytometry

Surface PrP\(^c\) was analyzed by flow cytometry using a fluorochrom-coupled mAb specific for human PrP\(^c\) (eBioscience, San Diego, CA) and a corresponding isotype-matched (mouse IgG1K) negative control antibody (eBioscience). Antibodies were titered to determine optimal concentration for staining (0.25ug/100ul). BMVEC were dissociated with TrypLE (Thermo Scientific) and 1 ×10\(^5\) cells were washed with FACS buffer, calcium and magnesium free PBS supplemented with 1% BSA (Thermo Fisher Scientific). Cells were incubated in the dark on ice for 30 min with antibodies. Following staining, cells were washed with FACS buffer and fixed with 2% paraformaldehyde. 1×10\(^5\) events were acquired with a BD FACS Canto II flow cytometer and analyzed with FlowJo software (TreeStar, Ashland, OR).

RNA isolation and qRT-PCR

Total RNA was isolated from BMVEC (1 ×10\(^6\)) by Trizol (Life Technologies, Carlsbad, CA) extraction according to the manufacturer’s instructions and quantified by Nanodrop (Thermo Scientific, Wilmington, DE). cDNA synthesis was performed using SuperScript VILO cDNA synthesis kit (Life Technologies). Relative mRNA expression of PrP\(^c\), 18S, and GAPDH was determined using a Taqman Gene Expression Assay (Life Technologies). Results are represented as relative expression of PrP\(^c\) normalized to 18S and/or GAPDH as housekeeping genes using 2^-ΔCt_. The ΔCt value was determined by subtracting the average Ct of the housekeeping gene from the average Ct of PrP\(^c\).
PrP<sub>c</sub> knockdown with shRNA

shRNAs corresponding to the human PRNP gene were synthesized by the shRNA Core at Albert Einstein College of Medicine. These shRNA clones were built on the pGIPZ vector<sup>53</sup> that encodes GFP and a puromycin selection marker making it possible to monitor and select transfected cells. To generate PrP<sup>c</sup> knockdown cell lines, BMVEC were infected with three lentiviruses carrying three different shRNA’s, with an MOI of 80 in the presence of polybrene (5ug/ml) (Sigma) for 48h. Cells were grown to confluence in fresh media for an additional 48h. Transfected cells were selected with puromycin (0.5ug/ml) (Sigma) and maintained in puromycin containing media. Three different cell lines with different shRNA sequences were generated. The specific shRNA sequences used were 5’ TGCATGTTCTTGTTTTGTT 3’ for PrP<sup>c</sup> KD1, 5’GACATATTCACAGTGAACA 3’ for PrP<sup>c</sup> KD2, and 5’ TGCGTCAATATCACAATCA 3’ for PrP<sup>c</sup> KD3. These three shRNAs target different regions in the 3’UTR of the PRNP gene. A lentivirus with a pGIPZ vector with no PRNP shRNA was used as a control. To assay for knockdown of PrP<sup>c</sup>, we determined the level of PrP<sup>c</sup> mRNA in the different cell lines and found mRNA reduction of 90%, 25% and 80% in PrP<sup>c</sup> KD1, PrP<sup>c</sup> KD2, and PrP<sup>c</sup> KD3, respectively. We also assayed for reduction in surface PrP<sup>c</sup> by flow cytometry and found 90%, 40% and 86% reduction in surface PrP<sup>c</sup> in PrP<sup>c</sup> KD1, PrP<sup>c</sup> KD2, and PrP<sup>c</sup> KD3, respectively.

Permeability measurement

Transfected BMVEC were plated on tissue culture inserts with 3μm pores (Corning, Corning, NY) at 4 x10<sup>4</sup> cells per insert and grown to confluence for 3–4 days. Cultures were maintained in puromycin containing media. Inserts were washed with phenol red-free DMEM (Thermo Fisher Scientific) and placed in 24-well tissue culture plates containing 400μL of phenol red-free DMEM/10% FBS in each well. Dextran-FITC (125 μg/mL, 70kDa, Sigma) (200µL) was added to the top of the insert, and after 5 min at 37°C, media was collected from the lower chamber and fluorescence was analyzed with a fluorescence-detecting plate reader (excitation λ 488 nm; emission λ 510 nm).

