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THE CHARACTERIZATION OF ARACHNOID CELL TRANSPORT II: PARACELLULAR TRANSPORT AND BLOOD–CEREBROSPINAL FLUID BARRIER FORMATION

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Abstract—We used an immortalized arachnoid cell line to test the arachnoid barrier properties and paracellular transport. The permeabilities of urea, mannitol, and inulin through monolayers were 2.9 ± 1.1 × 10^{-6}, 0.8 ± .18 × 10^{-6}, 1.0 ± .29 × 10^{-6} cm/s. Size differential permeability testing with dextran clarified the arachnoidal blood–cerebrospinal fluid (CSF) barrier limit and established a rate of transcellular transport to be about two orders of magnitude slower than paracellular transport in a polyester membrane diffusion chamber. The theoretical pore size for paracellular space is 11 Å and the occupancy to length ratio is 0.8 and 0.72 cm^{-1} for urea and mannitol respectively. The permeability of the monolayer was not significantly different from apical to basal and vice versa. Gap junctions may have a role in contributing to barrier formation. Although the upregulation of claudin by dexamethasone did not significantly alter paracellular transport, increasing intracellular cAMP decreased mannitol permeability. Calcium modulated paracellular transport, but only selectively with the ion chelator, EDTA, and with disruption of intracellular stores. The blood–CSF barrier at the arachnoid is anatomically and physiologically different from the vascular-based blood–brain barrier, but is similarly subject to modulation. We describe the basic paracellular transport characteristics of this CSF ''sink'' of the brain which will allow for a better description of mass and constitutive balance within the intracranial compartment. Published by Elsevier Ltd. on behalf of IBRO.

Key words: cerebrospinal fluid, hydrocephalus, arachnoid, blood–CSF barrier, blood–brain barrier.

INTRODUCTION

Cerebrospinal fluid (CSF) is important for the central nervous system's support and maintenance. It buffers the nervous tissues mechanically and chemically, serves as a vehicle for the transport of important substances, and removes waste and debris (Carpenter, 1991). Arachnoidal CSF egress is considered one of the major routes of CSF removal (Upton and Weller, 1985). How this occurs is unknown, but given the aqueous nature of CSF, the primary mean is thought to be paracellular transport. Paracellular transport is governed by a variety of mechanisms and components: cell height, paracellular space tortuosity, tight junctions, and gap junctions, of which the tight junction may be the most crucial. As a major component of the blood–CSF barrier (BCB) and ''sink'' for CSF absorption, the arachnoid stands at an important junction between the vascular system and the intracranial space. This gateway is similar to the blood–brain barrier (BBB) in that polar substances and dyes are restricted in their movement (Wolpow and Schaumburg, 1972). Arachnoidal tissue exists in multiple forms including membranes, dense granulation caps, and porous central cores (Kida et al., 1988). The degree of intracellular or extracellular matrix pathway involvement in transport is probably dependent on this local structure of the arachnoid tissue, but preliminary tracer studies have shown that permeabilities of low molecular weight substances in confluent monolayers parallel that of the BBB (Holman et al., 2010; Lam et al., 2011). Tight junctions crucial to the integrity of the BCB and regulation of paracellular transport have also been seen in the arachnoid cell–cell contacts with transmission electron microscopy (Hasegawa et al., 1997). Holman recently demonstrated these junctional complexes on cultured arachnoid monolayers as well. These complexes maintain the pathway integrity in conditions of osmotic and pressure gradient challenges. The geometric path and the chemical makeup in the paracellular space could be altered during these conditions, regulating the outflow of fluid out of the brain

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Abbreviations: BBB, blood–brain barrier; BCB, blood–CSF barrier; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid.

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and the intracranial cavity probably by a combination of complex feedback systems involving second messengers and physical alterations in the cells.

Because of the similarities in permeabilities of ionic substances in arachnoid cell-mediated blood–CSF barrier and BBB, the demonstration of regulated flow through the arachnoid cell, and the importance of arachnoid cells in the removal of CSF from the brain, we characterize in detail the paracellular transport in an immortalized cell line of the arachnoid cells (see (Janson et al., 2011) for cell line description) and investigate the physiologic mechanisms that alter this transport in this paper. Two important mechanisms in tight junction regulation are the calcium and cAMP second messenger pathways (Karczewski and Groot, 2000; Deli, 2009). They regulate the various intercellular junctional proteins, such as claudin, as well as the intracellular cytoskeletal systems important for transport. Given the cell's barrier capabilities, we expect the cellular and molecular dynamics to be similar to the BBB. However, an overarching element of contrast between the arachnoid cells and the brain endothelial cells is that the "neurovascular unit" is absent (see (Hawkins and Davis, 2005) for review). Lacking the astrocytic, neuronal, and pericytic influences on the BBB junctional complex, we believe the mechanisms of the BCB maintenance to be under different control and regulation. To that end, we believe the control mechanisms to be simpler and to be less finely controlled. Therefore, we probe the junctional complexes in arachnoid monolayers directly and test its integrity with well-known second messenger perturbation methods in these sets of experiments.

