Exp Brain Res (2003) 153: 356–365
DOI 10.1007/s00221-003-1620-4

RESEARCH ARTICLE

**Michael Török · Jörg Huwyler · Heike Gutmann · Gert Fricker · Jürgen Drewe**

# Modulation of transendothelial permeability and expression of ATP-binding cassette transporters in cultured brain capillary endothelial cells by astrocytic factors and cell-culture conditions

Received: 27 January 2003 / Accepted: 2 July 2003 / Published online: 12 September 2003


**Abstract** Confluent cell monolayers of brain capillary endothelial cells (BCEC) are used widely as an in vitro cell culture model of the blood–brain barrier. The present study describes the influence of cell-culture conditions on tight junctions, filamentous-actin cytoskeleton, and expression of ATP-binding cassette (ABC) transporters in primary cell cultures of porcine BCEC. Astrocyte as well as C6 glioma-conditioned cell culture medium was used in combination with retinoic acid, dexamethasone, cyclic adenosine monophosphate (cAMP) analogs, or 1,25-dihydroxyvitamin $D_3$. It was shown that C6-conditioned medium led to a reorganization of filamentous actin and to an improved staining of zonula occludens-associated protein-1 (ZO-1). Further optimization of these culture conditions was achieved with cAMP analogs and dexamethasone. Retinoic acid, as well as 1,25-dihydroxyvitamin $D_3$, did not improve cellular tight junctions as judged by filamentous actin, ZO-1 rearrangement, and transcellular electrical resistance (TER) measurements. However, these morphological changes did not influence the paracellular permeability of the extracellular marker sucrose. Expression of ABC transporters such as P-glycoprotein, multidrug resistance-associated protein-1 (MRP1), and MRP2 were compared by measuring messenger RNA (mRNA) levels in whole-brain tissue, isolated brain capillaries, and cultured cells. In freshly isolated BCEC, mRNA levels of MRP2 and P-glycoprotein dropped by two- to sevenfold, respectively, whereas MRP1 mRNA levels were slightly increased. During cell culture, mRNA levels of MRP1 and MRP2 decreased by up to fivefold, while P-glycoprotein levels remained constant. These results were unaltered by different cell-culture conditions. In conclusion, the present study suggests that paracellular permeability, as well as mRNA expression of the studied ABC transporters in primary cultures, of porcine BCEC are insensitive toward changes in cell-culture conditions.

**Keywords** Blood–brain barrier · P-glycoprotein · MRP · Brain capillary endothelial cells

**Abbreviations** *ACEM:* Astrocyte-conditioned endothelial medium · *BCEC:* Brain capillary endothelial cells · *C6CEM:* C6-conditioned endothelial medium · *TER:* Transcellular electrical resistance

M. Török · H. Gutmann · J. Drewe (✉)
Division of Clinical Pharmacology, University Hospital,
Petersgraben 4,
4031 Basel, Switzerland
e-mail: Juergen.Drewe@unibas.ch
Tel.: +41-61-2653848
Fax: +41-61-2658581

J. Huwyler
Pharmaceuticals Division, F. Hoffmann-La Roche Ltd,
Basel, Switzerland

G. Fricker
Institute for Pharmacy and Molecular Biotechnology,
University of Heidelberg,
Heidelberg, Germany

## Introduction

A continuous layer of brain microvessel endothelial cells is central to the integrity of the blood–brain barrier, which controls the passage of substances from the blood to the extracellular fluid environment of the brain. A key component of the majority of in vitro cell culture models of the blood–brain barrier developed during the last years is therefore a confluent cell monolayer of brain endothelial cells. Primary or passaged cultures of these cells can be obtained from mouse, rat, bovine, human, canine, or porcine brains (for a review, see Takakura et al. 1991). In some instances immortalized cell lines of brain endothelial cells have been established such as rat RBE4 cells (Durieu-Trautmann et al. 1993). A common problem associated with cell cultures of brain endothelial cells is the rapid dedifferentiation of these cells as soon as they are removed from their natural environment and placed in cell culture. A rationale for this is provided by observations that the microenvironment of the vasculature determines the endothelial phenotype (Stewart and Wiley 1981). As a

consequence, monolayers of brain endothelial cells have a tendency to be of low electrical resistance and are relatively leaky, which may lead to a poor correlation between permeability of brain endothelial cells to various compounds in cell culture and in vivo (Pardridge et al. 1990). During the last few years, however, much progress has been made with respect to the development of in vitro models of the blood–brain barrier, with an improved predictive value by the systematic modification of cell-culture conditions. Since astrocyte–endothelial cell interactions are sufficient to induce the essential properties of the blood–brain barrier (for a review, see Abbott et al. 1992), cocultures between brain endothelial cells and astrocytes have been established (Dehouck et al. 1995; Raub 1996) or media conditioned by astrocytes (primary cultures, Rubin et al. 1991; or astroglioma cell lines, Raub et al. 1992) have been used to induce blood–brain barrier properties in primary cultures (Meyer et al. 1991) or immortalized brain endothelial cells (Rist et al. 1997). Alternative methods comprise treatment of cell cultures with agents that elevate intracellular cyclic adenosine monophosphate (cAMP; Rubin et al. 1991), retinoic acid (El Hafny et al. 1997), or hydrocortisone (Franke et al. 1999). While the impact of such modifications of cell-culture conditions on electrical resistance and permeability of cell monolayers has been well documented, comparatively little is known about its consequences for ATP-binding cassette (ABC) proteins (Klein et al. 1999), such as the drug-efflux pumps P-glycoprotein and multidrug resistance-associated proteins MRP1 and MRP2.

Therefore, the aim of the present study was to systematically modify the cell-culture conditions used for primary cultures of porcine brain capillary endothelial cells (BCEC). Modifications included the use of two types of astrocyte-conditioned cell culture media as well as cell culture supplements, such as retinoic acid, dexamethasone, cAMP analogs and 1,25-dihydroxyvitamin $D_3$. The impact of these environment modifications upon cell morphology [i.e., organization of filamentous-actin (F-actin) cytoskeleton and tight junctions], permeability of the extracellular marker sucrose and mRNA expression of P-glycoprotein, MRP1, and MRP2 were determined and directly compared with freshly isolated brain microvessels.

## Materials and methods

### Isolation of porcine brain capillaries

Porcine brain capillaries were prepared as described previously for bovine brain capillaries (Pardridge et al. 1985) but with modifications. In brief, cortical gray matter of one porcine brain was minced and subsequently homogenized with five up-and-down strokes using a glass homogenizer with a clearance of 0.095–0.115 mm. The homogenate was brought to 15% (w/v) dextran and centrifuged for 10 min at 5,750*g* at 4°C. The pellet obtained was then passed through a 150-µm mesh to remove small arterioles and tissue fragments. Brain capillaries in the effluent were further purified by passing the filtrate over a 25-ml column containing 20-ml glass beads of 0.45–0.50 mm diameter. Trapped brain capillaries were dislodged from the glass beads by stirring, washed and collected by centrifugation (200*g* for 5 min at 4°C). Isolated capillaries were cryopreserved at –196°C in Dulbecco's modified Eagle medium, supplemented with 10% (v/v) dimethylsulfoxide (DMSO) and 100 mM sucrose.