Immunofluorescence

Primary BMVEC were grown to confluence on 0.2% gelatin (Fisher Scientific) coated 35 mm ibiTreat dishes (ibidi USA, Madison, WI). Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 min and permeabilized in 0.01% Triton X-100 for 1 min. Blocking was performed for 2 h at room temperature with 5 mM EDTA (Thermo Fisher Scientific), 1% fish gelatin (Sigma), 1% essentially immunoglobulin-free BSA (Sigma), 1% heat-inactivated human serum type AB (Lonza), and 1% goat serum (Vector, Burlingame, CA, USA) in deionized water. Cells were incubated overnight at 4°C in primary antibody anti-occludin, (Abcam, Cambridge, MA), and anti-claudin-5 (Santa Cruz Biotechnology, Dallas, TX) diluted 1:500 and 1:300, respectively. Cells were washed 3 times with PBS at room temperature and incubated with the appropriate secondary antibodies conjugated to Cy3, and labeled with phalloidin to identify the shape of the cells. ibiTreat dishes were then mounted using antifade reagent with DAPI to identify nuclei and the cells were then examined by confocal microscopy using a Nikon A1 confocal microscope with spectrum detection (Nikon, Tokyo, Japan). Due to the nature of the
distribution of occludin and claudin-5 in BMVEC, we identified XY areas that were positive for PrP<sup>c</sup> siRNA (areas that were GFP positive) and stained as described above. After identifying the cells that were positive for PrP<sup>c</sup> siRNA, we characterized the intracellular and membrane expression by positivity for actin filament and membrane expression by lack of positivity for actin. Using the previous acquired XY coordinates with GFP from the PrP<sup>c</sup> siRNA, we compared the expression in areas with or without knockdown using NIS elements by quantifying the numbers of positive pixels for each location.

Results

TNF-α and VEGF decrease surface PrP<sup>c</sup> from BMVEC

BMVEC monolayers are characterized by the presence of intercellular TJ and adhesion proteins, and are a major component of the BBB. The BBB contributes to cerebral homeostasis and controls leukocyte migration, as well as pathogen invasion, into the brain. Immunofluorescence, as detected by confocal microscopy, showed that PrP<sup>c</sup> was localized to junctions between adjacent endothelial cells similar to the adhesion protein platelet endothelial cell adhesion molecule-1 (PECAM-1), suggesting that PrP<sup>c</sup> may be part of endothelial adhesion complexes that maintain integrity of the endothelial monolayer. In intestinal epithelial cells, PrP<sup>c</sup> localizes to adhesion complexes as surface PrP<sup>c</sup> has been shown to regulate intestinal barrier functions.

TNF-α increases BBB permeability directly by downregulating TJ proteins occludin, claudin-5, and ZO-1 and indirectly by inducing the production of chemokines and cytokines that regulate TJ and AJ proteins and affect barrier integrity. VEGF decreases endothelial barrier permeability by downregulating claudin-5 and occludin. We hypothesized that another mechanism by which these inflammatory factors disrupt TJ formation and BBB integrity is by changing the surface expression of PrP<sup>c</sup>.

To determine the effect of TNF-α and VEGF on cell surface PrP<sup>c</sup>, human BMVEC were cultured to confluence on 0.2% gelatin coated tissue cultures plates. Cultures were treated with VEGF (100ng/ml) or TNF-α (10ng/ml) for 18h and 24h. Earlier time points, 30 min, 6h and 12h were examined and we determined that significant decreases in PrP<sup>c</sup> expression occur starting at 18h post treatment. Cells were dissociated from plates using TrypLE and stained with PrP<sup>c</sup> antibody or isotype matched control antibody and analyzed by flow cytometry.

