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SUBCELLULAR LOCALIZATION OF TRANSPORTERS ALONG THE RAT BLOOD–BRAIN BARRIER AND BLOOD–CEREBRAL-SPINAL FLUID BARRIER BY *IN VIVO* BIOTINYLATION

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Abstract—Nutrient transporters and ABC efflux pumps at the blood–brain barrier are major determinants of drug penetration into the brain. Immunohistochemical analysis of transporter subcellular localization is challenging due to the close apposition of the luminal and abluminal microvessel plasma membranes. We employed *in vivo* perfusion of biotinylation reagent through rat brain microvessels to domain-specifically label proteins exposed on the microvessel luminal surface. Using this approach, we analyzed the luminal/abluminal localization of a number of blood–brain barrier transporters identified by quantitative PCR profiling as being highly expressed and enriched in rat brain endothelial cells compared with whole brain. We also examined the apical/basal–lateral distribution of transporters in the choroid plexus, a secondary site for transport of nutrients between the blood and CNS. We detected P-glycoprotein (Pgp) (Abcb1), ATP-binding cassette (Abc) g2, multidrug resistance protein (Mrp) 4 (Abcc4), glucose transporter 1 (Glut1) (Slc2a1), Lat1 (Slc7a5), and monocarboxylate transporter-1 (Mct1) (Slc16a1) on the luminal surface of rat cerebral microvessels by both immunofluorescence staining and Western blotting of *in vivo* biotinylated proteins. Mrp1 (Abcc1) appeared primarily abluminal by immunofluorescence staining, and was barely detectable in the biotinylated protein fraction. Organic anion transporter (Oat) 3 (Slc22a8), organic anion transporter polypeptide (Oatp) 2b1 (Slco2b1, Oatpb), and Mrp5 (Abcc5) were not detected on the luminal surface using either method, while Oatp1a4 (Slco1a4, Oatp2) appeared to partially localize to the microvessel lumen by immunofluorescence staining, but was not detected in the biotinylated protein fraction by Western blotting. Lat1, Mrp1 and Mrp4 were detected on the basal–lateral surface of lateral ventricle choroid plexus epithelial cells. Mrp5, Oct3 and Oatp2b1 (Oatpb) were detected in the ependymal cells lining the ventricle. We did not detect Pgp expression in choroid plexus by immunofluorescence staining. *In vivo* biotinylation provides a method for domain-specific labeling of luminal surface proteins within the capillaries of the blood–brain barrier, allowing for biochemical analysis of protein localization and facilitating optical discrimination of the luminal and abluminal endothelial surfaces. **© 2008 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: cerebral microvessels, choroid plexus, luminal, abluminal, ABC transporter.

The blood–brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier denote the anatomical, biochemical, and transport mechanisms that regulate the access of molecules in the peripheral circulation to the CNS. Brain capillary microvessels make up the BBB, while choroid plexus epithelial cells form the blood–CSF barrier. Tight junctions between cells at these boundaries create physical barriers to diffusion of substances from the blood into the brain (Grant et al., 1998). Of equal importance is the repertoire of transporters, efflux pumps, and metabolic enzymes expressed by BBB microvessels and choroid plexus epithelial cells that protect the brain from toxic xenobiotics while selectively extracting nutrients and glucose from the bloodstream. Nutrient carrier systems actively transport small molecule drugs that are able to penetrate the brain efficiently, while efflux pumps create a biochemical barrier to drug penetration of the CNS (Pardridge, 2003). This compartmentalization is essential to protect the brain from substances in the peripheral circulation, yet presents a major impediment to the penetration of drugs into the brain (Pardridge, 2003). Brain microvessels of the BBB form the primary interface between the blood and the CNS, with a surface area of up to 5000 times that of the choroid plexus (Nag, 2003), yet drug transport across the choroid plexus into the CSF also can be a significant determinant of brain penetration (Pardridge, 1998).