### Isolation and cell culture of porcine brain capillary endothelial cells

Primary cultures of porcine BCEC were prepared as described (Huwyler et al. 1996). Cortical gray matter of six porcine brains were minced and digested enzymatically for 2 h using 0.5% (w/v) dispase. Cerebral microvessels were obtained after centrifugation in 13% (w/v) dextran and were subsequently incubated for 4.25 h in a buffer containing 0.1% (w/v) collagenase/dispase. The resulting cell suspension was supplemented with 10% (v/v) horse serum and filtered through a 150-µm nylon mesh. BCEC were isolated on a continuous 50% (v/v) Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). Endothelial cells were filtered through a 35-µm nylon mesh and cryopreserved at –196°C in endothelial cell culture medium (45% v/v MEM, 45% v/v Ham's F-12 nutrient mixture, 10% (v/v) heat-inactivated horse serum, 100 µg/ml streptomycin, 100 µg/ml penicillin G, 100 µg/ml heparin, 13 mM $NaHCO_3$ and 10 mM HEPES) supplemented with 10% (v/v) DMSO. Isolated endothelial cells were seeded at a density of 150,000 cells/$cm^2$ onto collagen/fibronectin-coated surfaces. Cells were grown either in endothelial cell culture medium or in medium consisting of 50% (v/v)-conditioned endothelial cell culture medium and 50% (v/v) endothelial cell culture medium. After cells had reached confluence (day 7), supplemented or unsupplemented medium, with or without serum, was added for 24 h. Supplements used were: 250 µm 8-(4-chlorophenylthio) cAMP (Sigma, Buchs, Switzerland) plus 35 µm phosphodiesterase inhibitor RO-20-1724 (Calbiochem, San Diego, Calif., USA), 10 µm dexamethasone (Sigma), 5 µm all-trans retinoic acid (Sigma), or 500 nM 1,25-dihydroxyvitamin $D_3$ (Calbiochem).

### Conditioned medium

Endothelial cell culture medium was conditioned by rat astrocytes or by the rat glioma cell line C6 (American Type Culture Collection, Rockville, Md., USA). Medium (10 ml per T-75 culture flask) was added for 2 days to the confluent cells and the removed medium was sterile filtered and stored at −20°C.

Primary cultures of rat astrocytes were prepared from 1-day-old rat cortex essentially as described by Lillien et al. (1988). Small pieces of gray cortex were rubbed across a 63-µm mesh. The homogenate was then cultured for about 10 days in astrocyte cell culture medium (90% (v/v) MEM, total 33 mM D-glucose, 13 mM $NaHCO_3$, 20 mM HEPES, 100 µg/ml streptomycin sulfate, 100 µg/ml penicillin G, 100 µg/ml heparin, 10% (v/v) heat-inactivated fetal calf serum). Culture flasks were then shaken overnight at 37°C and the remaining cells (enriched in type-1 astrocytes) were passaged on poly-D-lysine-coated flasks. Confluent cells were treated with astrocyte cell culture medium, supplemented with 10 µM cytosine arabinofuranoside, for 2 days to limit the growth of rapidly dividing contaminating cells. After renewed confluence, cells were treated again with cytosine arabinofuranoside. Confluent cells were subsequently used to condition endothelial cell culture medium. By immunocytochemical criteria, the cultures were approximately 95% type-1 astrocytes (Lillien et al. 1988).

C6 cells (passage 40 to 43) were plated at a concentration of 100,000 cells/$cm^2$ in C6 cell culture medium (41.25% v/v MEM, 41.25% v/v Ham's F-12 nutrient mixture, 13 mM $NaHCO_3$, 20 mM HEPES, 100 µg/ml streptomycin sulfate, 100 µg/ml penicillin G, 100 µg/ml heparin, 15% v/v heat-inactivated horse serum, 2.5% v/v heat-inactivated fetal calf serum). Confluent cells were used for up to 1 week to condition the medium.

### Transcellular electrical resistance measurements

Transcellular electrical resistance (TER) across endothelial cell monolayers was measured in the growth medium using the Millicell-ERS system with chopstick electrodes (Millipore, Bedford, Mass., USA). Values were corrected for resistances of collagen/fibronectin-coated, blank filters. TER was expressed as ohm·centimeters squared.

### Flux measurements

Transport experiments were performed using cell monolayers cultured on filter inserts (0.4 μM pore size, 24-well Transwell plates; Costar, Cambridge, Mass., USA) at 37°C on a rotary platform (120 rpm). Experiments were initiated by adding 0.5 ml transport buffer (142 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.5 mM $K_2HPO_4$, 4 mM D-glucose, 10 mM HEPES, pH 7.4) containing 0.6 μCi/ml $^{14}$C-sucrose (Amersham Pharmacia Biotech) to the apical chamber. The emergence of the radioactive compound in the basolateral chamber was measured after 10 min by taking a 200-μl sample from the basolateral chamber, which contained 1 ml transport buffer. Radioactivity was determined by scintillation counting. Transport was calculated from the initial concentration of tracer in the apical chamber and the final concentration of tracer in the basolateral chamber according to: $P_{app}=dQ/dt\cdot 1/A/C_0$, where $dQ/dt$ is the rate of translocation, $A$ is the surface of the membrane (here 1 cm$^2$) and $C_0$ is the initial concentration of the labeled drug.

### Immunocytochemistry

Confluent cultured BCEC were fixed and stained directly on the culture inserts after flux experiments. Brain capillaries were adhered to a glass slide by using a Cytospin 2 centrifuge (Shandon, Pittsburgh, Pa., USA) at 200 g for 5 min at room temperature. Tissues were fixed for 20 min in 4% (w/v) paraformaldehyde and then permeabilized in 0.5% (v/v) Triton X-100 for 5 min. Tissues were incubated for 1 h at 37°C in primary antiserum diluted in 3% (w/v) bovine serum albumin in phosphate-buffered saline (PBS), followed by incubation for 1 h at room temperature in fluorochrome-conjugated secondary antibody diluted in 3% (w/v) bovine serum albumin in PBS. Filamentous-actin was stained by addition of 3 U/ml phalloidin-rhodamine (Molecular Probes, Eugene, Ore., USA), nuclei were stained using 100 μg/ml 4',6-diamidino-2-phenylindole or 60 μg/ml propidium iodide. Microscopy specimens were mounted in FluoroSave (Calbiochem, San Diego, Calif., USA).

Primary antibodies used for these experiments included rabbit polyclonal anti-von Willebrand factor (6 μg/ml; Sigma), rabbit polyclonal anti-ZO-1 (10 μg/ml; Zymed, San Francisco, Calif., USA), murine monoclonal anti-glial fibrillary acidic protein (1 μg/ml; Boehringer Mannheim, Mannheim, Germany), and murine monoclonal antibody C219 directed against P-glycoprotein (5 μg/ml; Signet, Dedham, Mass., USA). Secondary antibodies used included Cy2-conjugated rabbit anti-mouse (15 μg/ml) and Cy2-conjugated goat anti-rabbit (15 μg/ml; both obtained from Jackson Immuno Research, West Grove, Pa., USA).