Fold changes in PrP<sup>c</sup> mean fluorescent intensity after TNF-α or VEGF treatments were compared with control untreated cells, which was set to one. Changes in PrP<sup>c</sup> mean fluorescence were determined by subtracting the mean fluorescent intensity of the isotype matched negative control antibody from the mean fluorescent intensity of the PrP<sup>c</sup> specific antibody. TNF-α treatment decreased surface PrP<sup>c</sup> on human BMVEC at 18h and 24h as shown by a representative histogram (Figure 1A). Quantification of 3 independent experiments shows that TNF-α decreases PrP<sup>c</sup> by 0.5 and 0.4 fold at 18h and 24h, respectively (Figure 1B, **p < 0.01). Similarly, VEGF decreased surface PrP<sup>c</sup> on human BMVEC at 18h and 24h as shown by a representative histogram (Figure 1C). Quantification of 3 independent experiments shows that VEGF decreases PrP<sup>c</sup> by 0.4 fold at 18h and by 0.5
fold at 24h (Figure 1D, **p < 0.01). For both mediators, this is a 40–50% reduction, that are highly significant.

**TNF-α and VEGF decrease total PrPc in BMVEC**

To determine whether TNF-α and VEGF decrease surface PrPc by changing the localization of this protein or by decreasing total protein levels of PrPc, we treated BMVEC with these factors and studied changes in total PrPc protein by Western blotting. Cells were grown to confluence and treated with TNF-α and VEGF for 18h or 24h, after which cell lysates were prepared and changes in total PrPc were quantified. Densitometry was performed in which total PrPc was compared to a loading control, GAPDH, and these values were compared between control and TNF-α treated cells, and control and VEGF treated cells. Results are reported as fold change relative to control. We found that TNF-α decreased total PrPc by 0.4 fold (40%) at 18h and that PrPc protein levels returned to baseline by 24h (Figure 2A and 2B, *p < 0.05). VEGF decreased total PrPc in BMVEC by 0.2 fold (20%) at 18h and by 0.6 fold (60%) at 24h (Figure 2C and2D,*p < 0.05). These results suggest that during neuroinflammation, total PrPc in BMVEC is downregulated by inflammatory factors. We propose that this downregulation in turn may negatively impact endothelial function by decreasing barrier integrity.

**TNF-α and VEGF decrease PrPc gene expression in BMVEC**

To examine the mechanism by which TNF-α and VEGF regulate PrPc, we treated BMVEC with these mediators and analyzed PrPc mRNA by qRT-PCR. We found that TNF-α decreased PrPc mRNA at 12h and 18h by 0.2 fold (20%) and 0.4 fold (40%), respectively. By 24h, PrPc message returned to baseline (Figure 3A, *p < 0.05). VEGF decreased PrPc mRNA significantly at 12h, 18h, and 24h in BMVEC by 0.4 fold (40%), 0.5 fold (50%) and 0.6 fold (60%), respectively. (Figure 3B, *p < 0.05 and ***p< 0.0005). These results indicate that TNF-α and VEGF decrease de novo synthesis of PrPc.

**shRNA interference decreases surface PrPc on BMVEC**

To examine the specific role of PrPc in endothelial barrier integrity and function and the consequences of its downregulation, we generated 3 different PrPc knockdown BMVEC cell lines using shRNA interference. Cells were infected with lentivirus carrying shRNA against PRNP (the PrPc gene) and GFP and puromycin selection markers (as described in Materials and Methods). Control cells were infected with a lentivirus vector that did not contain PRNP shRNA. Cells were grown to confluence in puromycin containing media. Infections were performed 2 independent times with the 3 shRNAs described.

