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Research Report

Expression of drug transporters at the blood–brain barrier using an optimized isolated rat brain microvessel strategy

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ABSTRACT

Quantitative RT-PCR (qRT-PCR) and Western blotting studies on transporters at the blood–brain barrier (BBB) of isolated brain microvessels have produced conflicting data on their cellular distribution. A major problem is identifying cells expressing the genes of interest, since isolated brain microvessels are composed of several cell types and may be contaminated with mRNA or proteins from astrocytes and neurons. We isolated rat brain microvessels and examined microscopically samples at each step of isolation to evaluate microvessel purity. The expression of specific markers of endothelial cells (Glut-1, Flk-1), pericytes (Ng2), neurons (synaptophysin, Syn) and astrocytes (Gfap) was measured by qRT-PCR in order to select the protocol giving the least astrocyte and neuron mRNAs and the most endothelial mRNAs. We also evaluated the gene expression of drug transporters (Mdr1a, Mdr1b, Mrp1–5, Bcrp and Oatp-2) at each step to optimize their location in cells at the BBB. The *Mdr1a*, *Mrp4*, *Bcrp* and *Oatp-2* gene profiles were similar to those of endothelium markers. The profiles of Mrp2 and Mrp3 closely resembled that of Ng2. Mrp5 and Mrp1 expression was not increased in the microvessel-enriched fraction, suggesting that they are ubiquitously expressed throughout the cortex parenchyma. We also evaluated by Western blotting the expression of P-gp, Mrp2, Gfap and Syn in the cortex and in the purest obtained microvessel fraction. Our results showed that P-gp expression strongly increased in microvessels whereas Mrp2 was not detected in any of the fraction. Surprisingly, Gfap expression increased in isolated microvessels whereas Syn was not detected. Our results showed that the strategy consisting of identifying gene expression at different steps of the protocol is useful to identify cells containing mRNA at the BBB and give overall similar results with protein expression.

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Abbreviations: ABC, ATP-binding cassette; BBB, blood–brain barrier; Bcrp, breast cancer resistance protein; Gfap, glial fibrillary acidic protein; Mrp, multidrug resistance-associated protein; Oatp-2, organic anion transporting-polypeptide 2; P-gp, P-glycoprotein; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Syn, synaptophysin

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1. Introduction

The blood–brain barrier (BBB) is the main interface between the bloodstream and the brain parenchyma controlling the passage of endogenous and exogenous substances into and out of the central nervous system (CNS). Brain capillaries have a variety of structural features, including a lack of fenestration and pinocytosis, tight junctions between capillary endothelial cells that reduces paracellular permeability of hydrophilic molecules (Gonzalez-Mariscal et al., 2003) and numerous polarized drug transporters (Golden and Pollack, 2003).

ATP-binding cassette (ABC) proteins, including P-glycoproteins (P-gp, Abcb1), multidrug resistance-associated proteins (Mrp or Abcc subfamily), the breast cancer resistance proteins (Bcrp, Abcg2), as well as several solute carrier (SLC) transporters such as Oatp-2 (Slc21a5) and Glut-1 (Slc2a1) are all present at the BBB and limit or facilitate the entry of solutes into the brain parenchyma, depending on their efflux or uptake transport properties. Expression and function of these drug transporters at the BBB have been widely investigated by measuring mRNA, protein and solute transport, but the exact cerebral vasculature cells containing them remain controversial. For example, P-gp was first found in the human brain capillary endothelial cells using brain slices (Cordon-Cardo et al., 1989), but Golden and Pardridge (2000) showed that it was on human astrocyte foot processes rather than on the luminal membrane of endothelial cells (Golden and Pardridge, 2000). P-gp has also been found in primary cultures of rat astrocytes (Decleves et al., 2000), but it now seems clear that its major site of brain expression lies at the luminal side of brain capillary endothelial cells (Beaulieu et al., 1997; Bendayan et al., 2006). The locations of Mrps at the BBB are still debated. There are several reasons for this uncertainty, including the use of poor specificity antibodies and possible species-specific differences in drug transporter profiles. The complexity of the BBB architecture, with brain microvessel endothelial cells surrounded by pericytes, smooth muscle cells and astrocyte foot processes complicates the picture. Many techniques are currently used to isolate brain microvessels; they may be mechanical (Betz et al., 1979), enzymatic (Bowman et al., 1981) or use laser microdissection (Emmert-Buck et al., 1996). One major problem common to all these techniques is that of microvessel purity, which is essential for experiments designed to identify and locate drug transporter genes or proteins in constituents of the BBB. The mechanical isolation of brain microvessels includes homogenization, density-gradient centrifugation, and the purification of capillaries by filtration (Betz et al., 1979). Several protocols of gradient centrifugation and filtration can be used to maximize the purity of the microvessel preparation.