To determine the staining morphology of F-actin and ZO-1 in brain capillary endothelial cells under different culture conditions, a scoring system was used. For F-actin, scores from 1 to 5 were assigned and, for ZO-1, scores from 1 to 3 (Figs. 2, 3). Scores were assigned independently by two scientists, in a blind manner and specimens were presented in a random order.

### Fluorescence microscopy

Confocal microscopy was performed with a Zeiss LSM 510 inverted laser scanning microscope, equipped with He/Ne 543 nm, He/Ne 633 nm, and Ar 488 nm lasers. Objectives used were a Zeiss Plan-Neofluar ×40 oil-immersion objective with a numerical aperture of 1.3 or a Zeiss Plan-Neofluar ×63 oil-immersion objective with a numerical aperture of 1.25. Optical sections 0.35 μM thick were scanned through the *z*-plane of the sample. Data analysis, three-dimensional reconstruction from series of consecutive optical sections, and shadow projections were calculated on an O2 Workstation (Silicon Graphics, Mountain View, Calif., USA) with Imaris software (version 2.7; Bitplane, Zürich, Switzerland). Classical fluorescence microscopy was performed with a Zeiss Axiophot fluorescence microscope.

### Real-time quantitative PCR

Real-time quantitative PCR analysis was performed with the TaqMan assay using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland), a combined thermocycler and fluorescence detector. A dual-labeled fluorogenic probe complementary to a sequence within each PCR product was added to the PCR reaction. The fluorescent dye at the 5' end of the probe (6-carboxyfluorescein) served as reporter, and its emission was quenched by the second fluorescent dye at the 3' end of the probe (6-carboxytetramethylrhodamine). During elongation, the 5' to 3' exonuclease activity of the Taq DNA polymerase cleaved the probe, thus releasing the reporter from the quencher. Primers and probe were designed according to the guidelines of Applied Biosystems, and with the help of Primer Express 1.0 software (Applied Biosystems). Primers were custom synthesized by Life Technologies (Paisley, Scotland), probes by Eurogentec (Seraing, Belgium). The primers and probes used were:

A. GAPDH: GGTGAAGGTCGGAGTGAACG and CGACAATGTCCACTTTGCCA with the probe CGCCTGGTCACCAGGGCTGC
B. pgp1A: GTGGAATGATCTTCAATGGTAGCA and AAGCGCTCATCAACTGTGACC with the probe TTGATTAAATGCCCAGATTTTAACACAAATATTAGACAAC
C. ZO-1: AAGGCGGATGGCGCTACAAG and TTCACTACCTGGGGCTGACAGG with the probe ACCTTGATTTGCATGATGATCGTCTGTCCT
D. MRP1: GACCCTTGATTGCCACGTG and TGGGCTGTGGGAAGTCGT with the probe CCTCCACTTTGTCCATCTCAGCCAAGAG
E. MRP2: TGTGGGCTTTGTTCTGTCCA and CAGCCACAATGTTGGTCTCG with the probe CTCAATATCACACAAACCCTGAACTGGCTG

Quantitative data was normalized to GAPDH. Results were expressed relative to the control mean value, which was set as 1.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After DNase I digestion, RNA was quantified using a GeneQuant photometer (Pharmacia, Uppsala, Sweden). Its integrity was checked by ethidium bromide agarose gel electrophoresis. The purity of the RNA preparations was high, as demonstrated by the 260 nm to 280 nm ratio (range 1.8–2.0). One microgram of total RNA was reverse transcribed by Superscript II (Gibco, Basel, Switzerland) according to the manufacturer's protocol using random hexamers as primers. Complimentary DNA (cDNA; 25 ng total RNA) was amplified in a 25-μl volume containing 12.5 μl of the ×2 TaqMan universal PCR master mix (Applied Biosystems), 225-nM probe, and 900 nM of each primer. Cycling conditions were 10 min, 95°C initial denaturation and activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15 s 95°C denaturation, 1 min 60°C combined annealing and primer extension. Mathematical analysis was performed as recommended by Applied Biosystems (see user bulletin 2, ABI PRISM 7700 Sequence Detection System, downloadable at http://www.appliedbiosystems.com/support).

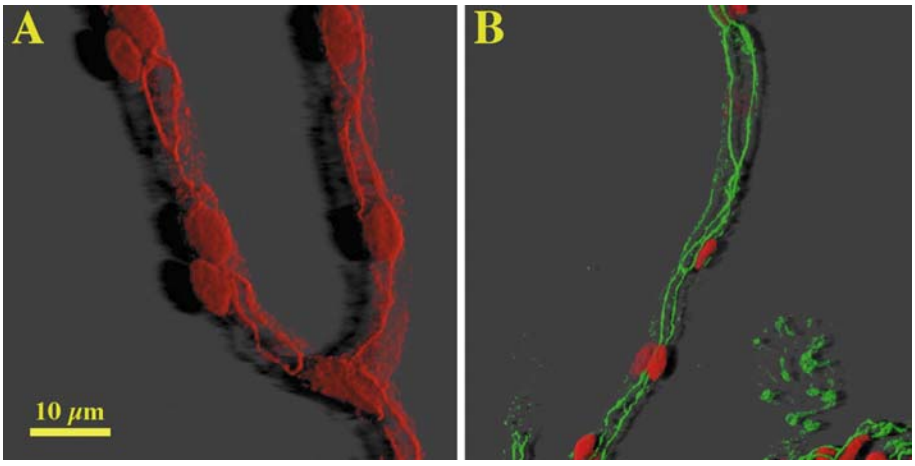


**Fig 1A, B** Distribution of tight junction-associated proteins in freshly isolated brain capillaries. Staining of **A** filamentous actin with phalloidin-rhodamine (*red*) and **B** zonula occludens-associated protein-1 (ZO-1; *green*). To visualize the location of individual cells in capillaries, nuclei were costained with propidium iodide (*red*) Filamentous actin and ZO-1 staining is predominantly organized at the cell borders. Images were rendered using three-dimensional image processing and shadow projection

### Statistical analysis

For statistical comparison, data groups were compared by analysis of variance (ANOVA). If this analysis revealed significance, pairwise comparisons between groups were performed by a two-tailed Student's *t*-test with Bonferroni's correction. Significance level was set to $P \leq 0.05$.

## Results

### Cell cultures and cytochemistry

Primary cultures of porcine brain microvessel endothelial cells were cultured on Transwell filter inserts under different cell-culture conditions (Table 1). Cell culture media were either used without pretreatment or conditioned using primary cultures of type-1 astrocytes or C6 astroglioma cells. Where indicated, supplements were added to the cell cultures 24 h prior to the experiment.