To confirm PrPc mRNA was reduced in the BMVEC, we analyzed PrPc mRNA in the 3 different cell lines by qRT-PCR. All cells lines were generated using the same donor of BMVEC, but unique shRNA sequences that target different regions of the 3'UTR of the PrPc gene. We found that shRNA knockdown of the PRPN gene resulted in a 90% reduction of PrPc mRNA in one cell line (PrPcKD1) as compared to the control cell line. In two other cell lines, shRNA knockdown resulted in 25% and 80% reduction of PrPc mRNA. These cell lines were termed PrPcKD2 and PrPcKD3, respectively (Figure 4A, *p < 0.05 and **p < 0.01).
We also analyzed changes in surface PrP<sup>c</sup> levels in the shRNA knockdown BMVEC lines by flow cytometry. BMVEC were dissociated from plates using TrypLE and stained with PrP<sup>c</sup> antibody or isotype matched control antibody and analyzed by flow cytometry. Changes in PrP<sup>c</sup> mean fluorescence were determined by subtracting the mean fluorescent intensity of the isotype matched negative control antibody from the mean fluorescent intensity of the PrP<sup>c</sup> specific antibody. We found that shRNA knockdown of the PRNP gene resulted in 90% reduction of cell surface PrP<sup>c</sup> in the cell line PrP<sup>c</sup> KD1. PrP<sup>c</sup> KD2 and PrP<sup>c</sup> KD3 showed 40% and 70% reduction of cell surface PrP<sup>c</sup>, respectively (Figure 4B and 4C, *p < 0.05 and **p < 0.01). Although all PrP<sup>c</sup> KD2 cells were GFP positive, indicating that they had lentivirus incorporated into their genome, only a percentage of these cells exhibited a decrease in PrP<sup>c</sup> as shown on the FACS histograms (Figure 4B). PrP<sup>c</sup> knockdown did not affect cell proliferation or viability as evidenced by counting of cells with trypan blue staining.

**PrP<sup>c</sup> contributes to barrier integrity in BMVEC**

To examine whether PrP<sup>c</sup> knockdown results in changes in permeability, control and PRNP shRNA transfected BMVEC were cultured on gelatin coated 3μm pore culture inserts placed in 24 well tissue culture plates. Changes in permeability of BMVEC monolayers of PrP<sup>c</sup> knockdown cell lines were assessed by the movement of dextran-FITC (70kDA) across the monolayers. Dextran-FITC (125 μg/mL) was added to the top of the insert, and after 5 min at 37°C, media was collected from the lower chamber and fluorescence determined. In cell lines that exhibited 90% knockdown of PrP<sup>c</sup> (PrP<sup>c</sup> KD1), permeability increased by 2 fold as compared to control cell lines. The cell line with a 25% decrease in PrP<sup>c</sup> mRNA (PrP<sup>c</sup> KD2) did not show any change in permeability, while PrP<sup>c</sup> KD3 showed a 1.5 fold increase in permeability (Figure 5, *p < 0.05 and **p < 0.0005). In previous studies, we have used other measurements of barrier integrity including transendothelial electrical resistance (TEER) and flux of albumin conjugated to Evans blue dye across monolayers. Measurement of movement of dextran-FITC across BMVEC monolayers is comparable to measurement with Evans blue dye conjugated to albumin, and therefore we used dextran-FITC to assess permeability in this study.

**PrP<sup>c</sup> knock down downregulates total and membrane associated expression of occludin and claudin-5.**

To examine the mechanism by which PrP<sup>c</sup> silencing leads to increased permeability of BMVEC monolayers, we studied the expression of occludin and claudin-5 in BMVEC which have decreased PrP<sup>c</sup>. In human Caco-2/TC7 enterocytes, downregulation of PrP<sup>c</sup> changes the localization and expression of tight junction proteins. We hypothesized that the absence of PrP<sup>c</sup> on BMVEC will lead to downregulation and redistribution of these TJ proteins on the cell surface which leads to a leaky barrier. To examine whether PrP<sup>c</sup> is required for expression and localization of occludin and claudin-5 in BMVEC, total expression and localization of these tight junction proteins in our KD lines was assessed by confocal microscopy and subsequent imaging analysis. We used actin as an intercellular marker. Proteins that did not localize with, but that were close to actin were considered membrane proteins. Control cells had 40–50% of their total occludin as membrane protein (Figure 6A, 6B). PrP<sup>c</sup> KD1 and PrP<sup>c</sup> KD3 had reduced total occludin expression ((Figure
Discussion

The blood brain barrier, consisting primarily of BMVEC and astrocyte foot processes, separates the brain from the periphery. TJ proteins, including occludin and claudin-5, connect adjacent BMVEC and restrict paracellular transmigration, rendering the barrier semi permeable.