This study was done to select a protocol for isolating rat brain microvessel that provided the least contamination with astrocyte and neuron mRNA and the highest yield of endothelial mRNA. We used qRT-PCR to evaluate expression of the genes encoding specific markers of brain endothelial cells (Glut-1, VEGFR-2 or Flk-1), pericytes (Ng2 proteoglycan), neurons (synaptophysin, Syn) and astrocytes (Gfap) at each step of isolation. This improved protocol was then used to compare the expression profiles of genes encoding the drug transporters Mdr1a, Mdr1b, Mrp1, Mrp2, Mrp3, Mrp4, Mrp5, Bcrp and Oatp2 and correlate them with the profiles of genes encoding specific cell markers. We also evaluated the protein expression of P-gp, Mrp2, Gfap and Syn in the cortex and in the purest microvessel-enriched fraction. The data were then analyzed to identify the BBB cells most likely to express them.

2. Results

2.1. *Purity and homogeneity of isolated brain capillaries*

Microscopic analysis (Fig. 2) revealed that the microvessel-enriched fraction after dextran centrifugation (S1) contained considerable cell debris that was removed by a single passage through a 20-μm mesh filter (S3). Passing the S1 fraction through a 100-μm mesh filter yielded a filtrate (S2) with small microvessels but did not remove small cell debris (probably nuclei). The S1 and S3 fractions also contained microvessels of varying size, but this was improved by passage through a 100-μm mesh filter (S4 and S5). S4 (without glass beads) and S5 (with glass beads) appeared to be microscopically the best, giving uniformly small microvessels not contaminated by cell debris.

2.2. *Expression profiles of genes encoding markers of endothelial cells, pericytes, astrocytes and neurons*

The expression (mRNA) of genes encoding markers of endothelial cells (Glut-1, Flk-1), pericytes (Ng2), astrocytes (Gfap) and neurons (Syn) was evaluated at each step of brain microvessel isolation by qRT-PCR, and the relative abundance of the mRNAs in each sample was compared to that of the whole cortex homogenate (S0) (Fig. 3, Table 1). Glut-1 and Flk-1 mRNAs were significantly enriched in all isolation steps as compared to S0 with a similar 7- to 11-fold increase (Table 1). Ng2 mRNA was also significantly increased 3- to 4-fold over S0 in all samples and the greatest increase (8-fold) was obtained in S5 where the amount of Ng2 was significantly higher than in other fractions.

In contrast to endothelial markers, the relative amounts of Gfap mRNA, representing contamination by astrocytes, was significantly lower in S3, S4 and S5 than in S0. The greatest decrease in Gfap mRNA expression (about 25-fold) occurred in S3 and S4. The relative amount of Syn mRNA was also significantly lower in S2, S3, S4 and S5 than in S0. The greatest decrease in Syn mRNA (327-fold) was observed in S4, showing that S4 gave the purest in brain capillary mRNA, with little contaminating astrocyte and neuron mRNA.

2.3. *Expression profiles of genes encoding drug transporters*

Expression of the genes for each drug transporter was determined in S1, S2, S4 and S5 and normalized to S0 (whole cortex homogenate) (Fig. 4, Table 1). Expression of the *Mdr1a*, *Bcrp*, *Mrp4* and *Oatp-2* genes in each sample was significantly much greater (10- to 30-fold) than in S0, suggesting their preferential expression in brain microvessels. The gene expression profile in each sample was very similar to that of the endothelial markers Glut-1 and Flk-1. *Mrp2* gene