Brain capillary endothelial cells in situ are known to form high-resistance tight junctions. Visualization of F-actin and the ZO-1, which are both associated with the junctional complex in freshly isolated brain microsvessels, reveals a characteristic belt-like distribution of these proteins (Fig. 1). This pattern of staining for F-actin (Fig. 2A) and ZO-1 (Fig. 3A) was only weakly retained in brain capillary endothelial cells under standard cell-culture conditions. However, modification of cell-culture conditions such as astrocyte-conditioned endothelial medium (ACEM) and C6-conditioned endothelial medium (C6CEM; Table 1) led to a substantial reorganization of F-actin and a reinforced staining of ZO-1. The degree of reorganization (reinforcement) was described by a score that ranged, for F-actin, between 1 (poor) and 5 (very high) and for ZO-1 between 1 (poor) and 3 (very high). Examples for the different scores and the associated cell-culture conditions are given for F-actin in Fig. 2 and for ZO-1 in Fig. 3. Figures 2 and 3 were obtained by conventional fluorescence microscopy and represent the situation encountered by the evaluating scientists. The scores that were obtained using different cell-culture conditions are summarized in Table 2. The higher the score, the higher the degree of differentiation was considered, since a situation closer to intact brain capillaries was reached (Fig. 1). F-actin scores showed that growing in C6CEM resulted in highest scores, whereby cells grown in ACEM still needed the addition of chlorophenylthio-cAMP/RO-20-1724 or dexamethasone to enhance the score further. All supplements in combination with endothelial medium (EM) did not lead to an improvement of the staining pattern compared with EM alone. Comparable results were also shown with ZO-1 scores. There was a statistically significant correlation between F-actin scores and ZO-1 scores, as demonstrated by least-square regression analysis (Pearson coefficient $R=0.833$). In summary, only chlorophenylthio-cAMP/RO-20-1724 and dexamethasone were capable of inducing changes in F-actin and ZO-1 morphology, but only in combination with ACEM (for F-actin) or alone (for ZO-1). Highest scores were observed in the group of cells grown in C6CEM.

**Table 1** Cell culture conditions

| Condition | Abbreviation | Exposure of cells |
|---|---|---|
| Standard endothelial cell culture medium | EM | 7 days |
| Astrocyte-conditioned medium | ACEM | 7 days |
| C6-conditioned medium | C6CEM | 7 days |
| 250 μM chlorophenylthio-cAMP + 35 μM ro-20-1724 | AMP | 24 h |
| 10 μM dexamethasone | DEX | 24 h |
| 5 μM all-trans retinoic acid | RET | 24 h |
| 500 nM 1,25-dihydroxyvitamin $D_3$ | VD3 | 24 h |

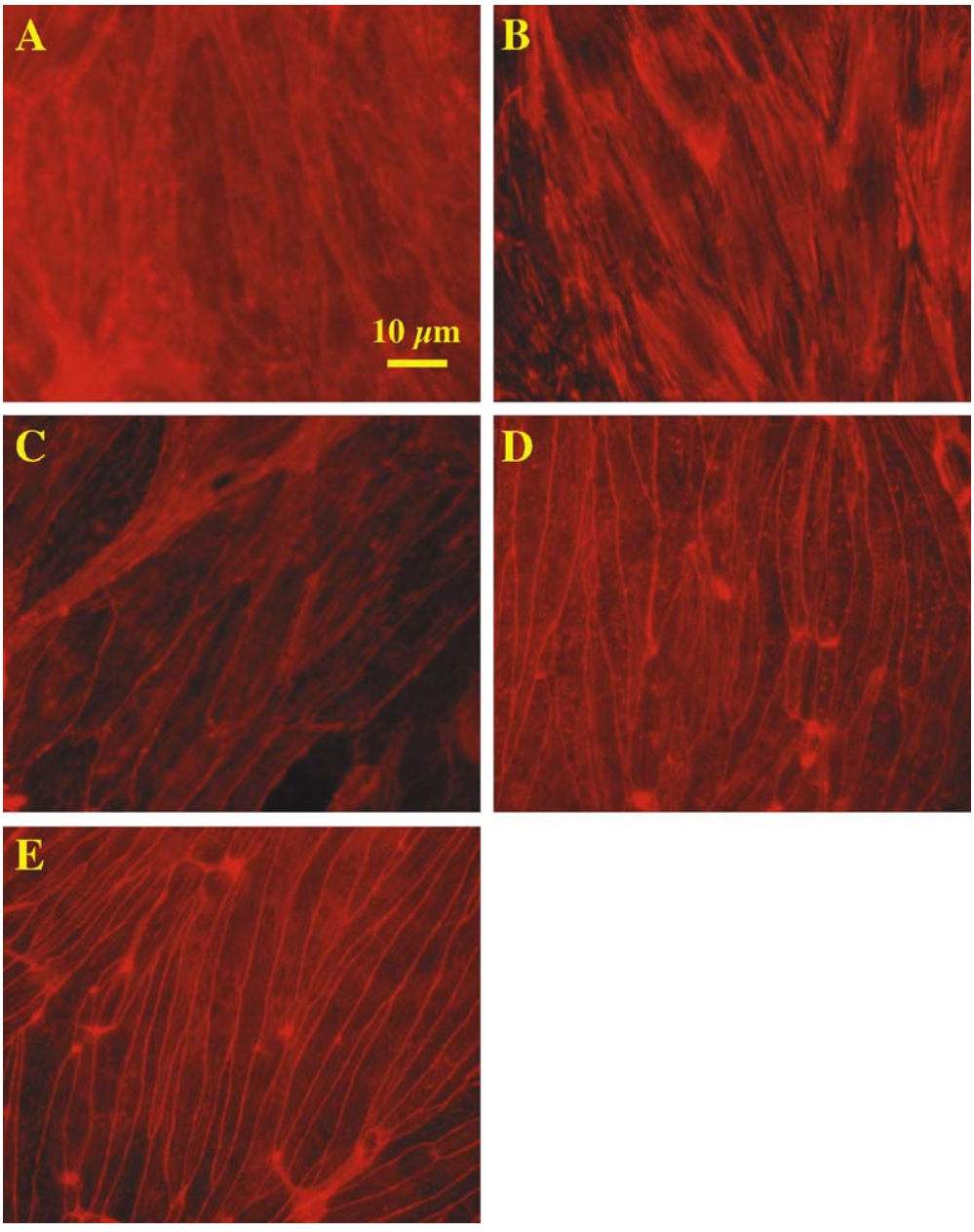


**Fig 2A-E** F-actin staining with phalloidin-rhodamine in confluent brain capillary endothelial cells. **A** Score 1 (cells grown in EM); **B** score 2 (cells grown in EM); **C** score 3 (cells grown in ACEM and 10 μM dexamethasone for 24 h); **D** score 4 (cells grown in C6CEM and 10 μM dexamethasone for 24 h); **E** score 5 (cells grown in C6CEM). *Size bar* is the same for all images