PrPc is an adhesion molecule expressed at cell junctions in endothelial and epithelial cells. In brain endothelial cells isolated from mice, rats, and humans, this protein was localized at intercellular junctions between two adjacent cells. In the same study, anti-PrPc antibodies, blocked the migration of U937 human monocytic cell line and freshly isolated human monocytes across a human endothelial monolayer, suggesting PrPc is a junctional protein in BMVEC and that it is essential for monocyte migration across the BBB. PrPc is also localized at cell junctions in human enterocytes and keratinocytes. This junctional localization of PrPc is dependent on the expression of PrPc on two adjacent cells. In cocultures of PrPc expressing brain endothelial cells (WT) and PrPc KO cells, PrPc was expressed only between adjacent WT cells, suggesting that this protein is involved in homotypic interactions and contributes to intercellular adhesion by that mechanism.

In this study we examined the effect of neuroinflammation on cell associated PrPc in the context of HIV infection of the CNS. HIV enters the CNS and induces neuroinflammation, BBB dysfunction, and production of toxic factors that can collectively lead to neuronal injury. This process results in neurocognitive impairment in 40–60 % of HIV infected individuals, despite antiretroviral therapy (ART). Adhesion molecules such as PrPc are expressed on cells of the CNS and the BBB, as well as infiltrating leukocytes, and regulate CNS homeostasis. They act as membrane receptors, transduce intracellular signals, and promote cellular communication and adhesion. During HIV infection of the brain, adhesion molecule expression is dysregulated and many physiological processes regulated by these proteins are disrupted, contributing to CNS pathology.

Previously, we showed that, shed (sPrPc) is increased specifically in the CSF of HIV positive people with cognitive impairment as compared to HIV positive people with no cognitive impairment. This increase in sPrPc correlated with CSF CCL2 levels, indicating that PrPc shedding is increased during neuroinflammation. In addition, we also demonstrated that PrPc shedding from cultured human astrocytes is increased in the presence of inflammatory factors CCL2 and TNF-α. We characterized the mechanism by which PrPc is cleaved, which is by the A disintegrin and metalloproteinase domain-containing protein 10 (ADAM10).
Shed PrPc in turn, increased the release of cytokines and chemokines from astrocytes while decreasing glutamate uptake, suggesting an additional mechanism by which shed PrPc may play a role in neuroinflammation.

In cerebellar granule cells, PrPc has been shown to be localized in lipid rafts in a specific detergent resistant domain known as Prion domain. These cholesterol and sphingolipid enriched areas of the cell membrane are suggested to be important to stabilize PrPc in its native (α-helical) conformation but don’t seem to affect its cleavage. In addition, the conversion of PrPc to the scrapie form (PrPsc ) seems to occur in lipid rafts. Studies implicate ADAM10 and ADAM17 in the shedding of PrPc in the brain and specifically from neurons. However, no studies have been conducted in astrocytes to study the role of lipid rafts in cleavage or conformational stability of PrPc.

In this current study we examined the effect of inflammation on BMVEC cell surface PrPc expression by treating cells with TNF-α and VEGF, and identifying potential consequences of changes in this expression. TNF-α and VEGF are increased during neuroinflammation and have been shown to decrease BBB integrity. We found that both TNF-α and VEGF decrease surface PrPc. To determine the mechanisms for this decrease, we examined changes in PrPc protein and mRNA expression in response to these inflammatory factors. Both TNF-α and VEGF decrease PrPc total protein in BMVEC as well as PRNP expression, suggesting that these factors regulate the de novo synthesis of PrPc.

In our experiments we used TNF-α at 10 ng/ml, which is the concentration used in many in vitro studies. This has been shown to elicit responses in cultured cells that often reflect what can be detected in vivo. Although this value is higher than the physiological TNF-α levels of serum, plasma, and tissue, in pathological conditions serum concentrations of TNF-α of up to 50 ng/ml have been quantified. Similarly, serum VEGF levels in normal physiological conditions are lower than the concentration used in our study (100ng/ml). However, serum VEGF values as high as 1208 ng/ml have been shown in pathological states.

Previously PRNP, the gene encoding PrPc, was thought to be a housekeeping gene because of the absence of a TATA box. However, later evidence showed that this gene has binding sites for several transcription factors including NFAT, AP1, AP2, MEF2, and p53, indicating that PrPc expression may be regulated by various cellular factors. In human neuroblastoma cells, TNF-α and IL-β cause increased PrPc transcripts while IFN-γ decreased PrPc mRNA. In addition to cytokines, heat shock and oxidative stress have also been shown to increase PrPc expression.