To understand the contribution of specific transporters to drug penetration into the brain, it is necessary to determine which transporters are expressed at the BBB and blood–CSF barriers, and whether these transporters are localized on the luminal (blood-facing) or abluminal (brain-facing) surface of the microvessel endothelium, or in the case of the choroid plexus, on the basal (blood-facing) or apical (CSF-facing) plasma membrane. Determination of transporter expression is relatively straightforward, however, analysis of subcellular localization in microvessels by

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Abbreviations: Abc, ATP-binding cassette; BBB, blood–brain barrier; BMEC, brain microvessel endothelial cell; CSF, cerebrospinal fluid; CT, cycle threshold; DTT, dithiothreitol; EM, electron microscopy; FITC, fluorescein-isothiocyanate; GFAP, glial fibrillary acidic protein; Glut1, glucose transporter 1; HBSS, Hank's balanced salt solution; Mct1, monocarboxylate transporter-1; Mrp, multidrug resistance protein; NA, Neutravidin biotin binding protein; Oat, organic anion transporter; Oatp, organic anion transporter polypeptide; PBS, phosphate-buffered saline; Pecam-1, platelet-endothelial cell adhesion molecule-1; Pgp, P-glycoprotein; qPCR, quantitative polymerase chain reaction; sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; TBS-T, 20 mM Tris pH 7.4, 500 mM NaCl, 0.1% Tween-20; TfR, transferrin receptor; TX-100, Triton-X-100; Zo-1, zonula occludens-1.

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immunofluorescence microscopy is challenging because the endothelial luminal and abluminal plasma membranes are closely apposed; the distance separating these endothelial surfaces is reported to be as small as 100 nM (Davson, 1998), but generally accepted to be between 200 and 500 nm (Cornford and Hyman, 2005; Pardridge, 2005). In addition, membrane proteins may partially colocalize in intracellular compartments, and thus confound attempts to determine transporter localization by immunofluorescence microscopy. To circumvent these challenges, we employed *in vivo* biotinylation by vascular perfusion, previously described for labeling of vascular proteins in a variety of tissues (De La Fuente et al., 1997; Hoya et al., 2001; Zhou et al., 2005; Rybak et al., 2005), to label the population of protein exposed on the luminal surface of the brain vasculature, and the basal surface of the choroid plexus epithelium. This approach allows for domain-specific staining of the BBB endothelial luminal plasma membrane and choroid plexus epithelial basal plasma membrane for transporter localization by immunofluorescence microscopy. In addition, luminal proteins can be purified from BBB microvessel protein extracts via the biotin tag and analyzed by Western blotting, providing a complementary approach to microscopy for detection of proteins on the luminal microvessel surface.

EXPERIMENTAL PROCEDURES

Rat brain perfusion

All protocols involving animals were approved by the XenoPort Animal Use Committee in accordance with the recommendations outlined in the NIH Guide for the Care and Use of Laboratory Animals. Experiments were designed to minimize the number of animals used and to prevent animal suffering. Eight-week-old male Sprague–Dawley rats were anesthetized by i.m. injection of a mixture of 66.6 mg/kg ketamine and 3.3 mg/kg xylazine. Room temperature solutions were perfused using a programmable syringe pump at a rate of 2.5 ml/min through the left ventricle of the heart, draining from the right atrium, with the abdominal aorta clamped. Biotinylation solution was prepared by dissolving 2 mg EZ-Link sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin) (Pierce Biotechnology, Rockford, IL, USA) in tissue culture grade Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA, USA). Biotinylation solution was perfused to covalently label capillary luminal proteins with biotin, followed by perfusion with Tris-buffered saline (30 ml of 50 mM Tris pH 7.4, 150 mM NaCl) containing 5 mg/ml glycine to quench and remove the biotinylation reagent. Immediately after perfusion, the brain was removed and placed in HBSS on ice.

Isolation of cerebral microvessels and brain microvessel endothelial cells (BMEC)

For immunofluorescence and Western blotting, a non-enzymatic method of cerebral microvessel isolation was used that preserves capillary morphology and protein integrity. For quantitative polymerase chain reaction (qPCR) analysis, a collagenase digestion protocol that reduces neuronal and glial cell contamination of endothelial isolates was used. For both methods, cerebral cortices from 8-week-old male rats were minced with a razor blade on ice and homogenized by five strokes of a type A glass pestle in 30 ml HBSS in a 40 ml glass dounce homogenizer on ice. Microvessels or BMEC was isolated from the brain homogenate as described below. Cerebral cortex homogenate was also used for qPCR analysis.