**Table 2** Filamentous actin (*F-actin*) and zonula occludens-associated protein-1 (*ZO-1*) morphology scores and transcellular electrical resistance (*TER*) measurements 24 h after confluence. TER values are expressed as mean ± SEM of $n \geq 4$ determinations

| Cell culture conditions | EM | | | ACEM | | | C6CEM | | |
|---|---|---|---|---|---|---|---|---|---|
| | F-actin | ZO-1 | TER ($\Omega \cdot cm^2$) | F-actin | ZO-1 | TER ($\Omega \cdot cm^2$) | F-actin | ZO-1 | TER ($\Omega \cdot cm^2$) |
| No supplement | 2 | 1 | 24±2 | 2 | 2 | 39±2* | 5 | 3 | 57±3* |
| AMP | 2 | 2 | 260±7* | 4 | 3 | 415±8** | 5 | 3 | 186±5*** |
| DEX | 2 | 2 | 58±1* | 3 | 2 | 82±5** | 4 | 2 | 106±15 |
| RET | 2 | 1 | 26±5 | 2 | 2 | 29±1** | 5 | 3 | 44±3 |
| VD3 | 2 | 1 | 33±3 | 2 | 1 | 45±4 | 4 | 3 | 58±6 |

Statistically significant ($P \leq 0.05$) by two-tailed Student's *t*-test and Bonferroni's correction, compared with *EM, **ACEM, ***C6CEM

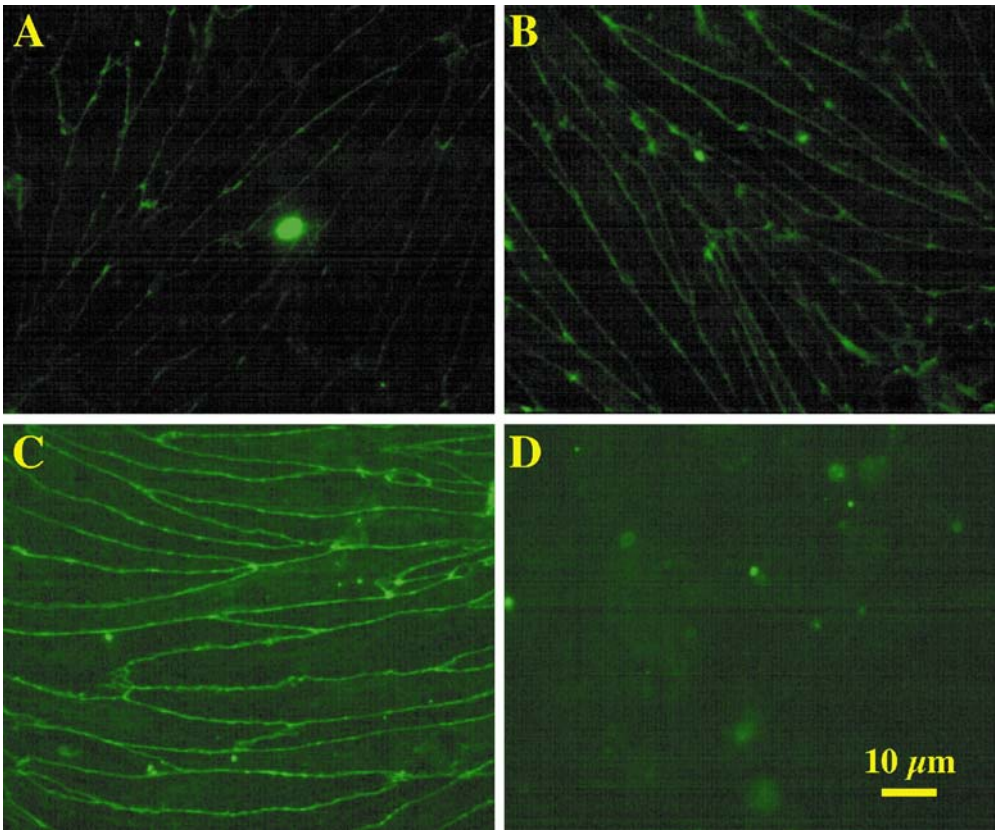


**Fig 3A-D** Immunocytochemical staining of the tight junctions-associated protein ZO-1 in confluent brain capillary endothelial cells. **A** Score 1 (cells grown in EM); **B** score 2 (cells grown in ACEM); **C** score 3 (cells grown in C6CEM); **D** control using only secondary antibody. *Size bar* is the same for all images

## Transcellular electrical resistance

Transcellular electrical resistance measurements 24 h after confluence (Table 2) showed that cells grown in ACEM or C6CEM, without supplement, showed approximately a doubling in their TER values compared with monolayers grown in EM. These alterations were statistically significant. Addition of chlorophenylthio-cAMP/RO-20-1724 significantly raised the TER value by a factor of ~10 for EM and ACEM and a factor of ~3 for C6CEM. To a lesser extent, dexamethasone led to a further increase of TER (factor ~2) compared with their respective media in absence of supplement. No alteration of the TER value was observed in the presence of all-trans retinoic acid or 1,25-dihydroxyvitamin $D_3$.

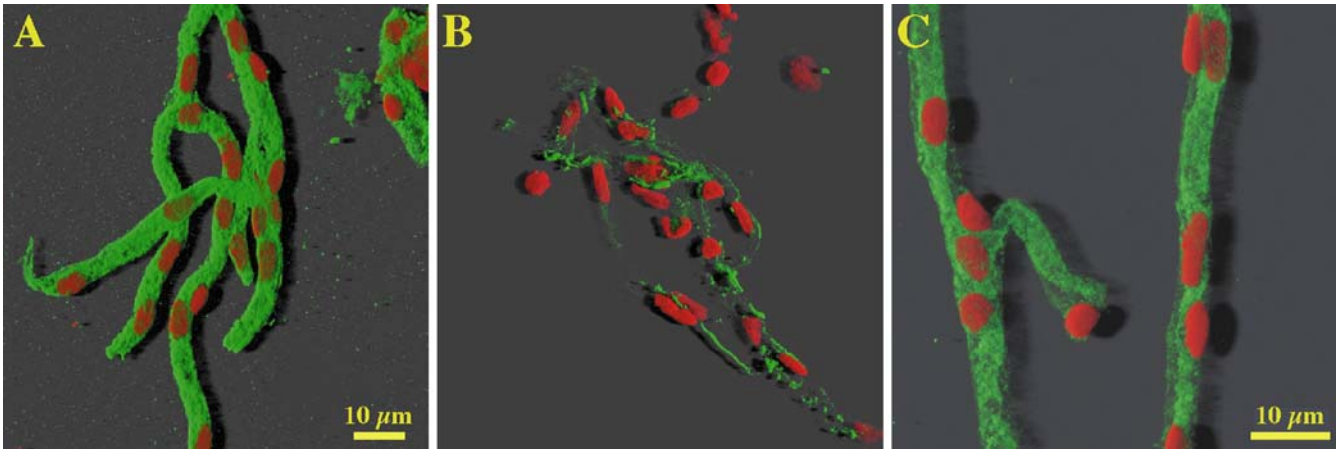


**Fig 4A-C** Colocalization of P-glycoprotein with brain capillary endothelial cells in freshly isolated porcine brain microvessels. Staining of **A** endothelial-specific von Willebrand factor (*green*); **B** astrocyte-specific glial fibrillary acidic protein (GFAP; *green*); and **C** P-glycoprotein antibody C219 (*green*). To visualize the location of individual cells in capillaries, nuclei were costained with propidium iodide (*red*). *Size bar* is the same for **A** and **B**. Images were rendered using three-dimensional image processing and shadow projection

### Transendothelial permeability of sucrose

Subsequent to TER measurements, permeability of the extracellular marker [$^{14}$C]sucrose across filters with confluent cells was determined. Interestingly, the apparent permeability ($P_{app}$) of sucrose did not improve using different cell-culture conditions (e.g., EM: $P_{app}$=7.1±0.6; ACEM: $P_{app}$=9.0±0.6; C6CEM: $P_{app}$=9.6±0.6; units are $10^{-4}$ cm/min; $n$=5, mean ± SE). Therefore all permeability coefficients were in the range of the standard cell-culture condition (EM).