The role of cell associated PrPc in intercellular adhesion and changes in the expression of this protein in response to inflammation have also been studied in other diseases such as irritable bowel syndrome (IBS). In intestinal epithelium PrPc localizes to cell junctions and in colonic epithelium of individuals with Crohn’s disease (CD) and ulcerative colitis (UC), hypothesized to be caused, in part, by disrupted barriers, levels of PrPc at cell junctions were decreased. These results suggest that PrPc is essential for barrier integrity and function of the intestine.
While the presence of PrP<sup>C</sup> at endothelial cell junctions was shown, its specific contribution to BMVEC monolayer integrity has not been determined. In the present study, we used lentivirus shRNA delivery to knockdown PrP<sup>C</sup> in BMVEC and examined the effect of PrP<sup>C</sup> loss on endothelial monolayer permeability. We found that cell lines that had low level surface PrP<sup>C</sup> formed monolayers that exhibited increased permeability as determined by a FITC-dextran permeability assay. In human Caco-2/TC7 enterocytes, PrP<sup>C</sup> silencing led to decreased barrier properties of this cell type by changing the localization and expression of junctional and adhesion proteins E-cadherin, claudin-5, and occludin. One potential mechanism by which PrP<sup>C</sup> knockdown increases BMVEC monolayer permeability is by changing the distribution and expression of tight junction and adhesion proteins. Both VEGF and TNF-α downregulate TJ proteins claudin-5, and occludin and increase endothelial barrier permeability. In this study we showed that VEGF and TNF-α decrease PrP<sup>C</sup> on BMVEC and that the absence of PrP<sup>C</sup> from these cells increases endothelial monolayer permeability. We propose that the downregulation of PrP<sup>C</sup> induced by VEGF and TNF-α will contribute directly to changes in TJ proteins affecting endothelial monolayer integrity.

Interestingly, in our studies, we found that only nearly complete knockdown of PrP<sup>C</sup> has an effect on the permeability of endothelial monolayers. These results suggest that during partial knockdown of this protein, the function of PrP<sup>C</sup> might be compensated by another redundant protein, possibly an adhesion or junction protein. However, when PrP<sup>C</sup> is completely absent, this compensatory mechanism might not be enough to compensate for the functional role of PrP<sup>C</sup> in barrier impermeability. Another possible mechanism is that there is excess PrP<sup>C</sup> on the cell surface and the amount of PrP<sup>C</sup> in cell lines that showed partial knockdown may be enough to maintain barrier integrity.

In intestinal epithelium, PrP<sup>C</sup> is important in maintaining the intestinal barrier by activating Src- signaling and regulating expression of TJ and adherence junctions (AJ) proteins including occludin, ZO-1 and E-cadherin. In epithelial cell lines including A431, MCF7, and Hela cells, PrP<sup>C</sup> regulates expression of E-Cadherin and B-catenin through a reggie-1 and EGF-R mediated pathway affecting cell-cell adhesion. However, in BMVEC, adhesion or tight junction proteins that are regulated by PrP<sup>C</sup>, or signaling molecules that are activated or modulated by this protein to affect endothelial barrier integrity have not yet been determined. Therefore, we examined whether knockdown of surface PrP<sup>C</sup> affects the expression and distribution of TJ proteins that will contribute to a more permeable barrier.

We found that PrP<sup>C</sup> knockdown results in decreased expression of occludin and claudin-5. Since PrP<sup>C</sup> is not a transmembrane protein, it is possible that it causes these changes in expression of proteins that are essential for membrane integrity by coupling with other adaptor proteins. In this study, we examined the effect of PrP<sup>C</sup> loss on the expression and localization of occludin and claudin-5. However, since both TNF-α and VEGF regulate the expression of ZO-1, we plan on expanding our studies to include the effects of PrP<sup>C</sup> loss on the regulation of ZO-1 expression.