Dextran solution was prepared by dissolving 180 g dextran from *Leuconostoc mesenteroides*, molecular weight 64,000–76,000 (Sigma-Aldrich, St. Louis, MO, USA) in 750 ml dH_2O, stirring with heat (85 °C) until completely dissolved. The volume was adjusted to 900 ml with dH_2O, the solution was autoclaved, then 100 ml 10× medium M199 (Invitrogen) and 10 ml 1 M Hepes were added, the pH was adjusted to 7.4, and the solution stored at 4 °C. Before use, the dextran solution was diluted in HBSS to a final concentration of 15% dextran for non-enzymatic microvessel isolation or 12.5% dextran for enzymatic BMEC isolation.

Non-enzymatic cerebral microvessel isolation

All steps were carried out on ice or at 4 °C. Homogenate from two to four rat brains was centrifuged at 1000×*g* for 5 min, and the cloudy supernatant decanted. The loose pellet was resuspended in 15% dextran (40 ml dextran solution for every two homogenized brains), and centrifuged at 2500×*g* for 20 min. The resulting floating myelin-rich layer and dextran supernatant were decanted into a clean tube, and the microvessel pellet was resuspended in 10 ml HBSS, transferred to a fresh tube, and kept on ice. The myelin layer was resuspended in the dextran supernatant by trituration and centrifugation step repeated once. The microvessels from each centrifugation were pooled in HBSS, then passed through 100 μm and 40 μm nylon mesh cell strainers (BD Biosciences, San Jose, CA, USA). Microvessels in the filtrate were pelleted at 1000×*g*, resuspended in 10 ml HBSS, and the number of recovered microvessel strands counted on a hemacytometer. The average yield was approximately 10^6 microvessel strands (two to five endothelial cells/strand) per rat brain. Substantial numbers of non-endothelial cells also were observed in the microvessel isolates. For immunofluorescence staining, a thin film of microvessel suspension was spread on untreated glass coverslips and incubated for 5 min on ice. The adherent microvessels were then fixed with either methanol for 5 min at −20 °C, or 4% paraformaldehyde in HBSS for 15 min at 4 °C, and then stored in HBSS at 4 °C until used for immunofluorescence staining. Alternatively, microvessels were pelleted, the supernatant was removed, and microvessel pellets were frozen on dry ice and stored at −80 °C until used for protein extraction.

Enzymatic isolation of BMEC

Homogenate from 10 rat brains was pelleted by centrifugation for 5 min at 1000×*g*, the cloudy supernatant was decanted, the loose pellet was resuspended in 100 ml warm medium M199 containing 1 mg/ml collagenase/dispase (Roche Applied Science, Indianapolis, IN, USA), and transferred to a 125 ml spinner flask. Tissue was incubated at 37 °C, 5% CO_2 with stirring at 50 RPM for 1 h. All subsequent steps were performed at 4 °C. Digested tissue was divided between two 50 ml conical tubes, pelleted by centrifugation at 1000×*g*, resuspended in 40 ml per tube of 12.5% dextran and centrifuged as described above for microvessel isolation. Pooled BMEC from each centrifugation were pelleted at 1000×*g* for 5 min, resuspended in HBSS, and filtered through 100 μm and 40 μm nylon mesh filters. BMEC in the filtrate were pelleted, resuspended in 2.5 ml HBSS, and layered atop a discontinuous 20%/50% discontinuous gradient of Percoll (Amersham Biosciences, Pittsburgh, PA, USA; 5 ml each concentration, prepared according to supplier's instructions). The gradient was centrifuged at 1000×*g* for 15 min, BMEC were collected from the 20%/50% Percoll interface, added to 10 ml HBSS, and pelleted. BMEC were resuspended in 5 ml HBSS and counted using a hemacytometer. The average yield was approximately 10^5 BMEC per rat brain. BMEC were pelleted, the supernatant was removed, and the cell pellet was frozen on dry ice and stored at −80 °C until used for qPCR analysis.

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