### Expression of ABC transporters

Additional studies were initiated to determine the impact of different cell-culture conditions on the expression of the ABC transporters P-glycoprotein and the multidrug resistance-associated proteins MRP1 and MRP2. Immunohistochemical analysis of freshly isolated brain capillaries using a monoclonal antibody (C219) specific for P-glycoprotein revealed colocalization of the protein with brain microvessel endothelial cells (Fig. 4).

To evaluate the level of expression of the ABC transporters, real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted from brain tissue, isolated brain capillaries, freshly isolated BCEC and BCEC grown for different time periods in standard EM. Expression levels of pgp1A, MRP1, and MRP2 were normalized to the internal standard GAPDH (Fig. 5). The mRNA levels of all three ABC transporters were markedly enriched in freshly isolated brain microvessels when compared with whole-brain tissue. There was a reduction in mRNA levels in isolated endothelial cells when compared with intact brain capillaries for P-glycoprotein and MRP2. There was a further decrease in mRNA levels of MRP2 only when freshly isolated endothelial cells were kept in culture, whereas the mRNA levels of P-glycoprotein and MRP1 did not change during cell culture. To assess whether the expression pattern of these transporters is altered under different cell-culture conditions, cDNA of cells cultured using different cell-culture conditions was assayed by quantitative real-time PCR. Surprisingly, endothelial cells expressed under all specified culture conditions demonstrated the same expression pattern as cells grown in standard EM.

## Discussion

Primary cultures of BCEC form confluent monolayers that retain many morphological and biochemical properties and are polarized like their in vivo counterparts as previously described (Huwyler et al. 1996, 1997; Török et al. 1998; Gutmann et al. 1999). However, the limitation of cultured primary brain capillary endothelia is higher than desirable paracellular permeability, attributed to alterations in the development of tight intracellular junctions. It has been shown that the microenvironment can influence the permeability properties of endothelial cells in situ (Stewart and Wiley 1981; Janzer and Raff 1987) There are at least four different cell types that constitute and/or are in close contact with the brain capillaries, i.e., endothelial cells, astrocytes, neurons, and pericytes (Pardridge 1993). The interactions between these cells are multifaceted and complex. Recent reports are indicative of a dynamic control of brain microcirculation as a consequence of neuron to astrocyte signaling (Zonta et al. 2003). Pericytes are present from the onset of blood–brain barrier formation (Korn et al. 2002) and can modulate the endothelial cell phenotype (Allt and Lawrenson 2001). Recent reports indicate that endothelial cells and pericytes reorganize in capillary-like structures when cocultured with astrocytes (Ramsauer et al. 2002), indicative of intimate interactions between these cell types. With respect to the aspect of cellular permeability of cultured endothelial cells, astrocyte-derived factors seem to play a central role (Janzer and

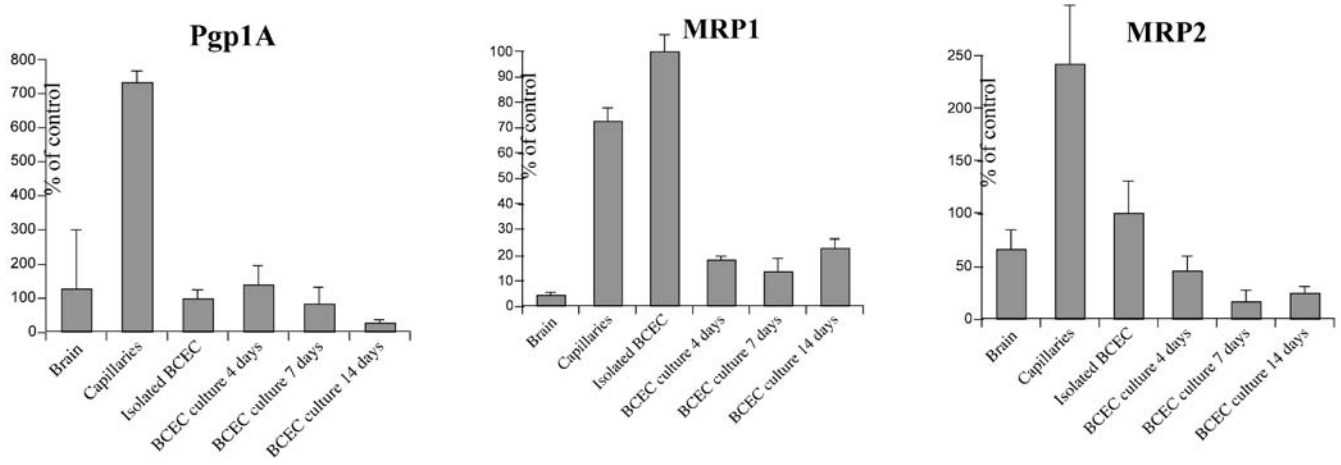


**Fig 5** Relative quantification of pgp1A, MRP1 and MRP2 mRNA by quantitative RT-PCR. GAPDH was used as an internal standard to correct for equal loading. Values were normalized to isolated brain capillary endothelial cells (BCEC), which was set at 100%. Values represent mean ± SD of four determinations

Raff 1987). We therefore decided to focus this report on astrocyte–endothelial cell interactions. ACEM (Rubin et al. 1991) and medium that was conditioned by C6 cells, a glioma cell line, were used (Raub et al. 1992). In addition, supplements were investigated, which have been shown to improve cultured BCEC monolayers. We used the cAMP analog chlorophenylthio-cAMP in combination with the cAMP-specific phosphodiesterase inhibitor RO-20-1724 (Rubin et al. 1991), dexamethasone (Grabb and Gilbert 1995), or all-trans retinoic acid (Lechardeur et al. 1995). 1,25-Dihydroxyvitamin $D_3$ was shown to regulate differentiation in many cell types, including normal, immortalized, and tumor cells (Reese et al. 1981; Tsao and Batist 1988; Bouillon et al. 1995). Supplement concentrations used corresponded to effective concentrations published in the previously cited publications.