EXPERIMENTAL PROCEDURES

Production of retrovirus containing SV40 LgTAg and hTERT and retroviral transduction

The generation of immortalized arachnoid cells has been previously presented.(Janson et al., 2011) Briefly, the retroviral constructs pBABE-neo-hTERT and pBABE-puro-SV40LT, containing hTERT and SV40 LTAg along with the G418 and puromycin resistance genes, were used to transfect the EcoPack2 cells (Clontech Mountain View, CA), which are 293HEK ecotropic feeder cells containing retroviral packaging genes. Cells were seeded 12–18 h prior to use at 5×10^5 cells in 25 cm^2 flasks. Then 5 mg of pBABE-puro-SV40LT was combined with the Fugene reagent (Roche Indianapolis, IN) and the OptiMem (Invitrogen) serum-free media in 250 ml total reaction volume with 3:1 ratio of Fugene:DNA. This was incubated for 30 min and added to each flask of 293HEK ecotropic feeder cells, with 3 ml of serum-containing media per flask.

Arachnoid cells were harvested from 21-to 23-day old male Sprague–Dawley rats. At passages 3 or 4, the arachnoid cells were replated into a Biocoat six-well plate (BD Biosciences San Jose, CA). Viral transduction was performed when the cells reached 60–80% confluence. Clarified viral supernatant containing BABE-puro-SV40LT was applied sequentially to the arachnoid cell primary culture. Target cells were initially at a density of 4×10^4 cells per well of a six-well plate. The media was aspirated and 3 ml virus-containing media was added per well. Polybrene (Millipore) was then added to a final concentration of 4 mg/ml. Cells transduced with pBABE-puro-SV40LT were selected over 14 days with puromycin (Sigma).

Histology and immunohistochemistry

Cells were permeabilized in 0.25% Triton X-100, blocked in 2% bovine serum albumin, and incubated with primary antibodies in PBS overnight at 4 °C. After rinsing with PBS (phosphate buffered saline), cells were incubated with appropriate secondary antibody and counterstained with DAPI. Images were analyzed using a Biorad MRC-1024 single photon confocal microscope 1024 (Biorad Cell Science, UK) (see Lam et al., 2011 for complete staining procedures). For establishing the presence of junctional proteins, the cells were stained with Claudin-1 (prod # RB-9209-PO, Thermo Scientific, West Palm Beach, FL) , connexin 45 (Prod #MAB3100, Millipore, Billerica, MA), ZO-1 (prod # ab59720 Abcam Inc., Cambridge, MA), and JAM-A (prod # 361700, Invitrogen, Carlsbad, CA). Actin (prod # 4970 Cell Signaling Technology, Danvers, MA) staining was also performed to show the cytoskeletal filaments.

TEM

The arachnoid cells were fixed on Transwell membranes with 3% glutaraldehyde solution in 0.1 M phosphate buffer. After glutaraldehyde fixation, the cells were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer containing 0.1 M sucrose (pH 7.4). They were then rinsed, stained (1% uranyl acetate overnight at 4 °C), and dehydrated in a graded series of ethanol.

The samples were incubated in two changes of hydroxypropyl methacrylate and placed in 100% resin overnight (Polybed 816), then embedded in fresh resin (Polybed 812; both from Polyscience, Warrington, PA) in a round silicon mold and polymerized at 60 °C for 24 h. The embedded samples were sectioned on an ultramicrotome (EM UC6; Leica) at 70 nm and stained with 2% uranyl acetate and Reynolds lead citrate. Samples were examined under a transmission electron microscope (Philips CM12 Transmission Electron Microscope) and photos taken with SIA L3C Digital Camera.

Functional transport assay: TEER, marker, and size differential transport study

4×10^5 immortalized rat arachnoid cells were plated in each of the 3 top wells of a 6-well Transwell (Corning Inc. Corning, NY; Cat No. 3450) (4.67 cm^2 per well and 0.4 μm pore size), and incubated for 3–5 days depending on when the cells became confluent. Three blank Transwells were used as negative controls. Separate 24-well plates Transwell (Corning Inc. Corning, NY; Cat No. 3470) (0.3 cm^2 per well and 0.4 μm pore size) were used for TEER (Trans Epithelial Electric Resistance) studies. Cells were plated at 3×10^4 cells per well and grown to confluency. Culture media in Transwell plates was removed and pre-warmed media was added into wells (apical 2 ml, basolateral 3 ml). The ^{3}H-mannitol stock solution was prepared as 1 ml radiolabeled mannitol per 1000 ml of assay buffer [LR1], for a final mannitol concentration of 82 nm and specific activity of 1 mCi/ml. The buffer was removed from the proximal (apical) compartments and pre-warmed radioactive solution was added to apical chambers, with 2 ml to each chamber. The plate was put on gentle shaker at 37 °C and 200 μl samples were taken from both compartments in each well at 15, 30, 60, 90, 120 min and replaced with an equal volume of respective buffer. Inulin-[Carboxyl-^{14}C] (50 mCi/mmol, Fisher Scientific Inc. Cat #1108650) and [^{14}C] Urea (54 mCi/mmol, Amersham, Piscataway NJ) were prepared as 1 ml of radiolabeled compound per 1000 ml assay buffer and tested in the same fashion. For the FITC (Fluorescein isothiocyanate)-labeled Dextran (10, 20, 40, and 70 kDa) (Cat No. 129K5304, 83797PJ, 069K5316. Sigma–Aldrich Co., St. Louis, MO) experiment, 1×10^5 immortalized rat arachnoid cells were seeded and tested in the same paradigm. All of the signals were measured