An intact BBB is essential for maintaining brain homeostasis and highly selective endothelial permeability, and its impairment has been shown to lead to neuroinflammation. Tight junction and adhesion molecules including claudin-5, occludin, and PrP<sup>C</sup>, are essential for regulating endothelial permeability. Thus, proper localization and expression of these
proteins are critical for BBB integrity.\textsuperscript{19, 20, 22, 23} Our study shows that TNF-\(\alpha\) and VEGF, factors associated with BBB disruption, decrease PrP\textsuperscript{c} in BMVEC. PrP\textsuperscript{c} on the cell surface of BMVEC is essential for monolayer integrity and its loss increases permeability. PrP\textsuperscript{c} also regulates the expression and localization of TJ proteins claudin-5 and occludin which are important in maintaining a proper barrier. Therefore, a possible mechanism by which TNF-\(\alpha\) and VEGF increase BBB permeability is by downregulating PrP\textsuperscript{c}, which in turn will affect TJ protein expression and localization. This may result in imbalance of ions and transmitters, less efficient prevention of toxin and pathogen infiltration, as well as increased leukocyte diapedesis across the barrier, which can subsequently lead to neuroinflammation.\textsuperscript{88} In an \textit{in vivo} setting, a complex interaction of several cell types including astrocytes, microglia and macrophages leads to the neuropathogenesis that is subsequent to HIV infection of the CNS. Our \textit{in vitro} system in this study enables us to elucidate individual mechanisms which lead to transient permeability of the endothelial layer and blood brain barrier integrity loss. Future studies will examine the effect of inflammation on BBB permeability and cognitive function in PrP\textsuperscript{c} knockout mice in the context of HIV infection.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TNF-α and VEGF decrease PrP<sup>c</sup> surface expression on endothelial cells. Surface PrP<sup>c</sup> was analyzed by flow cytometry after treatment with TNF-α (10ng/ml) or VEGF (100ng/ml) (A). Representative plot showing the change in surface PrP<sup>c</sup> determined by FACS analysis after treatment with TNF-α for 18h and 24h. Three independent experiments were quantified (B). The fold change in mean fluorescence intensity (MFI) of PrP<sup>c</sup> on BMVEC after TNF-α treatment as compared to control was calculated after subtracting the contribution of the isotype matched negative control antibody. Surface PrP<sup>c</sup> after 18h and 24h of TNF-α treatment decreased by 0.5 fold (50%) and 0.4 fold (40%), respectively. (C) Representative plot showing the change in surface PrP<sup>c</sup> determined by FACS analysis after treatment with VEGF for 18h and 24h (D). The fold change in MFI of PrP<sup>c</sup> on BMVEC after VEGF treatment as compared to control was calculated after subtracting the contribution of the isotype matched negative control antibody. Surface PrP<sup>c</sup> after 18h and 24h of VEGF treatment decreased by 0.4 fold (40%) and 0.5 fold (50%), respectively. Significance was determined using a two-tailed paired Student t test. *p < 0.05, **p < 0.01.
Figure 2.
TNF-α and VEGF decrease total PrPc in endothelial cells. (A). Representative blot showing PrPc decrease in BMVEC treated with TNF-α (10ng/ml) for either 18h or 24h and lysates prepared to examine total PrPc by Western blotting. GAPDH was used as a loading control. Four independent experiments were quantified. TNF-α decreased total PrPc by 0.4-fold (40%) at 18h with PrPc returning to baseline at 24h. (C) Representative blot showing PrPc decrease in BMVEC treated with VEGF (100 ng/ml) for either 18h or 24h and lysates prepared to examine total PrPc by Western blotting. GAPDH was used as a loading control. VEGF decreased total PrPc by 0.2 fold (20%) at 18h and by 0.6 fold (60%) at 24h. Significance was determined using a two-tailed paired Student’s t test. *p < 0.05, **p < 0.01.
Figure 3.