Tight junctions probably represent the major functional component of the blood–brain barrier and depend very much on adherens junctions. For example, it has been shown that antibodies against E-cadherin, the $Ca^{2+}$-dependent epithelial cell adhesion molecule, block tight-junction formation (Gumbiner and Simons 1986). The presumption is that the normal course of events after cell contact is cadherin-dependent cell adhesion followed by tight-junction assembly. To assess the degree of differentiation of cultured BCEC, the degree of tight- and adherens-junction formation was determined. To judge tight junctions, the ZO-1 pattern was assessed. Adherens junctions were rated by the distribution of F-actin, which closely interacts with the adherens junctions' catenin proteins (Staddon and Rubin 1996; Rubin and Staddon 1999). A scoring system was introduced in order to generate an ordinal mean to judge the staining patterns. A high score was assigned to a high degree of differentiation. Highest scores represented patterns such as the ones observed in intact brain capillaries. In these capillaries, distribution of ZO-1 showed an intense staining localized at the cell-to-cell contact area, whereas F-actin showed a weak intracellular distribution. This contrasts to cells grown in EM which showed a disordered F-actin staining that is associated with weak ZO-1 staining. Culturing in ACEM alone did not change the F-actin and ZO-1 patterns, whereas C6CEM resulted in a rearrangement of F-actin to the cell borders and enhanced ZO-1 signals.

BCEC cultures could be further improved by increasing intracellular cAMP levels and by addition of dexamethasone. These supplements were able to raise the TER value in EM- and ACEM-cultured BCEC and also to enhance the F-actin and ZO-1 score in cells grown in ACEM. This suggests that astrocytic factors and cAMP or dexamethasone use complementary pathways to tighten the intracellular junctions. The junction modulation by cAMP implies the existence of one or more proteins capable of being phosphorylated by protein kinase A, whose state of phosphorylation controls tight and adherens junctions (Rubin and Staddon 1999). It should be noted that F-actin scores were highly correlated with ZO-1 scores. No further improvement of tight junctions in BCEC could be observed when supplemented with all-trans retinoic acid or 1,25-dihydroxyvitamin $D_3$.

Surprisingly, when monolayer permeability was assessed, using the extracellular marker sucrose, cells grown under different culture conditions showed no statistically significant differences. This could be due to the fact that TER measures the flux of ions, whereas sucrose represents a larger molecule.

It has been shown that ABC transporters play a functional part in establishing a barrier between the blood and the brain (Schinkel et al. 1996; Regina et al. 1998; Miller et al. 2000; Fricker et al. 2002; Löscher and Potschka 2002). Our studies demonstrate colocalization of P-glycoprotein with the endothelial marker von Willebrand factor and not with fragments of astrocyte foot processes (Fig. 4). Recent publications, however, suggest some expression of P-glycoprotein and MRP1 by astrocytes (Declèves et al. 2000; Golden and Pardridge 2000). To determine the impact of different culture conditions on the expression of ABC transporters such as P-glycoprotein, MRP1, and MRP2, mRNA levels were assessed by quantitative RT-PCR. Compared with humans, pigs have four class I P-glycoprotein genes (pgp1A to pgp1D), but with only pgp1A being expressed (Childs and Ling 1996). pgp1A mRNA was highly enriched in cerebral capillaries compared with whole-brain tissue. However, in isolated BCEC, pgp1A mRNA was decreased. pgp1A expression did not substantially change during culture in EM, nor did it change in different cell-culture conditions. Therefore, an enhanced tight-junction morphology does not necessarily correlate with the preservation of P-glycoprotein. Our findings correspond with those obtained by a recent publication, where levels of immunoreactive P-glycoprotein were markedly decreased in cultures of BCEC. This effect was less pronounced in cocultures of BCEC with astrocytes (Gaillard et al. 2000). However, since P-glycoprotein is lost during the preparation of capillaries, in vitro experiments are likely to underestimate the influence of this carrier. The same holds true for MRP1 and MRP2, since mRNA is reduced during cell culture in the present study. It is interesting to note that other endothelial cell types, such as immortalized rat cell lines (El Hafny et al. 1997), or human umbilical vein endothelial cells (Hayashi et al. 1997) are more sensitive to cell-culture conditions, which results in upregulation of P-glycoprotein expression.

Expression of MRPs in BCEC has only been reported recently (Huai-Yun et al. 1998; Miller et al. 2000; Zhang et al. 2000; Fricker et al. 2002). Using primary cultured bovine brain capillary endothelial cells, RT-PCR shows the presence of MRP1, whereas MRP2 is absent (Zhang et al. 2000). However, using immunostaining of P-glycoprotein and MRP2 in isolated capillaries from rat, pig (Miller et al. 2000), and fish (Miller et al. 2002) brains, both multidrug transporters were localized to the luminal surface of the capillary endothelium. It is interesting to note that MRP2 mRNA levels are about 100 times lower than MRP1 mRNA levels (estimated by threshold-cycle differences on real-time quantitative RT-PCR).

The present study shows that the morphology of tight junctions and intracellular actin of cultured BCEC can be modulated by astrocyte-conditioned medium in combination with elevated intracellular cAMP levels or dexamethasone. These changes have an impact on the TER, but not on the paracellular flux of molecules, at least with the size of sucrose. ABC transporters such as P-glycoprotein, MRP1, and MRP2 are expressed in cultured BCEC and in intact brain capillaries of porcine origin. However, mRNA levels of these transporters are lower. This could lead to an underestimation of these carriers when assessing uptake or drug-interactions of different P-glycoprotein substrates. Monolayers of primary porcine BCEC can be used nevertheless as a robust cell culture model of the blood–brain barrier, since it is insensitive toward changes in cell-culture conditions.

**Acknowledgements** This work was supported by the Swiss National Science Foundation (grant 32-052918.97). We thank U. Behrens for excellent technical assistance. We would also like to thank S. Brill and Dr. P. Roberts for careful revision of this manuscript

## References

Abbott NJ, Revest PA, Romero IA (1992) Astrocyte-endothelial interaction: physiology and pathology. Neuropathol Appl Neurobiol 18:424–433

Allt G, Lawrenson JG (2001) Pericytes: cell biology and pathology. Cells Tissues Organs 169:1–11

Bouillon R, Okamura WH, Norman AW (1995) Structure–function relationships in the vitamin D endocrine system. Endocr Rev 16:200–257

Childs S, Ling V (1996) Duplication and evolution of the P-glycoprotein genes in pig. Biochim Biophys Acta 1307:205–212

Declèves X, Regina A, Laplanche JL et al. (2000) Functional expression of P-glycoprotein and multidrug resistance-associated protein (Mrp1) in primary cultures of rat astrocytes. J Neurosci Res 60:594–601

Dehouck MP, Dehouck B, Schluep C, Lemaire M, Cecchelli R (1995) Drug transport to the brain: comparison between in vitro and in vivo models of the blood–brain barrier. Eur J Pharm Sci 3:357–365

Durieu-Trautmann O, Federici C, Creminon C et al. (1993) Nitric oxide and endothelin secretion by brain microvessel endothelial cells: regulation by cyclic nucleotides. J Cell Physiol 155:104–111

El Hafny B, Chappey O, Piciotti M, Debray M, Boval B, Roux F (1997) Modulation of P-glycoprotein activity by glial factors and retinoic acid in an immortalized rat brain microvessel endothelial cell line. Neurosci Lett 236:107–111

Franke H, Galla HJ, Beuckmann CT (1999) An improved low-permeability in vitro-model of the blood–brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol. Brain Res 818:65–71

Fricker G, Nobmann S, Miller DS (2002) Permeability of porcine blood brain barrier to somatostatin analogues. Br J Pharmacol 135:1308–1314

Gaillard PJ, Sandt IC van der, Voorwinden LH et al. (2000) Astrocytes increase the functional expression of P-glycoprotein in an in vitro model of the blood–brain barrier Pharm Res 17:1198–1205

Golden PL, Pardridge WM (2000) Brain microvascular P-glycoprotein and a revised model of multidrug resistance in brain. Cell Mol Neurobiol 20:165–181

Grabb PA, Gilbert MR (1995) Neoplastic and pharmacological influence on the permeability of an in vitro blood–brain barrier. J Neurosurg 82:1053–1058

Gumbiner B, Simons K (1986) A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide. J Cell Biol 102:457–468

Gutmann H, Török M, Fricker G et al. (1999) Modulation of multidrug resistance protein expression in porcine brain capillary endothelial cells in vitro. Drug Metab Dispos 27:937–941

Hayashi Y, Nomura M, Yamagishi S et al. (1997) Induction of various blood–brain barrier properties in non-neural endothelial cells by close apposition to cocultured astrocytes. Glia 19:13–26

Huai-Yun H, Secrest DT, Mark KS et al. (1998) Expression of multidrug resistance-associated protein (MRP) in brain microvessel endothelial cells. Biochem Biophys Res Commun 243:816–820

Huwyler J, Drewe J, Klusemann C, Fricker G (1996) Evidence for P-glycoprotein-modulated penetration of morphine-6-glucuronide into brain capillary endothelium. Br J Pharmacol 118:1879–1885

Huwyler J, Fricker G, Török M, Schneider M, Drewe J (1997) Transport of clonidine across cultured brain microvessel endothelial cells. J Pharmacol Exp Ther 282:81–85

Janzer RC, Raff MC (1987) Astrocytes induce blood–brain barrier properties in endothelial cells. Nature 325:253–257

Klein I, Sarkadi B, Varadi A (1999) An inventory of the human ABC proteins. Biochim Biophys Acta 1461:237–262

Korn J, Christ B, Kurz H (2002) Neuroectodermal origin of brain pericytes and vascular smooth muscle cells. J Comp Neurol 442:78–88

Lechardeur D, Schwartz B, Paulin D, Scherman D (1995) Induction of blood–brain barrier differentiation in a rat brain-derived endothelial cell line. Exp Cell Res 220:161–170

Lillien LE, Sendtner M, Rohrer H, Hughes SM, Raff MC (1988) Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes. Neuron 1:485–494

Löscher W, Potschka H (2002) Role of multidrug transporters in pharmacoresistance to antiepileptic drugs. J Pharmacol Exp Ther 301:7–14

Meyer J, Rauh J, Galla HJ (1991) The susceptibility of cerebral endothelial cells to astroglial induction of blood–brain barrier enzymes depends on their proliferative state. J Neurochem 57:1971–1977

Miller DS, Nobmann SN, Gutmann H et al. (2000) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. Mol Pharmacol 58:1357–1367

Miller DS, Graeff C, Droulle L, Fricker S, Fricker G (2002) Xenobiotic efflux pumps in isolated fish brain capillaries. Am J Physiol Regul Integr Comp Physiol 282:191–198

Pardridge WM (1993) Brain drug delivery and blood–brain barrier transport. Drug Delivery 1:83–101

Pardridge WM, Eisenberg J, Yamada T (1985) Rapid sequestration and degradation of somatostatin analogues by isolated brain microvessels. J Neurochem 44:1178–1184

Pardridge WM, Triguero D, Yang J, Cancilla PA (1990) Comparison of in vitro and in vivo models of drug transcytosis through the blood–brain barrier. J Pharmacol Exp Ther 253:884–891

Ramsauer M, Krause D, Dermietzel R (2002) Angiogenesis of the blood–brain barrier in vitro and the function of cerebral pericytes. FASEB J 16:1274–1276

Raub TJ (1996) Signal transduction and glial cell modulation of cultured brain microvessel endothelial cell tight junctions. Am J Physiol Cell Physiol 271:495–503

Raub TJ, Kuentzel SL, Sawada GA (1992) Permeability of bovine brain microvessel endothelial cells in vitro: barrier tightening by a factor released from astroglioma cells. Exp Cell Res 199:330–340

Reese DH, Fiorentino GJ, Claflin AJ, Malinin TI, Politano VA (1981) Rapid induction of alkaline phosphatase activity by retinoic acid. Biochem Biophys Res Commun 102:315–321

Regina A, Koman A, Piciotti M et al. (1998) Mrp1 multidrug resistance-associated protein and P-glycoprotein expression in rat brain microvessel endothelial cells. J Neurochem 71:705–715

Rist RJ, Romero IA, Chan MW et al. (1997) F-actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell monolayers: effects of cyclic AMP and astrocytic factors. Brain Res 768:10–18

Rubin LL, Staddon JM (1999) The cell biology of the blood–brain barrier. Annu Rev Neurosci 22:11–28

Rubin LL, Hall DE, Porter S et al. (1991) A cell culture model of the blood–brain barrier. J Cell Biol 115:1725–1735

Schinkel AH, Wagenaar E, Mol CA, Deemter L van (1996) P-glycoprotein in the blood–brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest 97:2517–2524

Staddon JM, Rubin LL (1996) Cell adhesion, cell junctions and the blood–brain barrier. Curr Opin Neurobiol 6:622–627

Stewart PA, Wiley MJ (1981) Developing nervous tissue induces formation of blood–brain barrier characteristics in invading endothelial cells: a study using quail-chick transplantation chimeras. Dev Biol 84:183–192

Takakura Y, Audus KL, Borchardt RT (1991) Blood–brain barrier: transport studies in isolated brain capillaries and in cultured brain endothelial cells. Adv Pharmacol 22:137–165

Török M, Huwyler J, Drewe J, Gutmann H, Fricker G (1998) Transport of the β-lactam antibiotic benzylpenicillin and the dipeptide glycylsarcosine by brain capillary endothelial cells in vitro. Drug Metab Dispos 26:1144–1148

Tsao MS, Batist G (1988) Induction of γ-glutamyl transpeptidase activity by all-trans retinoic acid in cultured rat liver epithelial cells. Biochem Biophys Res Commun 157:1039–1045

Zhang Y, Han H, Elmquist WF, Miller DW (2000) Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. Brain Res 876:148–153

Zonta M, Angulo MC, Gobbo S. (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 6:43–50