TNF-α and VEGF decrease PrPc mRNA in endothelial cells. (A) BMVEC were treated with TNF-α (10ng/ml) for 12h, 18h, or 24h and mRNA levels of PrPc were evaluated using qRT-PCR. TNF-α decreased PrPc mRNA at 12h and 18h by 0.2 fold (20%) and 0.4 fold (40%), respectively, and PrPc mRNA returned to baseline at 24h, n=5. (B) BMVEC were treated with VEGF (100ng/ml) for 12h, 18h, or 24h and mRNA levels of PrPc were evaluated using qRT-PCR. VEGF decreased PrPc mRNA by 0.4 fold (40%), 0.5 fold (50%), and 0.6 fold
(60%) at 12h, 18h, and 24h respectively, n=3. Significance was determined using a two-tailed paired Student’s t test. *p < 0.05, **p < 0.01, ***p < 0.0005.
Figure 4.
Significant knockdown of PrP<sup>c</sup> in BMVEC with shRNA. (A) mRNA levels of PrP<sup>c</sup> were analyzed by qRT-PCR in control cells and 3 lines infected with PRNP shRNA. One cell line (PrP<sup>c</sup> KD1) showed 90% decrease in PrP<sup>c</sup> mRNA. The second cell line (PrP<sup>c</sup> KD2) and the third cell line (PrP<sup>c</sup> KD3) showed 25% and 65% fold decrease in PrP<sup>c</sup> mRNA, respectively. (B) Surface PrP<sup>c</sup> was analyzed by flow cytometry in control cell lines and 3 cell lines that were infected with lentivirus containing PRNP shRNA. (C) Change in the mean fluorescence intensity (MFI) of PrP<sup>c</sup> on BMVEC cell lines infected with PRNP shRNA as compared to control was calculated after subtracting the contribution of the isotype matched negative control antibody. PrP<sup>c</sup> KD1 showed a 90% decrease in surface PrP<sup>c</sup> while PrP<sup>c</sup> KD2 showed a 40% decrease in surface PrP<sup>c</sup> and PrP<sup>c</sup> KD3 showed a 70% decrease in PrP<sup>c</sup>.
Figure 5.
PrP<sup>c</sup> knock down results in increased BMVEC monolayer permeability. Permeability was measured by the passage of dextran-FITC (70kDA) across BMVEC monolayer. Transfected BMVECs were cultured on tissue culture inserts with 3 μm pores placed in 24-well tissue culture plates. Permeability of the barrier was measured by passage of dextran-FITC (70kDA) (125μg/ml) as described in the Materials and Methods section. The cell line with a 90% knock down of PrP<sup>c</sup> (PrP<sup>c</sup> KD1) showed a 2.0-fold increase in permeability as compared to control cell lines. PrP<sup>c</sup> KD2, which has a 40% knock down of PrP<sup>c</sup>, showed no increase in permeability while PrP<sup>c</sup> KD3, which has a 70% knock down of PrP<sup>c</sup>, showed a 1.6 fold increase in permeability. Significance was determined using a two-tailed paired Student’s t test. *p < 0.01, ***p < 0.0005.
Figure 6.
PrP<sup>c</sup> knock down downregulates total and membrane associated expression of occludin and claudin-5. Occludin and claudin-5 expression was measured by confocal microscopy and analyzed. Control and PrP<sup>c</sup> knock down cells were plated on ibdi dishes and fixed with PFA. Cells were stained using occludin and claudin-5 antibodies and analyzed for changes in expression and localization. (A) Cells were stained with DAPI to identify cell nuclei and labeled with phalloidin to identify the shape of the cells. PrP<sup>c</sup> KD1 and PrP<sup>c</sup> KD3 show reduced total occludin and cell membrane occludin. (B) To compare conditions, similar
number of cells (300 to 800 cells) and total area was analyzed. Control cells had 43% of their total occludin as membrane protein. PrPc KD1 had reduced membrane occludin (28%) while PrPc KD3 had 27% of their total occludin as membrane occludin. (C) Cells were stained with DAPI to identify cell nuclei and labeled with phalloidin to identify the shape of the cells. PrPc KD1 and PrPc KD3 show reduced total claudin-5 and cell membrane claudin-5. (D) To compare conditions, similar number of cells (300 to 800 cells) and total area was analyzed. Control cells had 32.64% of their total claudin-5 as membrane protein. PrPc KD1 had reduced membrane claudin-5 (25%) while PrPc KD3 had 22.65% of their total claudin-5 as membrane claudin-5. Significance was determined using ANOVA test. * p ≤ 0.001, # p ≤ 0.0002.